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Otx1 and Otx2 Define Layers and Regions in Developing Cerebral Cortex and Cerebellum

Gretchen D. Frantz, James M. Weimann, Margaret E. Levin, and Susan K. McConnell

Department of Biological Sciences, Stanford University, Stanford, California 94305

Within the cerebral and cerebellar cortices, neurons are organized in layers that segregate neurons with distinctive morphologies and axonal connections, and areas or regions that correspond to distinct functional domains. To explore the molecular underpinnings of pattern formation in layered regions of the CNS, we have characterized the patterns of expression of two homeodomain genes, Otx1 and Otx2, by *in situ* hybridization during embryonic and postnatal development in the rat. Otx1 and Otx2 are vertebrate homologs of the *Drosophila* gap gene *orthodenticle*, and are expressed during the development of the murine CNS (Simeone et al., 1992). Here we report that Otx1 mRNA is expressed in a subpopulation of neurons within cortical layers 5 and 6 during postnatal and adult life. This gene is also expressed by the precursors of deep-layer neurons within the developing cerebral ventricular zone, but is apparently downregulated by the progenitors of upper-layer neurons; Otx1 is never expressed by the neurons of layers 1–4. The spatial and temporal patterns suggest that Otx1 may play a role in the specification or differentiation of neurons in the deep layers of the cerebral cortex. Within the cerebellum, mRNAs for Otx1 and Otx2 are found within the external granular layer (EGL), but in three spatially distinct domains. During postnatal development, Otx1 is expressed within anterior cerebellar lobules; Otx2 mRNA is localized posteriorly, and a region of overlap in mid-cerebellum defines a third domain in which both genes are expressed. The boundaries of Otx1 and Otx2 expression correspond to the major functional boundaries of the cerebellum, and define the vestibulocerebellum, spinocerebellum, and pontocerebellum, respectively. Spatially restricted patterns of hybridization are observed early in postnatal life, at times that correspond roughly to the invasion of spinal and pontine afferents into the cerebellum (Arsénio-Nunes and Sotelo, 1985; Mason, 1987). These results raise the possibility that Otx1 and Otx2 play a role in cerebellar regionalization during early development.

[Key words: neurogenesis, homeodomain, areas, cerebral cortex, homeodomain, migration, pattern formation, layers]

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Correspondence should be addressed to Dr. Gretchen D. Frantz, Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020.

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Within the CNS of mammals, neurons are organized into discrete functional domains. The generation of a diversity of neurons from a simple neural tube requires that phenotypically distinct neurons be produced, organized into functional units, and interconnected through the formation of specific axonal and synaptic contacts. The mechanisms by which both pattern and diversity are achieved during development have been explored in a variety of CNS regions with differing types of organization, for example, dorsal-ventral patterning within the spinal cord (Basler et al., 1993; Yamada et al., 1993), segmentation within the hindbrain (Wilkinson et al., 1989; Fraser et al., 1990; Lumsden, 1990; Guthrie et al., 1992; Marshall et al., 1992), and lamination within the cerebral and cerebellar cortices (Altman, 1972a,b; Altman and Bayer, 1978; McConnell, 1991; McConnell and Kaznowski, 1991; Kuhar et al., 1993). We have been interested in how the developing brain constructs layers of cells that segregate neurons with characteristic dendritic morphologies, physiological properties, and both local and long-distance axonal connections (McConnell, 1988a, 1991). Two systems that use distinct developmental strategies for generating the neurons that constitute different layers are the cerebral cortex and the cerebellum. Cerebral cortical neurons are generated from the ventricular zone that lines the lateral ventricle of the telencephalon; postmitotic neurons migrate away through the intermediate zone and into the cortical plate, which will form the six-layered cerebral cortex of the adult animal (reviewed in McConnell, 1988a). It has long been apparent from ^{3}H-thymidine "birthdating" studies that precursors sequentially produce neurons destined for the six layers in an inside-first, outside-last manner (Angevine and Sidman, 1961; Berry and Rogers, 1965; Rakic, 1974). Early precursor cells are multipotent: although normally neurons generated early in development migrate to the deep layers of the cortex, transplantation of their progenitors into an older brain environment can result in the respecification of daughters to an upper-layer fate (McConnell, 1988b; McConnell and Kaznowski, 1991). Thus, cell–cell interactions or other environmental cues influence the lamina-specific phenotypes adopted by cerebral cortical neurons. Progenitor cells are only capable, however, of responding to such cues during a window of time during the cell cycle: by the time of mitosis, ventricular cells have undergone a commitment to generating daughters that migrate to the deep cortical layers (McConnell and Kaznowski, 1991). The nature of the environmental influences that specify fates, and the molecular changes that enable cells to migrate to an appropriate layer, are largely unknown.

The cerebellum, another layered structure, arises using a distinct and somewhat unusual strategy: although most types of

cerebellar neurons are generated from a true ventricular zone, the granule neurons arise from a separate proliferative zone, the external granular layer (EGL). The EGL is derived from a migratory population of cells that leave the ventricular zone of the alar plates and move around the rhombic lip to cover the pial surface of the cerebellum (Miale and Sidman, 1961; Altman and Bayer, 1978). Here cells proliferate, leave the cell cycle, and then migrate inward toward the ventricular surface to take up residence in the internal granular layer (IGL), subjacent to the Purkinje cell layer (Rakic, 1971). Fate mapping studies in avian systems have revealed that the cerebellum is derived from two regions of the neural tube: the caudal mesencephalon, which forms the mediodorsal cerebellum, and the rostral metencephalon, which generates more posterior and lateral structures (Martinez and Alvarado-Mallart, 1989; Hallonet and Le-Douarin, 1993; Otero et al., 1993). In contrast, the EGL is derived entirely from the metencephalon (Hallonet and Le-Douarin, 1993; Otero et al., 1993). Although in normal animals the EGL is restricted to the production of granule neurons (Hallonet et al., 1990; Gao et al., 1991), the developmental potential of these precursors has only recently been explored through transplantation experiments (Gao and Hatten, 1994). The two populations of cerebellar precursors, one residing in the ventricular zone proper and the other in the EGL, participate in neurogenesis at separate times during development (Altman, 1972a; Altman and Bayer, 1978) and show distinct patterns of gene expression (Kuhar et al., 1993; see also below), consistent with the possibility that the progenitor pools themselves are biased toward or committed to the production of separate cell types.

Both the cerebral and cerebellar cortices show a second aspect of pattern in addition to lamination. Each of these structures is also divided along the plane tangential to the pial surface into functionally distinct areas or regions. The cerebral cortex is composed of areas that subserve functions ranging from the processing of incoming sensory information to the generation of motor outputs. Areas are characterized by distinctive cytoarchitectonic features, including the density of neurons in different layers (Brodmann, 1909) and the origins of afferent axons that innervate each area (Kreig, 1946). The cerebellum is similarly divided into functional domains along the rostral-to-caudal axis: the spinocerebellum (the paleocerebellum or anterior lobe: lobules 1–5), which receives input from the spinal cord; the pontocerebellum (the neocerebellum or posterior lobe: lobules 6-9, crus 1 and 2 ansiform lobules), which obtains afferent input from cerebral cortical neurons via the pons and inferior olive; and the vestibulocerebellum (archicerebellum or flocculonodular lobule: lobule 10 and flocculus), which receives vestibular afferent input (Brodal, 1981; Carpenter, 1985).

Studies in mammals of the molecular mechanisms by which neurons are generated and patterned during development have been greatly aided by studies of the early development of the fruit fly *Drosophila*. Genes involved in the construction of the body plan in *Drosophila* have homologs in vertebrates that appear to be involved in the generation of anterior-posterior differences within the embryo and particularly in the neural tube. Many of these genes encode known or putative transcriptional regulators and contain DNA binding motifs such as homeoboxes. While much attention has focused on the potential roles for Hox genes, the homologs of genes that comprise the *Drosophila Antennapedia* and *Ultrabithorax* complexes, in the determination of segmental identities among the rhombomeres of the hindbrain (Marshall et al., 1992; Dolle et al., 1993), Hox genes are not expressed in more rostral CNS structures (Wilkinson and Krumlauf, 1990). Recent studies have identified a variety of other genes with homologs in *Drosophila* that are expressed in the forebrain and midbrain. For example, Dlx1 and Dlx2, homologs of *Distal-less* in *Drosophila*, have expression patterns that suggest an involvement in the formation of neuromeres within the forebrain, primarily in the ventral thalamus and basal telencephalon (Bulfone et al., 1993). Recently Simeone and colleagues (Simeone et al., 1992a,b) cloned four novel mammalian genes based on their homology to two *Drosophila* genes involved in head development, orthodenticle and empty spiracles. The mammalian counterparts, known as Otx1, Otx2, Emx1, and Emx2, encode proteins that contain homeoboxes (Simeone et al., 1992a,b); the Otx1 and Otx2 homeodomains are similar to that of bicoid and bind to the same DNA elements that are recognized by bicoid protein (Simeone et al., 1993). The mRNAs of the Otx and Emx genes are expressed in a "nested" pattern within the developing forebrain and midbrain of murine embryos (Simeone et al., 1992a). These earlier studies did not report on the pattern of Otx1 and Otx2 hybridization in postnatal life, mentioning only that the expression of both declines after E15 in the mouse (Simeone et al., 1993). The presence of Otx1 and Otx2 in areas of the CNS that generate layers of neurons prompted us to explore their expression in the developing and adult rat brain, with the goal of ascertaining whether their expression patterns suggest a role for these genes in the process of lamination. We chose the rat as a common model system for studies of both cerebral and cerebellar cortical development (Berry and Rogers, 1965; Altman, 1972a; Altman and Bayer, 1978; Miller, 1985), and have focused on later stages of embryogenesis and on postnatal life, periods during which neurons are generated and layers emerge.

Materials and Methods

Animals. Timed-pregnant Long-Evans rats (Simonsen) were used for the developmental studies. The morning after breeding (the day a vaginal plug was detected) was considered embryonic day (E) 0. A total of 32 developing and adult rats were used for *in situ* hybridization experiments: E13, two; E14, two; E15, one; E16, two; E17, one; E18, three; E19, two; E20, two; postnatal day (P) 1, one; P2, two; P5, one; P9, one; P11, one, P13, three; P14, one; P16, two; adult, five. For Northern analysis, one litter each was used at E14, E16, E18, and E20 (14–16 fetuses per litter); in addition, RNA was prepared from one adult animal.

In situ *hybridization.* Animals were anesthetized with an overdose of Nembutal (≥100 mg/kg body weight, i.p.). Whole embryos or brains were dissected out, immediately placed in OCT embedding compound, frozen on dry ice and stored at −70°C until use. Frozen sections were cut at 15 μm on a cryostat, mounted onto polylysine-coated slides, postfixed in 4% paraformaldehyde at pH 7.0, rinsed in PBS, dehydrated, and stored at −70°C. ^{35}S-labeled Otx1 (396 bp) and Otx2 (263 bp) antisense and sense riboprobes were transcribed from clones pMOTX 1.3/81 and pMOTX 2.10/32, respectively (provided by Dr. A. Simeone; see Simeone et al., 1993). Linearization with EcoRI and transcription with SP6 RNA polymerase produced antisense transcripts. Sense transcripts were synthesized from HindIII digested plasmid incubated with T7 polymerase. The *in situ* hybridization protocol was based on that of Simmons et al. (Simmons et al., 1989) with the modifications described below. Tissue pretreatments consisted of proteinase K digestions (3.7 μg/ml), followed by acetylation. Following overnight hybridization at 60°C, sections were treated with ribonuclease A (5 μg/ml) at 37°C. Sections were then treated with a high-stringency wash in 0.1× SSC at 60°C. Slides were dehydrated in a series of ethanols and xylenes, air dried, dipped in Kodak NTB2 autoradiographic emulsion, and exposed for 3 weeks before developing. Sense control probes revealed no hybridization above background (not shown).

Grain counts. In order to determine the fraction of cells in different layers of the cerebral cortex that express Otx1, we first determined the number of cells within the field of view of a 20× objective that had the lightly staining, medium- to large-sized nuclei that are characteristic of neurons in Cresyl violet stained sections. Neurons were considered positive for Otx1 expression if six or more silver grains were found overlying the cell soma; the background level of hybridization in layer 2/3 was only one grain per cell on average in all sections chosen for analysis. Cells were visualized at 40–100× magnification to determine the number of silver grains overlying each positive neuron. Counts were obtained from posterolateral regions of the P13 and adult cerebral cortex, in which the fractions of Otx1-expressing cells in layer 5 were highest. For counts within the ventricular zone, sections with equal background levels of hybridization were chosen for comparison. It was impossible to obtain reliable grain counts over individual ventricular cells because the cells were very closely packed and lacked much cytoplasm, and the grains generated by ^{35}S scattered over adjacent cells. Instead, the ventricular zones of E15 and E19 rats were viewed at 100×; all grains in the field were counted, then divided by the total number of cell bodies in the field to yield an average number of grains/ventricular cell. Note that these figures therefore represent an underestimate of the actual value, since some cells in each field may not express Otx1 at all.

Photography. Selected sections were photographed using a Nikon UFX camera system for color photomicrographs. For black and white figures, images were captured through an MTI CCD camera (Dage) using NIH IMAGE and processed on a Macintosh Quadra computer using Adobe PHOTOSHOP.

Preparation of RNA and Northern blots. At all ages except E14, brains were dissected in Hank's Balanced Salt Solution (HBSS) to separate out tissue from the cerebral cortex, which was then frozen in liquid nitrogen. At E14 the entire telencephalon was used. PolyA^{+} mRNA was prepared from each tissue sample separately using Micro-Fast Track kits (Invitrogen). RNA was denatured on 1% agarose/2.2 M formaldehyde gels, transferred to positively charged nylon membranes, and hybridized with random-primed ^{32}P-labeled probes (Sambrook et al., 1989) representing a 396 base pair region of the third exon of Otx1. Hybridization was carried out at 42°C for 36 hr, and high-stringency washes were done in 0.4× SSC with 0.5% SDS at 65°C for 30 min. Membranes were exposed to film and images of the resulting Northern were obtained using a Hewlett Packard gel scanner linked to a Macintosh computer; images were processed using Adobe PHOTOSHOP.

Cloning of a cDNA encoding the rat Otx1 gene. A rat brain cDNA library (λZapII, Stratagene) was screened at low stringency with a probe to the third exon of the mouse Otx1 gene (Simeone et al., 1992a, 1993). The library was plated, transferred in duplicate to nitrocellulose filters, and screened using ^{32}P-labeled cDNA probes generated by random priming (Sambrook et al., 1989). Positive clones were rescreened and the cDNA insert of those clones that hybridized on the second screen were sequenced. A DNA sequence analysis program (GCG, Genetics Computer Group, Madison, WI) was used to predict all possible amino acid sequences encoded by these sequences; these were compared to the published amino acid sequence of Otx1 (Simeone et al., 1993) to ascertain whether the correct cDNA had been obtained.

Results

Previous studies have described the expression of Otx1 and Otx2 in regionally restricted patterns during the embryonic development of the murine CNS (Simeone et al., 1992a, 1993), but the postnatal expression patterns of these genes were not reported. We were therefore interested in asking whether the expression of these genes was correlated with the acquisition of discrete cell fates later in life. Here we report that the expression of Otx1 and Otx2 in the rat forebrain and cerebellum define regionally distinct populations of neurons. First we describe the patterns of expression in the forebrain from embryonic stages through adulthood, focusing on the cerebral cortex. We then describe the localization of Otx1 and Otx2 mRNAs in the developing cerebellum, where they define spatially segregated groups of granule cell precursors. In a final section we present the cloning of a cDNA encoding the rat Otx1 gene.

Table 1. Distribution of Otx1 and Otx2 in P13 rat brains

	Otx1	Otx2
Telencephalon		
Cerebral cortex	+	−
Hippocampus	−	+
Subiculum	−	+
Piriform cortex	−	+
Striatum	−	+
Lateral septum	−	+ (weak)
Medial septum	−	−
Amygdala	−	+
Olfactory bulb	−	+
Anterior olfactory nucleus	−	+
Choroid plexus (lateral and third ventricle)	+	+
Diencephalon		
Dorsal lateral geniculate nucleus	−	+
Ventral lateral geniculate nucleus	+	+
Anterodorsal thalamic nucleus	+	+ (weak)
Mediodorsal thalamic nucleus, lateral	+	−
Medial habenula	+	+
Zona incerta	−	+
Lateral hypothalamic area	+	−
Mesencephalon		
Inferior colliculus	+	+
Superior colliculus	−	+
Anterior pretectal nucleus	+	+
Posterior pretectal nucleus	+	+
Medial pretectal nucleus	+	+
Zona incerta	−	+
Substantia nigra	+	−
Central gray	+	+
Interstitial nucleus medial longitudinal fasciculus	+	−
Nucleus Darkschewitsch	+	+
Ventral tegmental area	+	+
Dorsal tegmental nucleus	+	−
Laterodorsal tegmental nucleus	+	−
Prerubral field	+	−
Interpeduncular nucleus	+	−
Red nucleus	−	+
Metencephalon		
Cerebellum		
External granular layer	+	+
Internal granular layer	−	+
Choroid plexus (fourth ventricle)	−	+
Myelencephalon		
Dorsal cochlear nuclei	−	+
Raphe pontis nucleus	+	−
Cuneiform nucleus	+	−

Sections were analyzed in the coronal, sagittal, and horizontal planes, and regions identified using a rat brain atlas (Paxinos and Watson, 1986). Although as complete a series of sections was not analyzed in adult rat brains, the pattern in adults was essentially identical to that described here for P13 animals. + indicates the presence of label above background, and − indicates the absence of label for each probe.

Otx1 and Otx2 expression in developing forebrain

Embryonic expression. The patterns of Otx1 and Otx2 expression were characterized by *in situ* hybridization using probes to the third exon of the mouse Otx1 and Otx2 genes (kindly provided by Dr. A. Simeone). Although these probes were used on

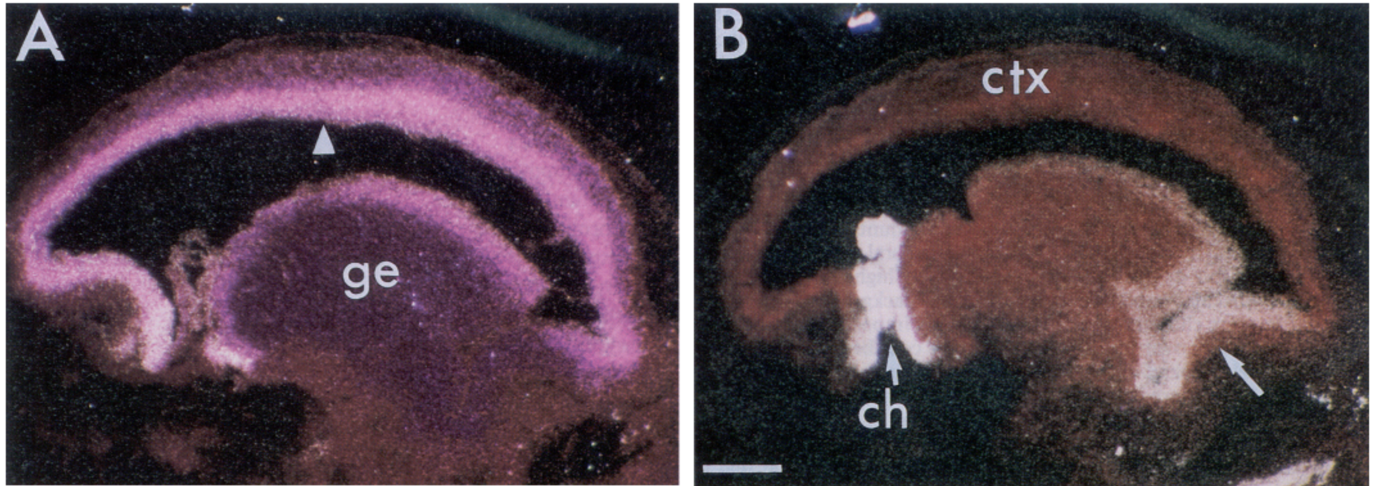

Figure 1. *In situ* hybridization for Otx1 and Otx2 mRNAs in the E16 rat forebrain. These are dark-field views of parasagittal sections counterstained with cresyl violet; anterior is to the right. *A*, Otx1 mRNA is detected in the cortical ventricular zone (*arrowhead*) and emerging cortical plate, choroid plexus of the lateral ventricle, and the ventricular zones of the ganglionic eminence (*ge*), olfactory bulb, and hippocampus. *B*, Otx2 mRNA is found in the choroid plexus (*ch*) of the lateral ventricle, and the ventricular zones for the septum (*arrow*), ventral olfactory bulb, and anterior ganglionic eminence. *ctx*, cerebral cortex. Scale bar, 0.5 mm.

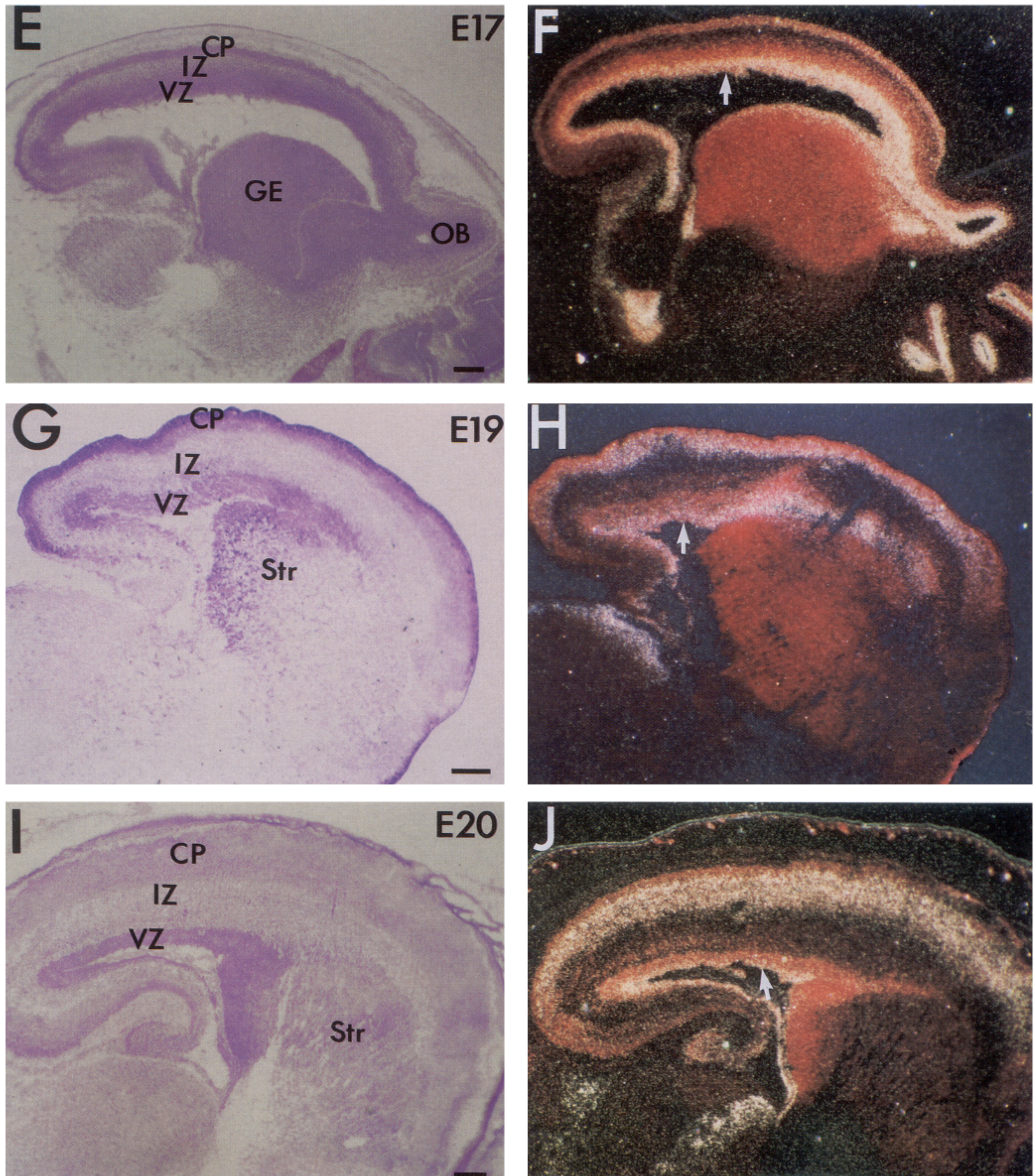

Figure 2. Developmental regulation of Otx1 expression within the embryonic rat forebrain. *A, C, E, G,* and *I,* Bright-field views of parasagittal sections counterstained with cresyl violet; anterior is to the right. *B, D, F, H,* and *J,* Dark-field views of Otx1 hybridization in the same or adjacent section to that shown on the left. *A* and *B,* E13. Otx1 is expressed in the cerebral cortical ventricular zone (*arrow*). The anterior-posterior extent of the cortex is marked by *arrowheads.* Hybridization is also present in the ventricular zone of the ganglionic eminence (the anlage of the striatum), olfactory bulb, as well as the olfactory and facial epithelia. *C* and *D,* E15. Otx1 hybridization is apparent throughout the entire cortical ventricular zone (*VZ, arrow*), olfactory bulb (*arrowhead*), and within the diencephalon and the olfactory epithelium. *E* and *F,* E17. Within the cerebral cortex, Otx1 mRNA is expressed in the ventricular zone (*arrow*) and emerging cortical plate (*CP*), as well as in the olfactory bulb (*OB*) and ventricular zone of the ganglionic eminence (*GE*). *G* and *H,* E19. Otx1 hybridization is present within the cortical plate, and is present within the ventricular zone (*arrow*) at decreased levels relative to earlier ages. *I* and *J,* E20. Within the cortex, the expression of Otx1 is strongest in deep regions of the cortical plate and weak, but present, in the ventricular zone (*arrow*). *IZ,* intermediate zone; *Str,* striatum. Scale bars, 0.3 mm (for each pair of adjoining panels).

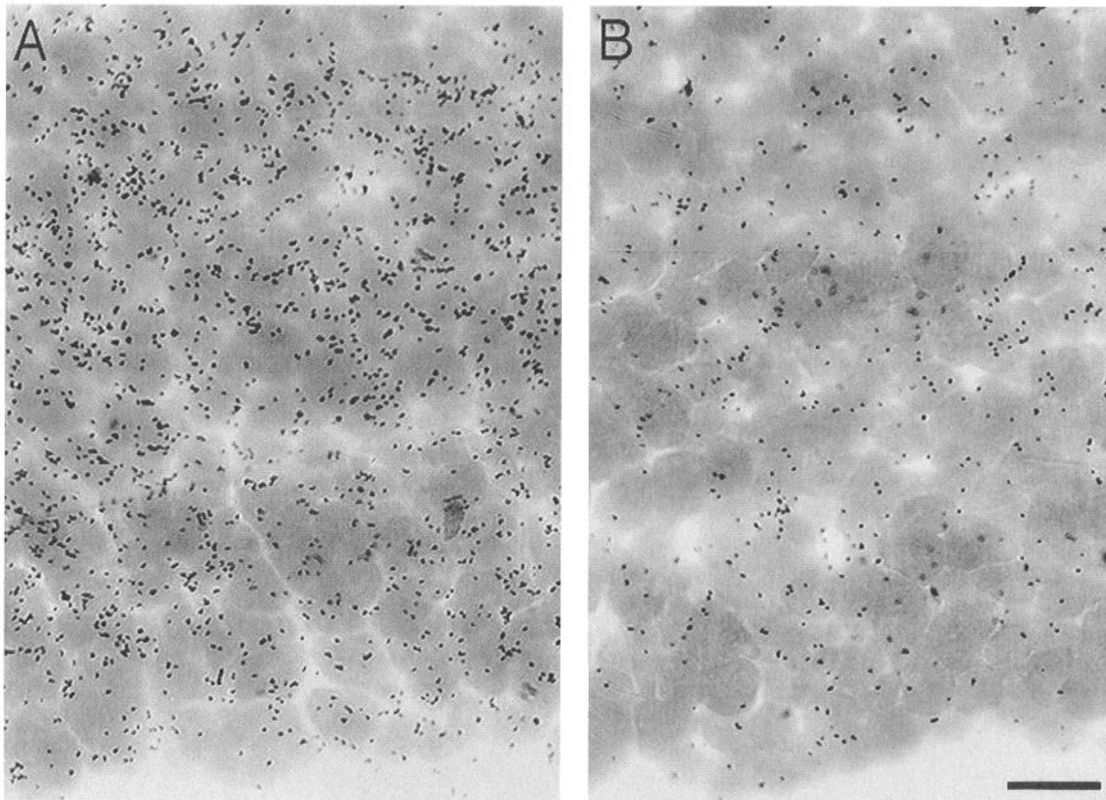

Figure 3. High-power bright-field views of Otx1 hybridization within the cerebral cortical ventricular zones of E15 and E19 rats. *A*, E15 ventricular cells show high levels of hybridization, and most cells appear to express Otx1. *B*, E19 cells show much lower levels of hybridization. Scale bar, 0.01 mm.

rat tissue, we emphasize that the amino acid sequence homology between mouse and human is roughly 97–99% for both genes (Simeone et al., 1993), and the nucleotide identity between the rat and mouse Otx1 genes in the region of this probe is 94% (see below). Thus, we are confident that the patterns reported here accurately reflect the distributions of the rat mRNAs for these two genes.

From E13 to E16, the earliest ages studied, the patterns of expression of Otx1 and Otx2 closely resemble those reported by Simeone et al. (1992a, 1993) for the mouse. Figure 1*B* shows the distribution of Otx2 hybridization in the E16 rat forebrain: hybridization is present in the choroid plexus and the ventricular zones of the ganglionic eminence and septum. Not shown but observed in other sections is the expression of Otx2 in distinct regions of the diencephalon, nasal epithelium, developing eye, otic capsule, choroid plexus of the fourth ventricle, and presumptive superior and inferior colliculi, as previously reported (Simeone et al., 1992a, 1993). Note that Otx2 is not expressed at levels above background within the cerebral ventricular zone or cortical plate at this age (Fig. 1*B*), although it was present at earlier ages in mice (Simeone et al., 1992a). In postnatal and adult animals, Otx2 expression is not observed in striatal neurons, which are produced by the ganglionic eminence, but expression is observed in the choroid plexus, lateral septum, thalamic and pretectal nuclei, superior and inferior colliculi, and cerebellum throughout adult life (see Table 1).

Although the expression of Otx2 in regions of the dorsal forebrain is transient, we observed Otx1 hybridization in this region not only during embryonic life, as reported by Simeone et al (1992a, 1993), but also throughout development and into adulthood. Figure 2*B* shows the distribution of Otx1 hybridization in the E13 rat forebrain. Hybridization is prominent in the cerebral ventricular zone, where expression appears of even strength throughout its anterior-posterior and medial-lateral extent. In addition, the ganglionic eminence shows Otx1 signal but in a pattern that is roughly complementary to that for Otx2 (E16, Fig. 1*A*; also see below). Otx1 mRNA is also expressed within the diencephalon, nasal epithelia, facial epithelia, epithelia lining the oral cavity, otic capsule, and developing eye (not shown), in patterns similar to those reported for mouse (Simeone et al., 1992a, 1993).

Expression of Otx1 remains essentially identical to this pattern at E14–E15. At E15 (Fig. 2*C*), the cerebral wall shows strong Otx1 hybridization in the ventricular zone, which at these ages is involved in the generation of neurons destined for the deep cortical layers 5 and 6 (Miller, 1985; Frantz et al., 1994). No signal is detectable in the cortical plate, which at this age consists primarily of subplate neurons (Bayer and Altman, 1990) because the neurons of the deep layers have not yet arrived in their final positions within the cortical plate proper (Berry and Rogers, 1965; Bayer and Altman, 1990). The pattern of Otx1 expression within the ganglionic eminence (Figs. 1*A*, 2*D*) is roughly complementary to that seen for Otx2 (Fig. 1*B*).

At E16–E17, Otx1 expression is apparent within several regions of the cerebral wall. Hybridization is strong in the ventricular zone, more moderate in the cortical plate, and weak but detectable in the cell-sparse intermediate zone (Figs. 1*A*, 2*E*). Notably, at these ages, the neurons of the deepest cortical layers 5

and 6 have largely completed their genesis, and many have already arrived at their final destinations within the cortical plate.

Between E18 and E20, Otx1 expression is prominent within the cortical plate, which consists of postmigratory neurons of layers 5 and 6 (Fig. *2H,J*). At these ages, upper-layer neurons are being generated in the ventricular zone, where the Otx1 signal is still present but has apparently weakened (Fig. *2H,J*). Figure 3 shows a higher-power view of the ventricular zones from E15 and E19 animals, revealing a lower number of grains per cell at the later age. Grain counts of ventricular cells from E15 and E19 sections show a threefold difference (average number of grains per cell at E15 is 12.0 grains/cell, $n = 79$ cells; at E19, average 3.9 grains/cell, $n = 89$ cells; see Materials and Methods, and note that although it is difficult to determine accurately the relative levels of hybridization in different sections, each section was exposed for an identical period of time and exhibited nearly identical background grain counts). Although the scattering of grains relative to individual cells prohibits us from determining directly whether all ventricular cells express Otx1, Figure *3A* suggests that most cells do so at E15 (although in other sections a few individual cells appeared to lack label). These results suggest that early deep-layer cortical progenitors express Otx1 at higher levels than do the progenitors of upper-layer neurons. We see no clear evidence of any cell cycle–dependent regulation of Otx1 mRNA expression within the ventricular zone, at least as assessed by the spatial patterns of hybridization: S-phase cell bodies are concentrated in the upper third of the ventricular zone (Fujita, 1963a), but Otx1 expression is observed throughout (Fig. 3).

Postnatal expression. Otx1 continues to be expressed specifically in neurons of the deep cortical layers 5 and 6 throughout postnatal life. Figure *4A* shows that at P2, when neurons begin populating the upper layers 2 and 3, hybridization is confined to the lower half of the cortical plate. Between P2 and P5 (Fig. *4A,B*), a prominent band of Otx1-expressing cells is apparent in layer 5; the level of hybridization appears to be somewhat weaker in layer 6. Cell counts in posterolateral regions of cortex show that at P13, 41% of neurons in layer 6 are labeled above background (≥ 6 grains/cell; $n = 51$), with an average of 13.1 grains/cell. In layer 5, 4.6% of all neurons are labeled ($n = 323$) with an average of 63.0 grains/cell, roughly a fivefold higher level of expression compared to layer 6.

Between P14 and adulthood (Fig. *4C,D*), hybridization is maintained in both layers 5 and 6, although the signal in layer 6 appears to weaken. A higher-power view of the adult rat cerebral cortex processed for Otx1 mRNA hybridization is shown in Figure *5A*. This shows clearly the lack of signal in layers 1–4 and the expression of Otx1 in what appears to be a subpopulation of neurons in layers 5 and 6; we have not identified the axonal projection patterns of Otx1-expressing neurons in either layer. These cells are shown in more detail in Figure 5, *B* and *C*. Layer 5 cells that express Otx1 have the large, pale nuclei characteristic of pyramidal neurons (Fig. *5B*); the small, dark nuclei of glial cells are unlabeled. In posterolateral cortical regions where layer 5 expression is strongest (see below), about 4.3% of layer 5 neurons express Otx1 message (≥ 6 grains/cell; $n = 394$), with an average of 97 grains/cell. The Otx1-expressing cells appear to be somewhat smaller and more superficially located within layer 5 than are those neurons that express another homeodomain gene, SCIP (Frantz et al., 1994). Within layer 6, Otx1 hybridization is found in a subpopulation of cells with medium-sized nuclei, typical of the polymorphic neurons of this layer, and not in cells presumed to be glial (Fig. *5C*). Roughly 27% of layer 6 neurons show clear hybridization above background levels (≥ 6 grains/cell; $n = 73$), with an average of 15.0 grains/cell; thus, Otx1 expression in layer 6 neurons is roughly 6.5-fold lower than in layer 5. Within layer 6, there was no obvious change in the average number of grains/cell between P13 and adulthood, but there was an overall decrease in the fraction of labeled neurons (from 41% to 27%). These data suggest that the apparent weakening of Otx1 hybridization in adults is attributable to a loss of Otx1-expressing neurons; however, we cannot determine whether this loss results from a downregulation of Otx1 by a subset of layer 6 neurons that survive into adulthood, or a loss of some Otx1-expressing cells through cell death.

The expression of Otx1 within layer 6 appears uniform throughout the postnatal rat neocortex (Fig. 6). In contrast, whether viewed in coronal, horizontal, or sagittal sections, the band of hybridization in layer 5 is most prominent in posterior and lateral cerebral cortex (Fig. *6A,D,E*). In anterior regions, Otx1 signal is absent from layer 5 of frontal, insular, and orbital cortices, even though layer 6 expression is maintained (Fig. *6B–E*). Thus, Otx1 expression shows regional heterogeneities across the areas of the adult cerebral cortex. These differences are also clearly visible during postnatal life (~P2–P5; Fig. *3A,B*). While no striking regional variations are evident at embryonic ages (Fig. *2B,D,F,H,J*), we do not know definitively whether Otx1 is expressed in layer 5 in all cortical areas during embryogenesis because layers 5 and 6 are not histologically distinguishable at these ages. By P2, thalamic afferents have already invaded the lower cortical plate (e.g., Wise and Jones, 1978; Catalano et al., 1991; Agmon et al., 1993); we therefore cannot distinguish whether area-specific patterns of Otx1 expression in layer 5 precede or follow thalamic invasion.

Otx1 and Otx2 expression in the cerebellum

Embryonic development. Otx1 and Otx2 mRNA can initially be detected in the cerebellar primordia of E17 animals in the "germinal trigone" region above the choroid plexus (not shown). By E19, Otx2 signal is apparent throughout the posterior cerebellum, within the external granular layer (EGL) and in the emerging internal granular layer (IGL) (Fig. *7B*). Figure *7A* shows that Otx1 expression at this age is restricted to the "germinal trigone," a region that has been suggested to be the source of external granular cells prior to their migration around the rhombic lip (Miale and Sidman, 1961; Altman and Bayer, 1978).

Postnatal development. By P5, spatially restricted patterns of Otx1 and Otx2 expression have emerged within the EGL. Figure *7C* reveals that Otx1 expression is found predominantly in anterior cerebellum and does not extend into the most posterior regions (note that not all the adult lobules have formed at P5). In contrast, Otx2 is expressed in posterior lobules in an apparent gradient that extends through the three most posterior lobules, with the highest level of expression seen at the most posterior extent of the cerebellum (Fig. *7D*). Thus, by P5 the expression of Otx1 and Otx2 defines three cerebellar domains: an anterior region in which only Otx1 is expressed, a middle domain in which hybridization for both genes is seen, and a posterior domain in which only Otx2 is expressed.

At P13, Otx1 and Otx2 mRNAs are expressed in distinct regions within the radial extent of the EGL and IGL: Otx1 mRNA is confined to the outer portion of the EGL, which contains mitotically active progenitor cells (Kuhar et al., 1993),

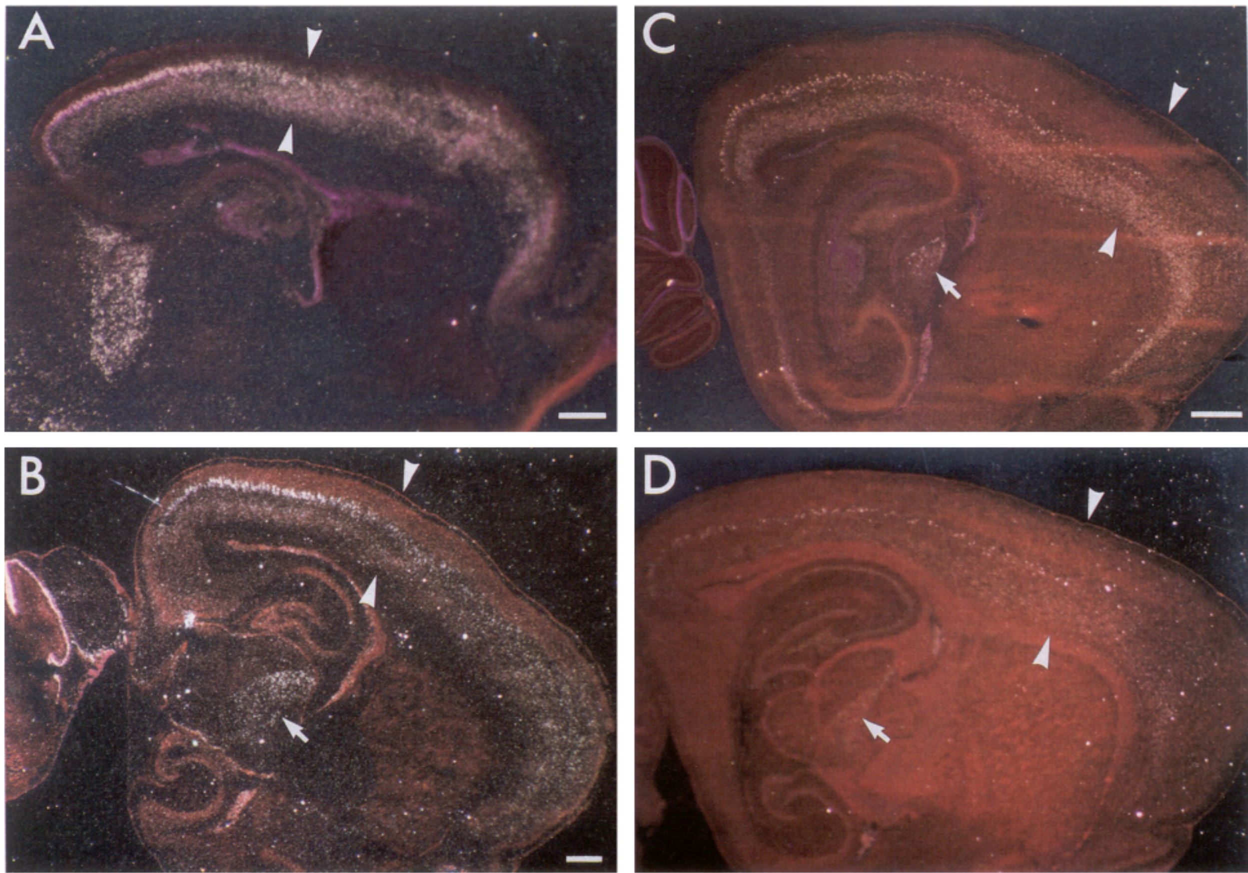

Figure 4. Regulation of Otx1 expression during postnatal development of the cerebral cortex. These are dark-field views of parasagittal sections counterstained with cresyl violet; anterior is to the right. In each panel the extent of the cerebral cortical layers is defined by *arrowheads*. In addition, the rostrocaudal position of the arrowheads denotes the anterior border of expression of Otx1 in layer 5 neurons. *A*, P2. Otx1 hybridization is apparent within deep layers of the cerebral cortex. At this age many upper-layer neurons have reached their destinations at the top of the cortical plate and are devoid of label. Intense signal appears over layer 5 cells in the posterior cortex (*bright band to left of arrowheads*), while layer 6 signal appears equivalent in posterior and anterior regions. *B*, P5. Otx1 signal is present throughout layer 6 and in layer 5 neurons in posterior regions. Note that upper cortical layers lack label, as do the striatum and the hippocampus. An *arrow* in this and subsequent panels identifies the ventral lateral geniculate nucleus, which shows hybridization. *C*, At P14, the patterns of Otx1 expression are similar to those seen at P5. *D*, Adult. Between P14 and adulthood, Otx1 signal appears to decrease in layer 6. As at earlier postnatal ages, Otx1 expression is absent from layer 5 neurons in frontal cortex (*to right of arrowheads*). Scale bars: *A* and *B*, 0.5 mm; *C*, 1.0 mm (for *D* also).

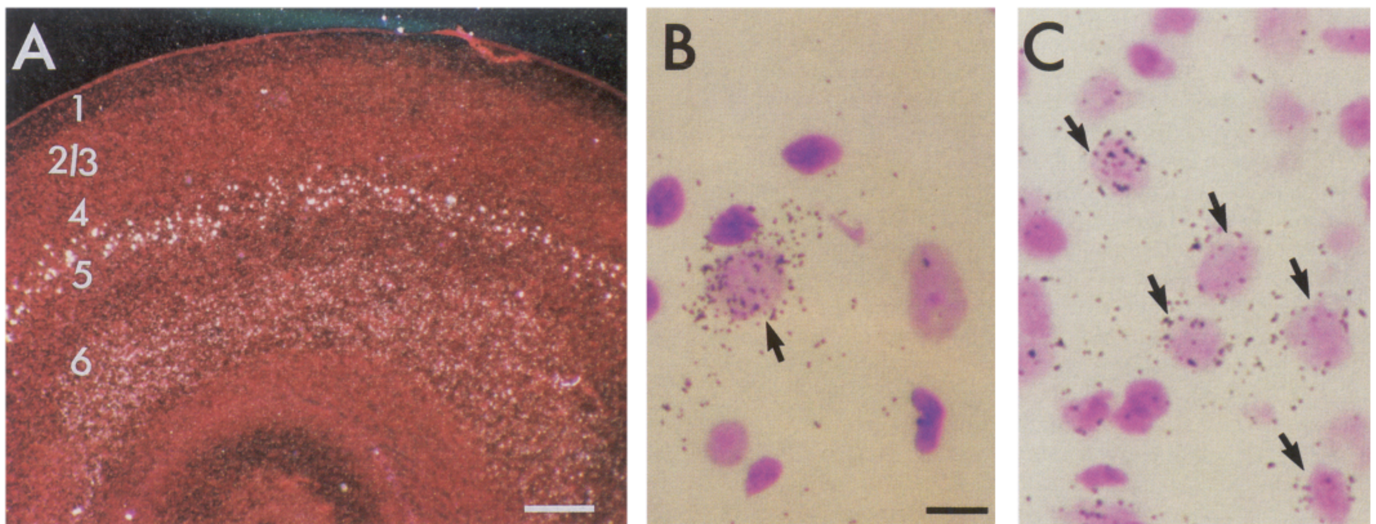

Figure 5. Higher-power views of Otx1 expression in layers 5 and 6 of the posterolateral cerebral cortex of the adult rat. *A*, Dark-field view of all cortical layers, showing intense hybridization in the superficial half of layer 5, and somewhat weaker hybridization in layer 6. There is no Otx1 expression above background in layers 1–4. *B*, Otx1 expression in layer 5 cells. An *arrow* identifies a cell heavily labeled for Otx1. This cell has the large, pale-staining nucleus characteristic of a pyramidal neuron. Note that a neighboring neuron is unlabeled (at right), as are the small dark somata of glial cells (above and below). *C*, Otx1 expression in cells of layer 6. A far higher fraction of neurons in layer 6 are labeled than in layer 5, but the intensity of hybridization per cell is lower. The *arrows* point out cells with medium-sized pale nuclei that show positive hybridization for Otx1. Scale bars: *A*, 0.5 mm; *B*, 0.01 mm (for *C* also).

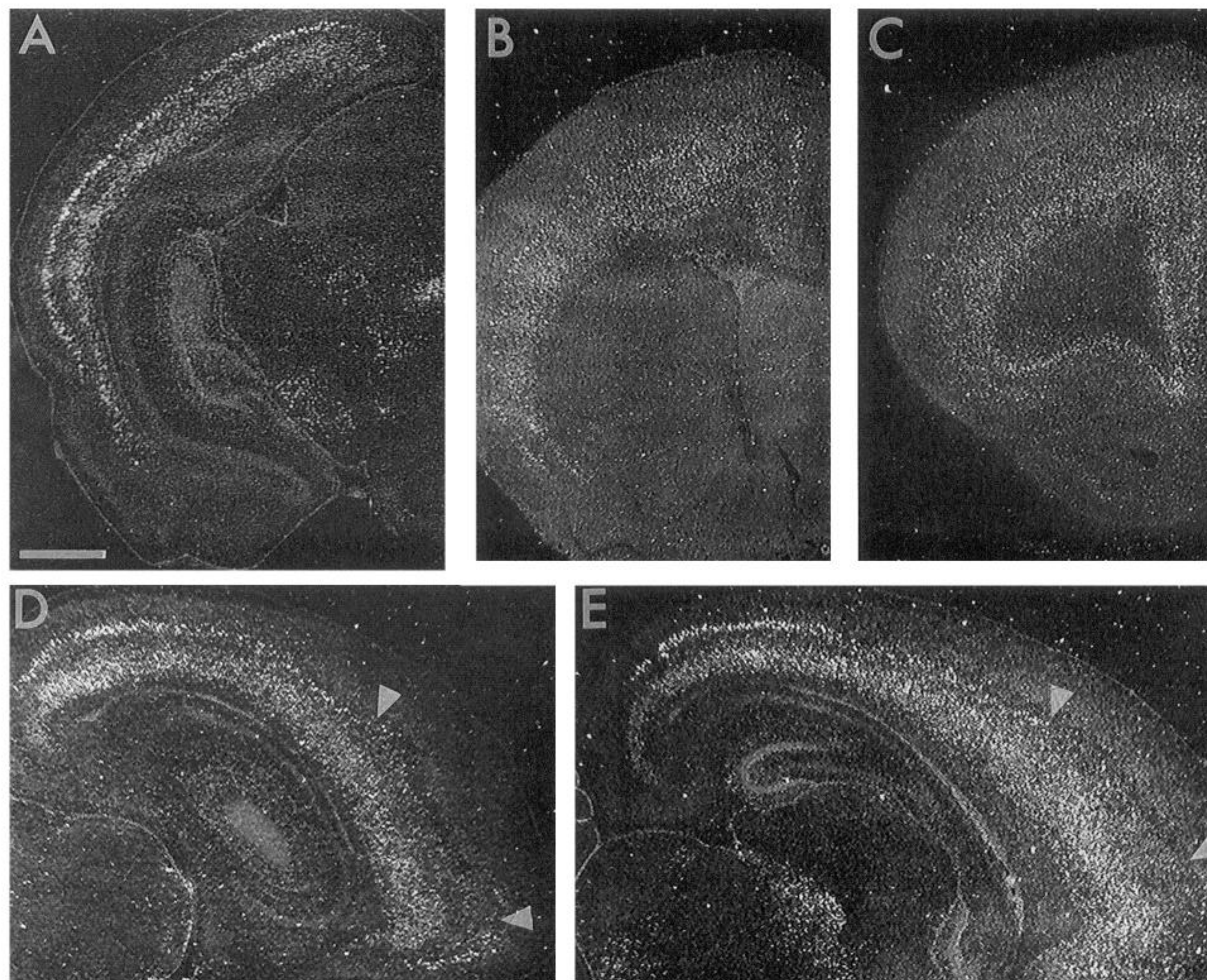

Figure 6. Otx1 is strongly expressed in a band of layer 5 cells in posterior and lateral cortical regions, but expression in layer 5 decreases in the frontal cortex. *A–C*, Coronal sections of P13 rat brains viewed in dark field; dorsal is up and medial is to the right. Each panel shows a progressively more anterior section. *A*, Otx1 expressing cells form a prominent band in layer 5 in posterior cortical regions (occipital and temporal cortices). *B* and *C*, Otx1 expression declines in layer 5 cells in anterior cortical regions (frontal and parietal cortices), but levels in layer 6 are similar to posterior regions. *D* and *E*, Horizontal sections of P13 rat brains viewed in dark field; medial is down and anterior is to the right. *D* is located more dorsally than *E*. The *arrowheads* delineate regions in anterior cortex in which layer 5 lacks Otx1 expression. A prominent band of hybridization in layer 5 is visible posterior to the *arrowheads* in both panels. Scale bar, 2.0 mm.

and is absent from differentiating granule neurons in the inner EGL and the IGL (Fig. 8*A*). In contrast, Otx2 is expressed by cells throughout both the EGL and IGL, suggesting that both granule neurons and their precursors express this gene (Fig. 8*B*).

In P11–P16 rat cerebella, Otx1 and Otx2 continue to show opposing gradients of expression within the tangential plane of the EGL, with Otx1 expression strongest in anterior lobules and Otx2 strongest in posterior regions. Otx1 expression appears to follow an anterior to posterior gradient. In Figure 9, *A, C,* and *E,* highest levels of Otx1 expression are visible in the outer portion of the EGL of lobules 1–6 of the vermis, and in the simple, paramedian, crus 1 and 2 ansiform lobules, and copula pyramis of the hemispheres. Somewhat weaker expression is seen in lobules 6–8 of the vermis. Very low levels of expression were observed in lobule 9 of the vermis and the paraflocculus; no hybridization was found in lobule 10 and the flocculus. No expression of Otx1 was observed in the adult rat cerebellum (not shown).

Otx2 was also expressed in a gradient, but this mRNA was found at the highest levels in the most posterior cerebellar regions and declined anteriorly (Fig. 9*B,D,F*). The strongest expression was found throughout the EGL and IGL of lobules 9 and 10 of the vermis, and in the flocculus and paraflocculus; moderate levels of expression were found in lobules 6–8 of the vermis, and in the paramedian, crus 1 and 2 ansiform lobules, and copula pyramis of the hemispheres (Fig. 9*B,D*). No hybridization was observed in lobules 1–5 or the simple lobule. Gradients of Otx2 expression were detected within the IGL even within a given lobule, with posterior/ventral regions of the lobule showing stronger signal than more anterior/dorsal regions (Fig. 9*B,F*). In addition, Otx2 signal was present at moderate levels in the posterior half of lobule 6 but hybridization was absent from the anterior portion; likewise expression was observed only in the posterior region of the crus 1 ansiform lobule. Expression of Otx2 is retained in adult animals within those regions of the IGL that show postnatal hybridization (not shown).

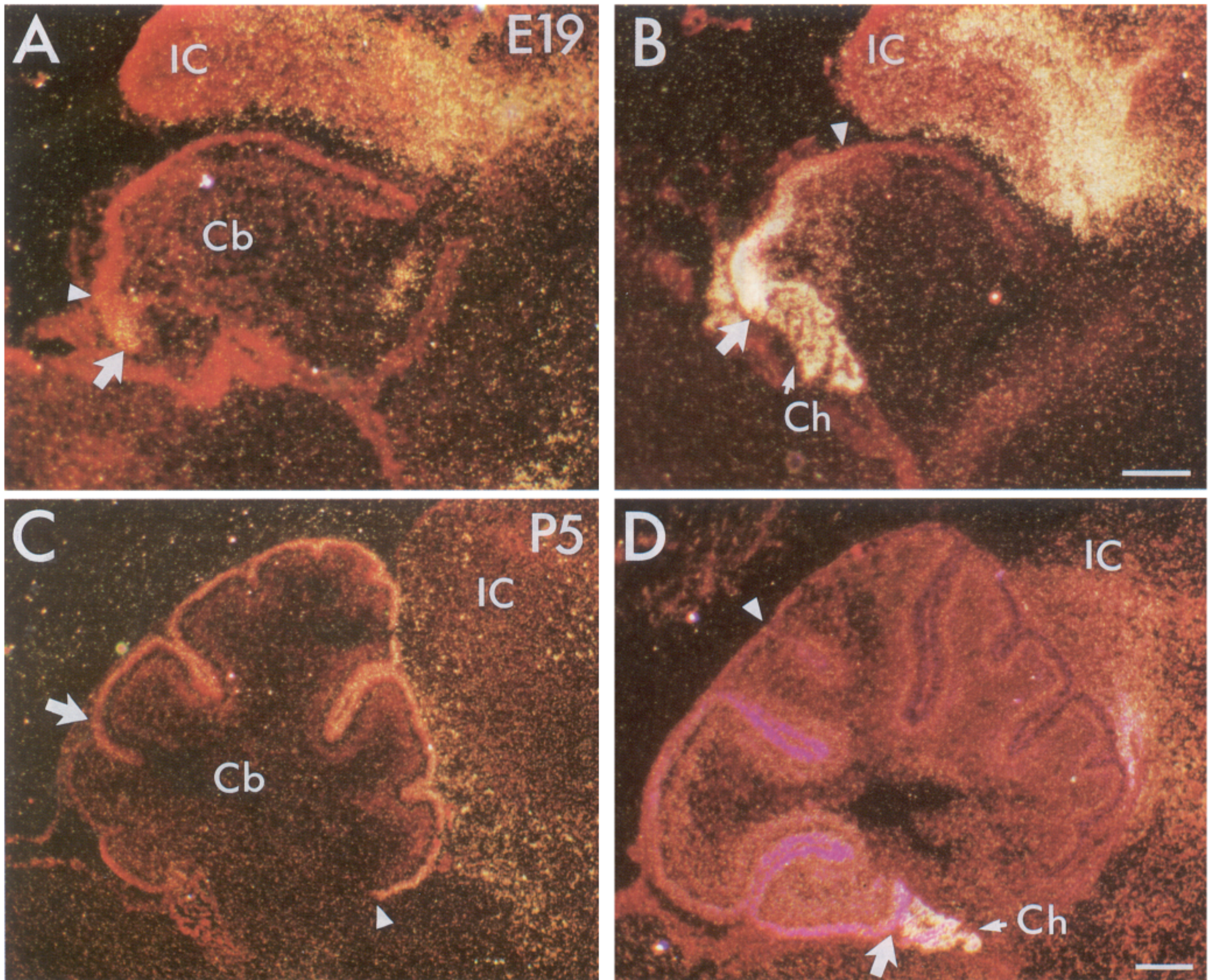

Figure 7. Otx1 and Otx2 expression in the developing cerebellum. These are dark-field views of parasagittal sections counterstained with cresyl violet; anterior is to the right. In each panel, the *large arrow* indicates the posteriormost extent of gene expression and the *arrowhead* delineates the anteriormost extent of gene expression. *A*, Otx1 in the cerebellum (*Cb*) of an E19 rat. Note that at this age Otx1 is expressed in the posterior cerebellum, within the region defined by Otx2 expression in *B*. *B*, Otx2 in the cerebellum of an E19 rat. High levels of Otx2 message are detected in the choroid plexus (*Ch, small arrow*) of the fourth ventricle and in the "germinal trigone" immediately overlying it. *C*, Otx1 expression in the cerebellum of the P5 rat. Otx1 hybridization is in the EGL of all but the most posterior lobules. *D*, Otx2 expression in the cerebellum of the P5 rat. High levels of Otx2 are expressed in the EGL and IGL of the most posterior lobules, but hybridization is decreased in anterior regions. *IC*, inferior colliculus. Scale bars: *B*, 0.2 mm for *A* and *B*; *D*, 0.3 mm for *C* and *D*.

Cloning of a cDNA encoding the rat Otx1 gene

All of the *in situ* hybridization results reported above were obtained using mouse probes to Otx1 and Otx2 (Simeone et al., 1992a, 1993) on rat tissue. While the high degree of conservation between the rat and human genes suggests that each probe should specifically recognize its appropriate rat homolog, we were interested in cloning the rat Otx1 gene, both to confirm the expected similarity and to obtain a full-length cDNA for future studies of cerebral cortical development. The rat Otx1 gene was cloned by screening a rat brain cDNA library (Stratagene) with a probe to the third exon of the mouse Otx1 gene (kindly provided by Dr. A. Simeone). We obtained and sequenced a 1.7 kb cDNA that encodes the complete amino acid sequence of the rat Otx1 gene (Fig. 10) and shows a 98.6% identity to the mouse sequence at the amino acid level (GenBank access number L32602). Within the homeodomain itself, the nucleotide sequences are 98% identical and the amino acid sequences are completely conserved. Within the region of the mouse probes used for *in situ* hybridization, the rat and mouse cDNAs are 94.4% identical at the nucleotide level, thus supporting our expectation that the mouse probes accurately reveal the distribution of the rat Otx1 gene; comparison of the rat and mouse probes by in situ hybridization on developing and adult rat brain reveals identical patterns of Otx1 expression (not shown). A single prominent mRNA transcript for Otx1 is detectable by Northern blot analysis of E20 rat embryos (Fig. 11); the transcript has a size of about 2.9 kb. The published size of Otx1 in the mouse is 3.2 kb (Simeone et al., 1993) for comparison.

Discussion

We have explored the embryonic and postnatal patterns of expression of two vertebrate homologs of the *Drosophila orthodenticle* gene, Otx1 and Otx2, originally cloned by Simeone et

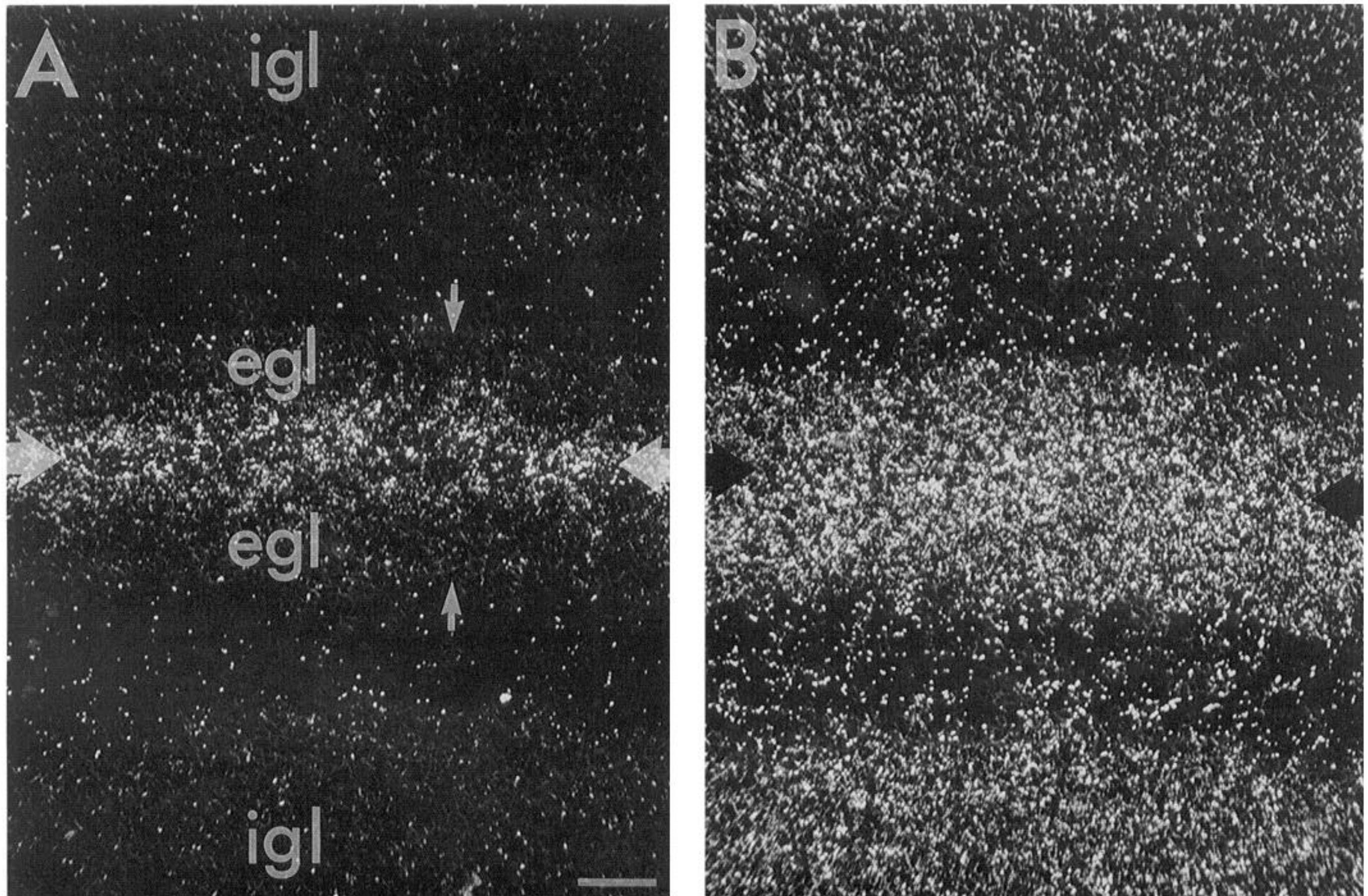

Figure 8. High-power views of Otx1 and Otx2 expression in the P13 cerebellum showing two adjacent lobules, 7 and 8. *Large arrows* indicate the outermost (pial) edges of the adjoining external granular layers (*egl*) of each lobule. *Small arrows* delineate the inner extent of each EGL. *A*, Otx1 is expressed only in cells of the outer (pial) portion of the EGL, which contains proliferating precursors; there is no hybridization above background levels in either the inner EGL or the internal granular layer (*igl*), which contain postmitotic granule neurons. *B*, Otx2 is expressed throughout the entire radial extent of the EGL and within the IGL. Scale bar, 0.06 mm.

al. (1992a, 1993). As previously reported (Simeone et al., 1992a, 1993), Otx1 and Otx2 are expressed in roughly complementary patterns throughout the forebrain and midbrain during development. In addition, however, we have found that Otx mRNAs are also expressed in discrete spatial and temporal domains within the developing cerebral cortex and cerebellum, two CNS structures that are composed of both layers and functionally distinct areas or regions. Within the adult rat cerebral cortex, hybridization for Otx1 mRNA is confined to a subpopulation of neurons in layers 5 and 6. During development, Otx1 expression is high in the progenitors of these deep-layer cells, early in cortical neurogenesis; by the time that upper-layer neurons are being born, the ventricular zone has apparently downregulated Otx1 expression. These findings suggest that Otx1 may play a role in the specification or differentiation of deep-layer neuronal phenotypes. Within the developing cerebellum, Otx1 and Otx2 are expressed in spatially restricted regions of the EGL; each gene is found in a rough gradient, from anterior to posterior in the case of Otx1, and from posterior to anterior in the case of Otx2. These expression patterns define three domains within the cerebellum: an anterior region in which only Otx1 is expressed at high levels, a middle region in which both Otx1 and Otx2 mRNAs are present, and a posterior region in which only Otx2 hybridization is apparent. These domains appear to correspond roughly to the three functional domains of the cerebellum: the spinocerebellum, pontocerebellum, and vestibulocerebellum, respectively (Brodal, 1981; Carpenter, 1985). Notably, spatially restricted patterns of Otx expression emerge at a time that corresponds to the invasion of these three regions by distinct sets of mossy fiber afferents (Arsénio-Nunes and Sotelo, 1985; Mason, 1987). These observations raise the possibility that Otx1 and Otx2 play a role in setting up the functional subdivisions of the cerebellar anlage.

Expression of Otx1 in the developing layers of the cerebral cortex

Otx1 expression within the cerebral cortex of postnatal and adult rats is restricted to a subset of neurons within layers 5 and 6, with the highest levels in medium- to large-sized layer 5 cells. No expression is detectable in glia. We do not know whether the subpopulations of neurons that express Otx1 are characterized by common features such as neurotransmitter content or axonal projection pattern. However, in previous studies we have defined a subpopulation of layer 5 neurons that express mRNA for the POU-homeodomain gene SCIP: these very large neurons form predominantly subcortical axonal projections and are located in the upper two-thirds of layer 5 (Koester and O'Leary, 1992; Frantz et al., 1994). In contrast, Otx1-expressing neurons

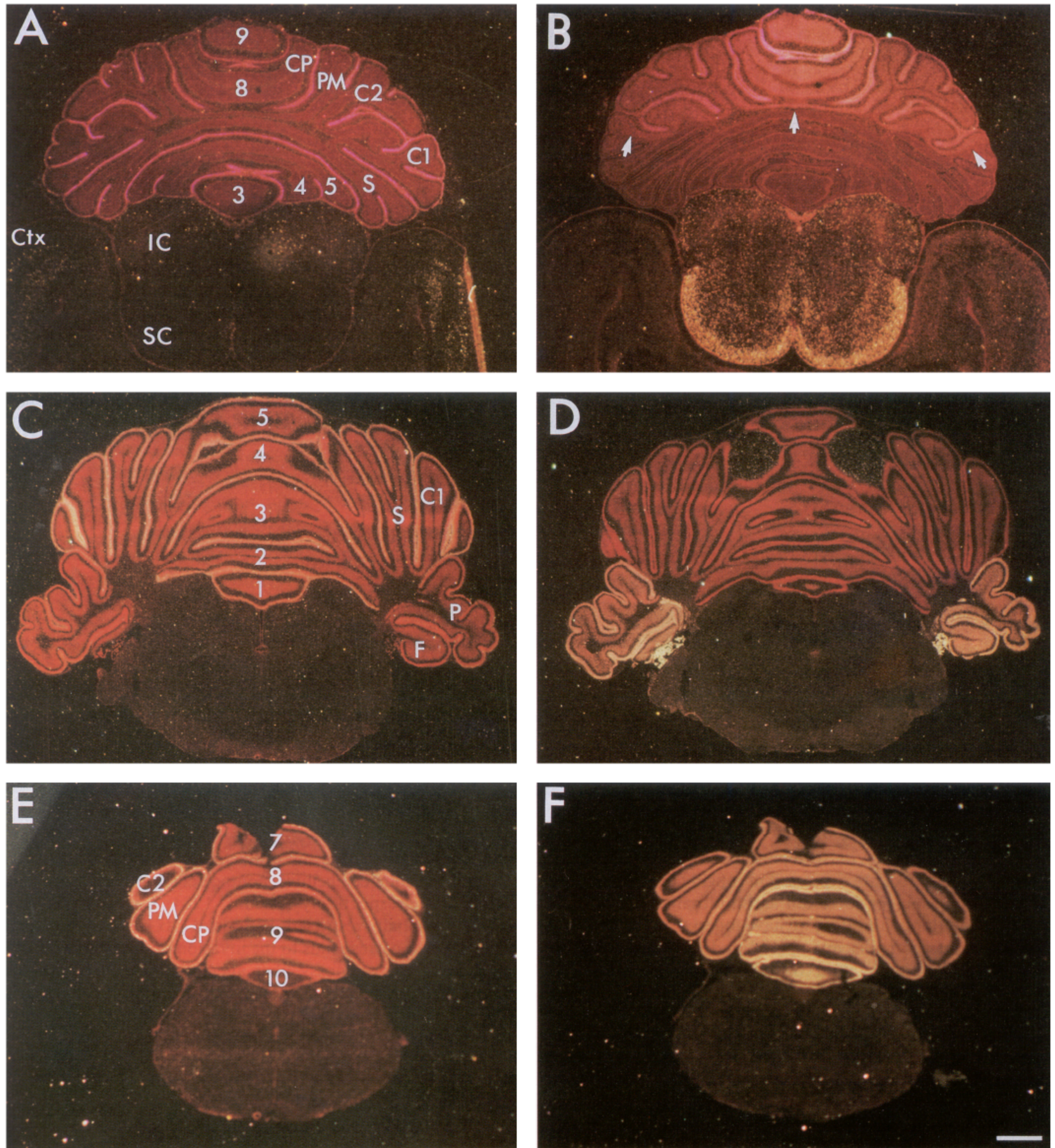

Figure 9. Otx1 and Otx2 expression in the P13 cerebellum. *A* and *B*, Horizontal sections counterstained with cresyl violet and viewed in dark field; posterior is up. *A*, Otx1 mRNA is expressed within the EGL of anterior cerebellar lobules; expression is weak in lobule 8 and only very low levels of expression are detectable in lobule 9. *B*, Otx2 expression is confined to the EGL and IGL of posterior regions, with the highest levels of expression visible in the most the posterior lobules. *Arrows* delineate the anteriormost boundary of Otx2 expression. Hybridization is absent from lobules 3–5 and the simple lobule (*S*) in this section. *C* and *D*, Coronal sections through anterior cerebellum; dorsal is up. *C*, Within anterior cerebellum, Otx1 is present within the EGL of all regions shown, with the exception of the flocculus (*F*), which lacks expression. Hybridization in the paraflocculus (*P*) is present but very weak. *D*, In this view of anterior cerebellum, hybridization for Otx2 is observed only in the flocculus and paraflocculus; no expression is seen in lobules 1–5 or the simple lobule. *E* and *F*, Coronal sections through posterior cerebellum. *E*, Otx1 is expressed weakly in lobules 7 and 8, at very low levels in lobule 9, and is absent from lobule 10. *F*, Otx2 expression is apparent in all regions shown of the posterior cerebellum, including lobules 7–10, the crus 2 ansiform lobule, the paramedian lobule, and the copula pyramis. *1–10*, vermal lobules 1–10; *S*, simple lobule; *C1*, crus 1 ansiform lobule; *C2*, crus 2 ansiform lobule; *P*, paraflocculus; *F*, flocculus; *CP*, copula pyramis; *PM*, paramedian lobule. Scale bar, 1.0 mm.

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rat     1 MMSYLKQPPYGMNGLGLAGPAMDLLHPSVGYPATPRKQRRERTTFTRSQL 50
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mouse     MMSYLKQPPYGMNGLGLAGPAMDLLHPSVGYPATPRKQRRERTTFTRSQL

       51 DVLEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQQQSG 100
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          DVLEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQQQSG

      101 NGTKSRPVKKKSSPVRESSGSESSGQFTPPAVSSSASSSSSASSASANPA 150
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          NGTKTRPVKKKSSPVRESSGSESSGQFTPPAVSSSASSSSSASSASANPA

      151 AAAAAGLGGNPVAAASSLSTPTASSIWSPASISPGSAPASVSVPEPLGAP 200
          |||||||||||||||||||||||||||||||||||||| |||||||| ||
          AAAAAGLGGNPVAAASSLSTPTASSIWSPASISPGSAPTSVSVPEPLAAP

      201 SNASCMQRSVAAGAATAATSYPMSYGQGRSYGQGYPAPSSSYFGGVDCSS 250
          ||||||||||||||||| |||||||||| |||||||||||||||||||||
          SNASCMQRSVAAGAATAAASYPMSYGQGGSYGQGYPAPSSSYFGGVDCSS

      251 YLAPMHSHHHPHQLSPMAPSSMAGHHHHHPHAHHPLSQSSGHHHHHHHHH 300
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          YLAPMHSHHHPHQLSPMAPSSMAGHHHHHPHAHHPLSQSSGHHHHHHHHH

      301 HHQGYGGSGLAFNSADCLDYKEPAAAAASSAWKLNFNSPDCLDYKDQASW 350
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          HHQGYGGSGLAFNSADCLDYKEPAAAAASSAWKLNFNSPDCLDYKDQASW

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Figure 10. Deduced amino acid sequence of the rat Otx1 gene product, compared with that previously published for the mouse (Simeone et al., 1993). The two aligned sequences are shown using single-letter codes for each amino acid; *lines between letters* indicate identical residues. The Otx1 homeodomain is encoded by amino acids in positions 38–98 (Simeone et al., 1993). The rat Otx1 sequence shows a 98.6% identity to the mouse sequence at the amino acid level; the full mouse nucleotide sequence is not available for comparison.

Figure 11. Northern blot analysis of Otx1 mRNA expressed in the developing rat cerebral cortex. PolyA+ mRNA was prepared from E20 cortex. The positions of RNA size markers are shown at *right*. The Otx1 mRNA transcript has a size of about 2.9 kb. Northern analysis of mRNA from E14, E16, E18, and adult cortex showed a similar transcript size (not shown).

in layer 5 are somewhat smaller in size and are found in regions of layer 5 that do not contain SCIP-expressing neurons, consistent with the possibility that these cells are a distinct subset of neurons that might preferentially form cortical axonal connections. This possibility remains to be examined by combining retrograde axonal tracing with *in situ* hybridization.

In rat, neurons of the deepest cortical layers 5 and 6 are generated from E14 to E16 (layer 6) and from E15 to E17 (layer 5); upper-layer neurogenesis peaks at E18–E19 and shuts down on about E20 (Berry and Rogers, 1965; Hicks and D'Amato, 1968; Miller, 1985; Frantz et al., 1994). Expression of Otx1 mRNA in ventricular zone cells is high between E13 and E17, but levels are markedly lower late in neurogenesis, particularly between E18 and E20. Thus, the expression of Otx1 in cortical precursors is correlated temporally with the production of the neurons that continue to express Otx1 in adult life. Deep-layer neurons appear to continue to express Otx1 during their migration through the intermediate zone, and levels of hybridization are strong once cells enter the cortical plate, although there is some suggestion that neurons in layer 6 may downregulate this gene product to lower levels during postnatal development. We observed no Otx1 expression in cells of layers 1–4 during their migration or differentiation. These results suggest that Otx1 mRNA is found specifically in deep-layer neurons and their progenitors, and raises the possibility that Otx1 may play a role in the generation or differentiation of layers 5 and 6.

The presence of high levels of Otx1 mRNA in the ventricular zone during the time of deep-layer neurogenesis is particularly tantalizing given the timing of commitment decisions that young neurons make to deep-layer fates. Transplantation studies of the developing cerebral wall have revealed that deep-layer progenitor cells are multipotent if transplanted during S phase of the cell cycle: their daughters, born in an older environment, take on novel fates and migrate to the upper cortical layers, along with newly generated host neurons (McConnell and Kaznowski, 1991). Thus, environmental cues can exert a determinative effect on laminar phenotypes. The multipotency of progenitors, however, is lost as cells progress through the cell cycle: precursors transplanted late in the cell cycle produced daughters that migrated specifically to layer 6, appropriately for their birthday (McConnell and Kaznowski, 1991). Laminar commitment therefore occurs prior to the mitotic division that generates a postmitotic cortical neuron. These findings prompt the question of whether Otx1 expression in the ventricular zone might be correlated with the acquisition of commitments to a deep-layer fate. Were this the case, one might predict that Otx1 would be expressed preferentially in the deepest two-thirds of the ventricular zone, in which cell bodies in the G2 and M phases of the cell cycle are found (Fujita, 1963b), and that Otx1 would not be expressed in the upper third of the ventricular zone, in

which the somata of multipotent S-phase progenitors are localized. This does not, however, appear to be the case: although it does not seem that Otx1 is expressed in every ventricular cell (see Fig. 3), the majority of cells appear to be positive, and hybridization is distributed throughout the ventricular zone. Thus, there is no overt evidence for regulation of Otx1 transcript in a cell cycle–dependent manner, although it remains possible that Otx1 is regulated posttranscriptionally.

Developmental potential of early and late cerebral ventricular zone cells

The patterns of Otx1 expression within the cerebral wall raise two interesting possibilities: first, that deep-layer precursors may be intrinsically biased to produce deep-layer neurons, and second, that early and late progenitor cells may have distinct developmental potentials that are related to their molecular differences. Although the transplantation experiments discussed above have provided evidence that early ventricular cells are multipotent, the expression of Otx1 by early progenitors raises the possibility that, in the absence of environmental instructions to the contrary, these cells are biased to generate deep-layer neurons—in other words, deep-layer fates may constitute a default pathway for neurons born early in cortical development. To address this hypothesis, one must ascertain the developmental outcome of neurons that have been deprived of environmental cues or cell–cell interactions during periods in which phenotypic decisions are made. We do not know whether early progenitors will generate deep-layer neurons in the complete absence of cell–cell interactions (as judged, e.g., by maintaining cells in low-density monolayer cultures in which neither cell contacts nor diffusible factors can affect the differentiation of multipotent cells); thus, it remains a plausible hypothesis that early Otx1 expression biases early-generated cells toward a deep-layer differentiation pathway.

With regard to the possibility that early and late progenitor cells may have distinct developmental potentials, the present study reveals that the molecular characteristics of ventricular cells change over the time of neurogenesis, with early progenitors expressing Otx1 at higher levels than late cells. Transplantation experiments have generally supported the hypothesis that the layered patterning of cerebral cortical neurons arises through a progressive specification of cell types, presumably accomplished through changes in environmental cues over time in development. It should be noted, however, that these experiments have not ruled out intrinsic changes in cortical progenitors over time, since we have so far only examined directly the developmental potential of early precursor cells (McConnell, 1988b; McConnell and Kaznowski, 1991) that are normally fated to produce cells of multiple cortical layers (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988; Austin and Cepko, 1989, 1990). It remains possible that late progenitors, which normally produce only upper-layer neurons, may have a more restricted developmental potential than early cells. Such a result would imply that the competence of ventricular cells to respond to environmental signals changes over time in development. A classic example of such a change in competence has been documented for the animal cap ectoderm of the *Xenopus* embryo: early animal cap cells respond to mesoderm-inducing factors (such as fibroblast growth factor and activin; Kimelman and Kirschner, 1987; Smith et al., 1989; Albano et al., 1990) by differentiating into mesodermal structures. At later times in development, however, these cells lose their competence to respond to mesoderm-inducing peptide growth factors and instead gain the ability to respond to neural inducers (Gurdon et al., 1985; Gurdon, 1987). A test of whether upper-layer progenitors have maintained or lost the potential to produce deep-layer neurons will entail the transplantation of late precursors into an earlier embryonic environment.

Expression of Otx1 and Otx2 in the developing cerebellum

Both vertebrate orthodenticle homologs, Otx1 and Otx2, are expressed in distinct patterns within the developing rat cerebellum. Neither gene is expressed by cells of the cerebellar ventricular zone, the source of most cerebellar cell types in development (Hallonet et al., 1990). Instead, both Otx1 and Otx2 are expressed within the EGL, a second proliferative region at the pial surface that is responsible for the production of granule neurons (Rakic, 1971; Altman and Bayer, 1978; Hallonet et al., 1990). Analysis of cell fates in chick-quail chimeras has suggested that during normal development, precursors in the EGL are restricted to the production of granule neurons whereas ventricular zone cells produce the remainder of cerebellar phenotypes (Hallonet et al., 1990). In support of this view, purified EGL cells produce only granule neurons both *in vitro* (Gao et al., 1991) and when transplanted back into the EGL of host mice (Gao and Hatten, 1994), whereas cells derived from the embryonic cerebellar anlage will produce all cerebellar phenotypes when transplanted into the EGL (Gao and Hatten, 1994). The expression of Otx1 and Otx2 in the EGL but not in the cerebellar ventricular zone provides molecular evidence that these two progenitor populations are indeed distinct, and adds two more genes to a growing list of mRNAs that are expressed by granule neurons and their precursors during distinct windows of development (Kuhar et al., 1993).

Molecular domains and the formation of functional regions in cerebellum

Within the cerebellum, we identified three spatially distinct populations of granule cell progenitors: those expressing only Otx1 (lobules 1–5, simple lobule), those expressing moderate to low levels of Otx1 and moderate to high levels of Otx2 (lobules 6–9, paramedian, copula pyramis, crus 1 and 2 ansiform lobules, paraflocculus), and those expressing no Otx1 and high levels of Otx2 (lobule 10 and flocculus). Two fissures, the primary and the posterolateral, roughly delineate these three populations. Interestingly, these fissures also subdivide the cerebellum into three functionally distinct lobes, the anterior, posterior, and flocculonodular lobes, respectively, which are thought to have emerged at different times during evolution (Ghez, 1991). The predominant inputs to the IGL in each region are distinct and correspond to the functions that have been ascribed to each lobe (Brodal, 1981; Carpenter, 1985): the anterior lobe receives the majority of its afferents from the spinal cord; the posterior lobe receives inputs from the spinal cord, auditory, and visual centers and from the pontine nuclei; finally, the flocculonodular lobe or vestibulocerebellum receives its input from the vestibular nuclei and projects back to the vestibular nuclei to regulate balance and eye movements (Ghez, 1991). All three sets of afferents are known as mossy fibers; they synapse on granule cell dendrites within structures called glomeruli. The three major cerebellar subdivisions can thus be identified anatomically, phylogenetically, and by their differing afferent inputs. Here we have shown that the patterns of Otx1 and Otx2 expression appear to correspond closely to these three subdivisions.

Spatially distinct patterns of Otx1 and Otx2 hybridization are apparent within the cerebellum in early postnatal life, with three domains of expression apparent by P5. The timing of the formation of these molecular domains is tantalizing with respect to the formation of afferent connections within each of the three functional domains. Mossy fibers normally grow into the cerebellar anlage by about P0, but their progress is arrested in the white matter until P3 (Arsénio-Nunes and Sotelo, 1985). The ingrowth of mossy fibers into the IGL of the cerebellar cortex takes place over the first 2 postnatal weeks in rodents (Arsénio-Nunes and Sotelo, 1985; Mason, 1987). Although the absence of postmitotic granule neurons at late embryonic and early postnatal ages has suggested that the granule cells themselves cannot attract mossy fibers into the cerebellum (Arsénio-Nunes and Sotelo, 1985), it is possible that granule cell precursors in the EGL play a role in regulating the invasion of mossy fibers into functionally appropriate cerebellar regions. The expression patterns of Otx1 and Otx2 raise the intriguing possibility that these genes might specify regional distinctions within the EGL, which in turn direct specific mossy fiber afferents to distinct functional domains of the cerebellum. Alternatively, the parcellation of Otx1 and Otx2 expression within the cerebellum may occur in response to preexisting regional signals, which might also regulate the ingrowth of mossy fibers.

Cerebellar mutations affecting the development of the anterior cerebellum

Recent studies in mutant mice and in the developing avian cerebellum reveal that the cerebellum consists of two developmentally distinct regions, composed of an anterior and a posterior domain. Transplantation studies in quail-chick chimeras have revealed that the rostromedial cerebellum derives from the mesencephalon whereas the posterior cerebellum derives from the metencephalon, with the exception of the EGL, which derives solely from the rostral metencephalon (Martinez and Alvarado-Mallart, 1989; Hallonet and LeDouarin, 1993; Otero et al., 1993). Several mutations in mice, including *swaying*, *leaner*, and *meander tail*, preferentially affect anterior but not posterior cerebellar regions. The *meander tail* (*mea*) mutation, for example, results in abnormal foliation and cytoarchitecture in folia I–V, leading Ross et al. (1990) to propose that the *mea* mutation reveals a compartmental structure within the developing cerebellum, and others to suggest that *mea* affects only those mesencephalic derivatives that contribute to the anterior cerebellum (Napieralski and Eisenman, 1993). In *swaying* mice, a mutation resulting from a frameshift mutation in the protooncogene *Wnt-1*, the development of the cerebellum anterior to the primary fissure fails completely, while posterior regions appear normal (Thomas et al., 1991). Interestingly, in the *swaying*, *leaner* and *meander tail* mutants, the demarcation between posterior (unaffected) and anterior (affected) regions corresponds closely to the anterior boundary of Otx2 expression (within the declive); cells in the EGL anterior to this boundary (folia I–V) express only Otx1. These findings raise the question of whether Otx1 and/or Otx2 might be involved in setting up the domains that are subsequently affected by mutations such as *meander tail* and *swaying*. The observations in mutant mice generally reinforce the suggestion that the anterior cerebellum may form a compartment or domain that is developmentally distinct from posterior cerebellum, and lend credence to the possibility that Otx1 and Otx2 may play a role in the very early parcellation of the cerebellum into functionally distinct domains. These hypotheses remain to be tested by altering the expression of Otx1 and Otx2 during cerebellar development.

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