Transient Calbindin-D_{28K}-Positive Systems in the Telencephalon: Ganglionic Eminence, Developing Striatum and Cerebral Cortex

Fu-Chin Liu and Ann M. Graybiel

Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Calbindin-D_{28K} (calbindin) is a member of the superfamily of calcium-binding proteins implicated in the regulation of intracellular calcium. In the mature brain, calbindin is widely expressed in neurons of the forebrain and the hindbrain, and in the telencephalon calbindin-like immunoreactivity is particularly strongly expressed by medium-sized neurons of the striatum and by certain other neurons in the cortex and subcortex. We have traced the development of calbindin expression in the forebrain of the rat, and report here that in addition to the steady development of these calbindin-positive neuronal systems, transient waves of calbindin expression occur in cells of the ventricular zones of the basal ganglia and cortex and in cells of the telencephalic regions derived from these ventricular zones including radial glia of the developing striatum.

In the striatum and its ventricular zone (the ganglionic eminence, or GE) we identified four transient calbindin-positive systems in the perinatal period. First, calbindin-immunoreactive cells began to appear in the GE by embryonic day (E)18, and by E20 an extensive dorsal and lateral part of the GE was marked by dense calbindin-like immunoreactivity in the ventricular zone. This calbindin system peaked at postnatal day (P)0–P3 and disappeared by P15. Its presence suggests that the GE is divisible on a molecular basis into lateral and medial districts that may correspond to derivatives of the lateral and medial ventricular ridges.

Second, a system of calbindin-positive processes appeared in the dorsal and lateral caudoputamen with temporal and spatial distributions matching the germinal zone system. Many of these processes could be traced from calbindin-positive cells in the ventricular zone of the GE, including processes stretching across the full width of the dorsal caudoputamen. Double-staining experiments demonstrated that these radial processes were Rat.401-positive, suggesting that they form a subset of radial glia in the developing telencephalon. These findings demonstrate that during devel-

opment calbindin is expressed in glial as well as neural cells. They further suggest that the radial glia associated with the GE form heterogeneous populations, the transient calbindin-positive radial glia being associated with the lateral ridge of the GE and its derivatives.

Third, a scattered population of calbindin-positive cells with morphologies different from the common medium-sized calbindin-immunoreactive neurons of the striatum appeared in the dorsal and lateral striatum from about E20 to P15. Some of these cells were close to the transient calbindin-positive radial processes in the same region, but others were not. Often they formed small clusters. The absence of such cells at maturity suggests that the developing dorsolateral striatum contains a population of cells that either migrate through the region, change phenotype, or undergo cell death.

Fourth, from about P0, a system of transient calbindin-positive neuropil patches appeared, then became very prominent by P3, and finally disappeared by P15. These transient patches were in spatial register with tyrosine hydroxylase-positive “dopamine islands,” forerunners of the developing striosomal system of the striatum. They were frequently, but not invariably, associated with small groups of the transient calbindin-immunoreactive cells. Thus, with respect to the neurochemical striosome/matrix compartments of the striatum, calbindin-positive systems of two sorts are present during development: a transient one associated with dorsal striosomes, and a permanent one associated with the matrix.

In the developing cortical primordium and its ventricular zone, calbindin was expressed with a prominent gradient in which the strongest early expression was dorsal and medial. Calbindin-positive cells first appeared in the ventricular zone and then appeared in the single-cell thick plexiform plate. This plate subsequently split into calbindin-positive subplate and marginal zones, known to contain transient cell populations. These bands could be followed as distinct calbindin-enriched layers into the early postnatal period as calbindin-positive neurons began to inhabit the developing cortical layers in between. Thus, in the cerebral cortex, as in the developing striatum, early waves of calbindin expression in the ventricular zone are followed by the emergence of transient calbindin-immunoreactive systems that ultimately give way to the permanent calbindin-positive neuronal systems of maturity.

We conclude that the early calbindin-positive telencephalic systems identified here could participate in phases of cell proliferation, migration, and differentiation as well as in the early development of compartments in the striatum and layers in the cerebral cortex. Given the calcium-binding

Received May 9, 1991; revised Sept. 24, 1991; accepted Sept. 27, 1991.

This work was funded by NSF Grant BNS-8720475. We are grateful to Drs. P. C. Emson, C. R. Gerfen, and S. Christakos for their generous gifts of calbindin-D_{28K} antiserum, Dr. S. Christakos for calbindin-D_{28K} protein, and Drs. R. D. G. McKay, E. L. Gustafson, W. D. Matthew, and M. B. Kennedy for their generous donations of antisera against nestin (Rat.401), DARPP-32, synaptic vesicle-associated protein (SV48), and α -subunit of calcium/calmodulin-dependent protein kinase type II (6G9), respectively. We thank D. Major and A. Rosales for their help with the histology, and H. F. Hall for help with the photography. F.-C.L. was supported by the Markey fellowship and by the Cerebral Palsy Research Fund.

Correspondence should be addressed to Dr. Fu-Chin Liu, Massachusetts Institute of Technology, Department of Brain and Cognitive Sciences, E25-618, 45 Carleton Street, Cambridge, MA 02139.

Copyright © 1992 Society for Neuroscience 0270-6474/92/120674-17\$05.00/0

capacity of calbindin, it is possible that such functional participation involves the control and redistribution of calcium ions.

Calcium ions act as the targets of second messenger systems in neurons and as first and second messengers themselves (Rubin et al., 1985). They participate in critical biochemical events in mature neurons including signal transduction, neurotransmitter synthesis and release, ion channel opening, and activation of kinases, proteases, and other enzymes (for review, see Evered and Whelan, 1986; Hidaka et al., 1988). The regulation of intracellular calcium levels is thus essential for normal cellular function in the nervous system, and indeed, prolonged increases in Ca^{2+} have been implicated in cell death in the nervous system (Mayer and Westbrook, 1987; Choi, 1988). Calcium ions also play important roles in the molecular events underlying neural development such as cell proliferation, migration, outgrowth of growth cones, and cellular differentiation (Walaas and Nairn, 1985; Takeichi, 1988; Rasmussen and Means, 1989; Kater and Mills, 1991).

Little is yet known about how calcium ions are regulated during maturation of nervous system, but a natural approach to this problem is to trace the developmental expression of members of the superfamily of calcium-binding proteins, candidate molecules for control of intracellular calcium levels at adulthood (for review, see Welsh, 1988; Rogers, 1989). Many of these calcium-binding proteins, which include calbindin- $\text{D}_{28\text{K}}$ (calbindin), calmodulin, oncomodulin, parvalbumin, S-100 proteins, troponin C, and calretinin, are expressed in regionally specific distributions in the brain and spinal cord at maturity and during ontogeny, suggesting that they function in relation to regional specifications in neurotransmission and development (Welsh, 1988).

In the study reported here, we determined the development of calbindin expression in the telencephalon, and, in particular, in the striatum and cerebral cortex. In both systems, calbindin expression characterizes subsets of neurons at maturity. We were especially interested in the striatum, where calbindin has a focus for study because of two unique properties of its distribution. First, calbindin expression distinguishes the common medium-sized spiny neurons, the main cell type in the striatum, from other striatal neurons (DiFiglia et al., 1989). Second, the expression of calbindin distinguishes between neurons in the two main neurochemical compartments of the striatum, the striosomes and the matrix (Graybiel and Ragsdale, 1983); calbindin is constitutively expressed by medium-sized neurons in most of the striatal matrix, but it is expressed by very few striosomal neurons (Gerfen et al., 1985).

For the striatum, we found that the expression of calbindin is characterized not only by a gradual appearance of calbindin in medium-sized neurons of the striatal matrix (Liu and Graybiel, 1991), but also by four transient calbindin-positive systems including (1) a subset of cells of the germinal zone of the basal ganglia, the ganglionic eminence (GE); (2) a subset of radial glial fibers extending from the calbindin-positive part of this germinal zone into the striatum; (3) a population of aspiny cells in the caudoputamen; and (4) patches of calbindin-positive neuropil corresponding to dopamine islands, which mark the sites of future striosomes. For the developing neocortex, we show that there are also waves of calbindin expression in its ventricular zone and that calbindin is expressed by cells of the transient subplate and marginal zones as well as in a gradually emerging

set of cells in the cortical plate. These patterns of expression strongly suggest that calbindin could participate in developmentally regulated cellular events in the striatum and neocortex during phases of development ranging from neurogenesis and migration to the formation of compartments and layers.

Materials and Methods

Embryos and pups from nine time-pregnant Sprague-Dawley rats (Taconic Farm) were used for brain tissue harvesting. The day of sperm positivity was counted as embryonic day (E)1 and the day of birth as postnatal day (P)0. Prenatal specimens were obtained from pregnant rats deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (Nembutal), and brain tissue was obtained by immersion fixation of heads of embryonic day (E)13 embryos ($n = 3$) or by perfusing embryos (E14, $n = 3$; E15, $n = 3$; E16, $n = 4$; E18, $n = 4$; E20, $n = 5$) through the transverse sinus or the heart. For all cases the fixative was ice-cold 4% paraformaldehyde containing 5% sucrose in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). For postnatal materials, P0 ($n = 7$), P3 ($n = 12$), P7 ($n = 6$), and P15 ($n = 3$) rat pups and adult rats ($n = 2$) were anesthetized by cooling on ice (P0, P3) or by intraperitoneal injection of sodium pentobarbital (P7, P15) and were then perfused transcardially with the same fixative. Heads (E13–E18) or brains (E20–P15) were postfixed in the same fixative at 4°C for 2–12 hr and then cryoprotected at 4°C for 24–36 hr in 20% sucrose in PBS. Brains or whole heads were cut on a freezing microtome at 40 μm (E13–E20) or 20–30 μm (P0–adult) in the coronal plane. The adult brain tissue ($n = 4$) was obtained from another study (Graybiel et al., 1990).

Immunostaining was performed by peroxidase-antiperoxidase (Sternberger, 1979) or avidin-biotin-peroxidase method (Hsu et al., 1981; Vector Laboratories). Free-floating sections were pretreated with 3% H_2O_2 and 10% methanol in 0.1 M Tris buffer saline (TBS) (pH 7.4), then rinsed sequentially in 25%, 40%, 25%, and 10% ethanol diluted in TBS. Sections were incubated in 3.3% or 5% normal goat serum (NGS) in TBS for 30–60 min and washed in TBS several times before being placed into primary antisera. The concentrations of primary antisera were as follows: 1:1000–4000, 1:500, and 1:800, respectively, for rabbit polyclonal anti-calbindin- $\text{D}_{28\text{K}}$ antisera kindly donated by Drs. P. C. Emson (Institute of Animal Physiology, Barabraham, Cambridge, UK), C. R. Gerfen (NIMH, Bethesda, MD), and S. Christakos (University of Medicine and Dentistry of New Jersey, Newark); 1:240–500 for rabbit polyclonal anti-tyrosine hydroxylase (TH) antiserum (Eugene Tech, Allendale, NJ); 1:20,000 for mouse monoclonal anti-dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein-32,000 (DARPP-32; antibody kindly provided by Dr. E. L. Gustafson of The Rockefeller University, New York); 1:1 for mouse monoclonal anti-synaptic vesicle-associated protein (SV48; antibody kindly provided by Dr. W. D. Mathew of Duke University Medical Center, Durham, NC); 1:500 for mouse monoclonal anti- α subunit of calcium/calmodulin-dependent protein kinase type II (CCPK II) (6G9; antibody kindly provided by Dr. M. B. Kennedy of the California Institute of Technology, Pasadena); and 1:1 to full strength for mouse monoclonal anti-nestin (Rat.401; antibody kindly provided by Dr. R. D. G. McKay of MIT, Cambridge, MA). The complete pre- and postnatal calbindin immunostaining series was carried out with the antiserum provided by Dr. P. C. Emson.

All primary antisera were diluted with TBS containing 1% NGS and 1% normal rat serum (NRS) with or without 0.2% Triton X-100. Sections were incubated in primary antisera at 4°C for 24–48 hr. For peroxidase-antiperoxidase immunocytochemistry, sections were washed several times in TBS and incubated in 1:50 goat anti-rabbit IgG (Antibodies Incorporated) or goat anti-mouse IgG (ICN Immuno-Biologicals) containing 1% NGS and 1% NRS in TBS for 1–2 hr and 1:30–50 rabbit or mouse peroxidase-antiperoxidase (Sternberger-Meyer) containing 1% NGS and 1% NRS in TBS for 1–2 hr, with several intervening rinses in TBS. For older tissue (P0, P3, P7), a double-bridge procedure was followed. Sections were developed in 0.05% diaminobenzidine (DAB) in 0.1 M phosphate buffer (PB) by adding H_2O_2 to a final concentration of 0.0024%. For avidin-biotin-peroxidase complex immunocytochemistry, sections were rinsed in the same buffer, incubated in 1:500 biotinylated goat anti-rabbit IgG or horse anti-mouse IgG (Vector Laboratories) containing 1% NGS or 1% normal horse serum and 1% NRS in TBS for 1–2 hr, rinsed several times in TBS, and incubated in avidin-biotin complex (6 μl A and 6 μl B/ml) (Vector Laboratories) in TBS for 1–2 hr. Sections were developed in 0.05% DAB containing 0.2% $\beta\text{D}(+)\text{glucose}$ and 0.04% NH_4Cl in 0.1 M PB by adding

glucose oxidase (Sigma) to a final concentration of 0.0004%. Alternating sets of serial sections from P0, P3, and P7 brains were processed for calbindin and TH immunostaining. Some sections of prenatal brains were stained with cresylecht violet to demonstrate cytoarchitecture.

Double immunostaining for calbindin and Rat.401 was carried out on sections from E20, P0, and P3 brains by first immunostaining for calbindin (Emsen) with the avidin-biotin-peroxidase method as described above and then, after extensive rinsing in TBS, incubating in Rat.401 primary antiserum at 4°C for 18–24 hr followed by incubating in 1:200–400 goat anti-mouse IgG conjugated to Texas Red (Molecular Probes Inc.) at room temperature for 2 hr. After incubation, sections were rinsed (TBS) and mounted on slides with glycerol/PB buffer containing DABCO (5 mg/ml). Controls for double immunostaining for Rat.401 and calbindin were carried out by omitting the secondary antibodies in the staining procedure as described above. Cellular colocalization of calbindin and Rat.401 antigen (nestin) was studied with the aid of a confocal microscope (Bio-Rad) by comparing the calbindin staining pattern viewed in laser-Nomarski light field optics with the Rat.401 immunofluorescence pattern observed with a rhodamine filter (Bio-Rad).

Immunostaining controls for the calbindin immunohistochemistry were performed on prenatal as well as postnatal material by incubating selected sections without primary antibodies (sections from all prenatal and postnatal brains) or (sections from E20, P0, and P3 brains) in calbindin antiserum (1:4000; from Dr. P. C. Emsen) preabsorbed with calbindin-D_{28k} protein purified from rat kidney (3.6×10^{-4} M; generously provided by Dr. S. Christakos). A series of antibody dilution tests was also carried out (E20, P0, P3 brains) with calbindin antiserum (Dr. P. C. Emsen) using avidin-biotin-peroxidase immunocytochemistry. The dilutions were 1:1000, 1:2000, 1:4000, 1:8000, 1:12,000, 1:20,000, and 1:40,000. In this article we refer to tissue elements showing calbindin-like immunoreactivity as “calbindin positive” or “calbindin immunoreactive.” The terms reflect only the staining patterns observed with the three calbindin antisera we used.

Results

Calbindin-like immunoreactivity in the forebrain during early telencephalic development (E13–E15)

Calbindin-like immunoreactivity was already distributed in the ventricular zone of the E13 telencephalon along pronounced mediolateral, dorsoventral, and caudorostral gradients (Fig. 1*A,A'*). The highest levels of calbindin expression were in the mediadorsal part of the ventricular zone, where the entire epithelium was intensely stained. Dorsolaterally, calbindin-immunoreactive cells in the ventricular zone were piled up in rows perpendicular to the ventricular surface, and a few tangentially oriented calbindin-immunoreactive cells began to form a thin layer (plexiform primordium or preplate; Uylings et al., 1990). Farther ventrally in the ventricular zone, calbindin-like immunoreactivity was very weak.

In the germinal zone of the GE at E13 and E14, only a few sporadic cells (Fig. 2*D*) and fibers (Fig. 2*A,C,D*) were as strongly immunostained as cells in the more dorsal part of the ventricular zone. By E14, the medial and lateral ridges of the GE had formed, and a few chains of weakly calbindin-positive cells were found stretching from the ventricular and subventricular zones toward the base of the lateral GE (Fig. 2*B*). These rare calbindin-positive columns were not obviously associated with other calbindin-immunoreactive elements. The lateral GE had perceptibly more diffuse immunostaining than the medial GE, but both were very pale.

Just lateral to the developing lateral GE, intensely immunoreactive cells began to appear in the developing cortical plate, and as early as E13 the ventral part of the preplate had swollen to form a prominent group of calbindin-positive cells (Fig. 1*A,A',B,B'*). This group probably corresponds to the anlage of the pyriform cortex. Ventral to the medial GE, another prominent group of calbindin-positive cells piled up (Fig. 1*B'*). This medial group lay near the base of the third ventricle, apparently

in the hypothalamic primordium. Some of the cells associated with this group were extremely large and highly ramified (Figs. 1*A',2E*). Such calbindin-positive giant cells were found as early as E13.

Starting from E15, many tangentially oriented calbindin-immunoreactive fibers appeared in the intermediate zone of the cortical primordium, and increasing numbers of calbindin-immunoreactive fibers could be traced from the lateral cortical anlage toward the growing striatal anlage (Fig. 1*C,C'*). When these fibers reached the striatal anlage associated with the lateral GE, they became intermingled with a group of fusiform calbindin-immunoreactive cells that were present there (Fig. 2*F*). A number of the calbindin-immunoreactive fibers appeared to turn at acute angles to invade the subventricular zone of the lateral GE (Fig. 2*C,D*). Some but not all of these fibers emerged from cell bodies in this region. Other calbindin-immunoreactive fibers extended as far as the medial GE (Fig. 1*C'*). Calbindin-immunoreactive fibers were continuously present in the striatal anlage from E16 on (Fig. 3*A*) and began to form fiber fascicles at E16–E18 (see below and Fig. 3*B*).

Emergence of calbindin expression in the GE and in radial processes in the striatal anlage

A sharp increase in expression of calbindin-like immunoreactivity in the ventricular zone of the GE began after E15. At E16 (Fig. 3*A*), most of the GE still had very little calbindin, but a transition to heightened expression began to appear at the dorsal margin of the lateral GE. In the following days a wave of calbindin expression spread from this dorsal zone farther ventrally along the ventricular and subventricular zones of the GE, maintaining strong dorsoventral and rostrocaudal gradients in the GE as described below. Ventrally, at the foot of the lateral ventricle, a second transient wave of calbindin expression developed in the germinal epithelium lining the medial side of the lateral ventricle. A striking dorsoventral gradient of calbindin-like immunoreactivity was present in the ventricular zone of GE at E18 (Fig. 3*B*). By this time, lateral and medial bulges could no longer be distinguished, but in the dorsal part of the ventricular zone of the GE, throughout its anteroposterior extent, intensely calbindin-immunoreactive cells appeared singly and in vertically aligned pairs or, rarely, in multicellular radial columns (Fig. 3*C*). The dorsal cells of this calbindin-immunoreactive population were associated with weakly stained calbindin-immunoreactive processes that extended from the ventricular zone through the subventricular zone, across the striatal anlage, and beyond it into the deep white matter separating the developing striatum from the cortical primordium (Fig. 3*B,C*). These calbindin-immunoreactive fibers gave the impression of forming a sharp border between the pallial epithelium and white matter above, and the GE and striatal anlage below. Along more ventral parts of the ventricular zone of the GE, there were progressively fewer calbindin-positive cells, and they were progressively more restricted to the superficial part of the ventricular zone. Very little calbindin-like immunoreactivity was present in the ventral one-third to one-half the ventricular zone (Fig. 3*D*).

By E20 (Fig. 4*A*), there was intense calbindin-like immunoreactivity in the ventricular zone of the GE and a strong development of radially organized calbindin-immunoreactive processes stretching away from the epithelium into the striatal anlage. The dorsoventral gradient in calbindin expression in the germinal zone was still prominent. In the dorsal part of the ventricular zone, there were layers of intensely calbindin-immu-

noreactive cells, but both the numbers and staining intensity of the epithelial cells and their depth distribution in the epithelium decreased ventrally. There was also a pronounced rostrocaudal gradient in calbindin expression in the ventricular zone. Rostally the calbindin-positive cells were more piled up within the zone, and they extended farther ventrally than calbindin-positive cells at caudal levels.

The long calbindin-immunoreactive processes emerging from the ventricular zone were also best developed dorsally and rostrally. They appeared through most of the height of the rostral striatal anlage, but farther caudally they were restricted primarily to its dorsal half. In these dorsal regions, the immunostained processes stretched through the subventricular zone and across the dorsal striatal anlage, bending in parallel with the curving dorsal surface of the striatum (Fig. 4A). Some of these calbindin-immunoreactive processes could be traced from the ventricular epithelium as far as the external capsule. The dorsal-to-ventral and rostral-to-caudal decline in the number of such calbindin-immunoreactive processes paralleled the decline in staining of cells in the ventricular zone. The calbindin-immunoreactive processes were best stained in sections treated with Triton X-100. Without Triton X-100 treatment, they tended to have vague outlines, could not be traced well in continuity, and had a reduced spatial extent.

The calbindin-immunoreactive radial processes were fully developed at P0. As shown in Figures 4D and 5, they formed dense parallel arrays and fascicles emerging from the ventricular epithelium, where there were numerous cells immunoreactive for calbindin, and some could be traced through the full width of the caudoputamen. The calbindin-positive germinal cells and radial processes were still most numerous in the rostral and dorsal caudoputamen. Even at levels where the processes were numerous, they did not appear in the ventral part of the caudoputamen in which a latticework of calbindin-immunoreactive medium-sized striatal cells was forming (Liu and Graybiel, 1991).

The calbindin-positive radial processes were still prominent at P3 (see Fig. 7A), and some occurred far enough ventrally to pass through the calbindin-positive band located at the ventrolateral edge of the rostral caudoputamen. At mid-striatal levels,

however, only a few weakly stained calbindin-immunoreactive processes remained, and they disappeared altogether in the caudal caudoputamen. The immunostaining for calbindin in the ventricular epithelium also progressively decreased from rostral to caudal and dorsal to ventral.

Demonstration that the transient calbindin-immunoreactive processes in the developing striatum form a subpopulation of radial glia

To test whether the distributions of the calbindin-positive radial processes were the processes of radial glial cells in the developing striatum, we carried out double immunostaining of sections from E20, P0, and P3 brains with calbindin (provided by Dr. Emson) and Rat.401 antiserum, which is a marker for radial glial cells and precursor cells for neurons and glial cells (Hockfield and McKay, 1985; Frederiksen and McKay, 1988; Lendahl et al., 1990). Rat.401-positive radial processes emerged from the entire extent of the ventricular zone, and they were distributed throughout the developing striatum (Fig. 4B).

The double staining experiments showed unequivocally that a subpopulation of these Rat.401-positive radial glia, primarily in the dorsal part of caudoputamen, also expressed calbindin-like immunoreactivity (Fig. 6). In these zones, almost all calbindin-positive processes could be shown to express Rat.401-like immunoreactivity. In the zones of densest calbindin expression, the majority of Rat.401-positive radial processes also expressed calbindin-like immunoreactivity, but not all Rat.401-positive processes were calbindin-positive. For co-labeled processes it was not always possible to detect each antigen along the entire length of a given process. Presumably, this reflected a technical problem (e.g., incomplete antibody penetration or veering of the process out of the focal plane), but incomplete expression along the length of such processes cannot be discounted. The transient calbindin-positive cells distributed in the same regions as the calbindin-positive processes (see below) were not Rat.401 positive, nor were the occasional Rat.401-positive cells calbindin positive. These single-labeled cells served as internal controls for the double immunostaining of the radial

Figure 1. Photomicrographs of calbindin immunostaining in coronal sections through the telencephalon of E13 (A, A'), E14 (B, B'), and E15 (C, C') rat brains at rostral (A–C) and caudal (A'–C') levels. Regions shown at higher magnification in other figures are indicated by corresponding figure numbers (2A, 2E, 2F, 8A, 8B). A and A', At E13, there is a distinct dorsomedial-to-ventrolateral gradient of calbindin immunostaining in the cortical primordium (C in A'). Ventrally, two groups of calbindin-immunoreactive cells are present. One lies in the lateral cortical primordium (arrowhead, presumably pyriform cortical primordium); the second (2A arrow) is at the base of GE (see also Fig. 2A). The GE itself has very little immunostaining. At caudal levels (A'), a group of large calbindin-positive cells with ramified processes (2E arrow) appears ventrally (presumably in the hypothalamic primordium; see also Fig. 2E). B and B', At E14, a cleft divides the GE into medial (GEm) and lateral (GEL) bulges. The calbindin-positive pyriform cortical anlage (arrow) now appears lateral to the lateral GE. The mediolateral gradient of calbindin expression is still evident in the cortical primordium. C and C', Calbindin expression appears throughout the mediolateral extent of the E15 cortical primordium (see Fig. 8B), and many calbindin-immunoreactive fibers appear in the intermediate zone of the cortical primordium. Laterally, they extend ventrally away from the cortical primordium and appear just under the lateral GE (C'). The striatal anlage (St) now appears beneath the GE (in C). In this region, many calbindin-positive cells are intermingled with calbindin-immunoreactive fibers that appear to emerge from the cortical primordium above (see also Fig. 2F). V, lateral ventricle; ssi, sulcus subpallii intermedius; ssd, sulcus subpallii dorsalis (see Lammers et al., 1980). Scale bar (for all photographs), 500 μ m.

Figure 2. Photomicrographs in A–D illustrate calbindin-immunoreactive fibers and cells in the GE. The ventricular zone is shown at the top in each photograph. A, High-magnification view of the zone indicated by arrow in Figure 1A to show a single calbindin-positive fiber (double arrows) stretching across the E13 GE. Note its swollen end in GE. B, A column of calbindin-positive cells (curved arrows) appearing in the subventricular zone in the E14 lateral GE. C, A bifurcated calbindin-positive fiber can be traced from the ventricular zone (arrowhead) of the E15 lateral GE toward the striatal anlage that lies below. D, Two curving calbindin-positive fibers in the E15 lateral GE in which a calbindin-immunoreactive cell (curved arrow) is also present. E, Calbindin-positive cells in the region indicated by an arrow in Figure 1A'. These calbindin-positive cells have large perikarya (~15 μ m) and thick, highly ramified processes. F, High-magnification view of the zone indicated by arrow in Figure 1C (E15 embryo). Note the numerous calbindin-positive cells intermingled with calbindin-immunoreactive fibers. This zone lies just ventral to the GE where the developing striatal anlage merges with the cortical primordium and its underlying white matter. VZ, ventricular zone; SVZ, subventricular zone; St, striatal anlage; C, cortical anlage. Scale bars: B, 50 μ m for A–D; E, F, 50 μ m.

A

E13

A'

B

E14

B'

C

E15

C'

Figure 3. Calbindin-like immunoreactivity in the E16 (*A*) and E18 (*B–D*) telencephalon. Cortical regions shown at higher magnification are indicated by figure numbers (8*C*, 8*E*, and for medial cortex out of field of view, 8*D*). Brackets in *B* show positions of photomicrographs shown in *C* and *D*. Very little calbindin-like immunoreactivity is detectable in the germinal epithelium of the GE at E16, but by E18, there is a distinct dorsoventral gradient of calbindin expression in the GE. Calbindin expression has begun to emerge in a group of cells (arrowhead in *B*) in the dorsal part of germinal epithelium of the GE (*B*, *C*). Some weakly stained fine calbindin-immunoreactive processes appear to stretch from this calbindin-positive zone across the junction between the cortical primordium and the GE (see arrows in *C*). Decreasing levels of calbindin-like immunoreactivity appear in the ventral part of the germinal epithelium of the GE, to the foot of the lateral ventricle. A second gradient of ventricular zone expression appears along the ventricular wall of the septal primordium (*B*, *D*). Note that many calbindin-positive fibers (*f*) now extend from the dorsolateral cortical primordium through the base of the striatal anlage (*St*). By E18 (*B*), these fibers come to form distinct fascicles. *V*, lateral ventricle; *ssd*, sulcus subpallii dorsalis. Scale bars: *B* (for *A* and *B*), 500 μ m; *D* (for *C* and *D*), 100 μ m.

glial fibers. Colocalization of Rat.401-like and calbindin-like immunoreactivity was not detected in the Rat.401-positive radial glia distributed in the ventral part of caudoputamen.

Transient appearance of calbindin-immunoreactive cells and calbindin-positive patches in the perinatal striatum

As the development of the calbindin-positive GE cells and radial processes was reaching its peak, the two other transient systems of calbindin-immunoreactive elements in the striatal anlage appeared for the first time. The first of these was a set of calbindin-immunoreactive cells first detected at E20 primarily in the lateral striatal anlage (Fig. 4A) and found as late as P15. At E20, some of these cells seemed to be associated with the radial calbindin-positive processes described above. By P0, many calbindin-immunoreactive cells were scattered in the rostradorsal caudoputamen and in the dorsal and lateral parts of the middle and caudal caudoputamen (Figs. 4D, 5). Some cells were small-to medium-sized cells (diameter, $\sim 6\text{--}12\ \mu\text{m}$), whereas others had larger perikarya (diameter, $\sim 13\text{--}18\ \mu\text{m}$) with ramified immunoreactive processes lacking detectable spines. Few such larger cells were found in the ventral part of the caudoputamen. These calbindin-positive cells were scattered between and alongside the calbindin-immunoreactive radial processes, and some of the calbindin-positive cells were attached to the processes (Figs. 4C, 5). We could not, however, find a consistent relationship between the orientations of the processes of calbindin-immunoreactive cells and the calbindin-positive processes. These cells were prominent dorsally until P3, and then gradually disappeared.

The second transient calbindin-immunoreactive system was made up of calbindin-immunoreactive patches approximately $75\text{--}150\ \mu\text{m}$ wide dispersed in the same regions as the calbindin-immunoreactive cells. The patches were first visible at P0 (Fig. 4C), peaked at P3 (Fig. 7A), and disappeared after P15. They appeared to be made up largely of a fine-fibered calbindin-immunoreactive neuropil, but many of the scattered calbindin-immunoreactive cells were also associated with these patches (Fig. 4C). Some of the calbindin-immunoreactive radial processes ran through the calbindin-immunoreactive patches. Both

the scattered calbindin-immunoreactive cells and the calbindin-immunoreactive patches were prominent in the dorsal and lateral parts of caudoputamen at P3. The calbindin-positive patches were clearly set off from the now intensely immunostained permanent calbindin-positive mosaic of medium-sized striatal cells developing ventrally (Fig. 7A,C). Altogether, the transient calbindin-positive patches formed a dorsolateral system that was roughly co-distributed with the region containing the transient calbindin-positive cells.

It was necessary to treat sections with Triton X-100 to obtain clear staining of the calbindin-immunoreactive patches (Fig. 7A). Without Triton X-100 treatment, the calbindin-immunoreactive patches were at most weakly stained and in some sections not visible at all.

Demonstration that the transient calbindin-positive patches correspond to dopamine islands

The system of calbindin-positive patches was strongly reminiscent of the "dopamine island system" of the developing caudoputamen even in the detail of including the rim of calbindin-immunoreactivity along the dorsolateral margin of the caudoputamen. To compare the locations of the calbindin-immunoreactive patches and dopamine islands directly, we studied serially aligned pairs and triplets of sections consecutively stained for calbindin-like immunoreactivity and for TH-like immunoreactivity (P0, $n = 7$; P3, $n = 12$; P7, $n = 6$; P15, $n = 3$) or, in P3 and P7 cases ($n = 1$ for each age), for three other markers of dopamine islands (DARPP-32, SV48, and CCPK II immunoreactivity) (Foster et al., 1987; Newman-Gage and Graybiel, 1988). In the P0 brain, the structure of the patches changed too rapidly in adjoining sections to allow secure comparisons, but serial-section analysis was possible beginning at P3. The calbindin-immunoreactive patches were closely aligned with TH-positive patches in adjacent sections, and there were few TH-positive patches that lacked corresponding calbindin-immunoreactive patches (Fig. 7A,B). Similar alignments were found for the calbindin patches and islands stained for DARPP-32, SV48, and CCPK II (data not shown).

TH-positive dopamine islands were still visible at P7, and

Figure 4. A, A coronal section through an E20 forebrain, stained for calbindin. Cortical region shown at higher magnification in Figure 8F is indicated by arrow in A. There is a pronounced dorsoventral gradient of calbindin expression in the ventricular and subventricular zones of the GE. Dorsally, many radial calbindin-immunoreactive processes stretch from the ventricular zone into the dorsal part of developing striatum (St), some reaching as far as the external capsule (EC). These processes are fully developed by P0. Note that by E20, numerous calbindin-immunoreactive bundles appear in the ventral striatum (see arrowhead in A). B, Pattern of immunostaining for Rat.401 in the E20 telencephalon. In contrast to calbindin-positive cells in the GE (A), which are concentrated dorsally, Rat.401-positive cells are distributed throughout the germinal epithelium. Rat.401-positive radial glia also appear both dorsally and ventrally in the developing striatum. The Rat.401-positive radial glia extend from the ventricular zone across the developing striatum to the external capsule. C, High-magnification photomicrograph showing a patch of calbindin-positive neuropil in the dorsal P0 caudoputamen. The calbindin-immunoreactive radial processes (straight arrows) arch through the calbindin-positive patch. A few calbindin-positive cells (curved arrows) are associated with the patch. D, Calbindin expression pattern in the P0 striatum at the rostral level shown, the calbindin-positive processes have invaded more ventral parts of the caudoputamen than at earlier developmental stages. AC, anterior commissure. Scale bars: A (for A, B, and D), 500 μm ; C, 20 μm .

Figure 5. A montage of photomicrographs from a P0 brain illustrating transient calbindin-immunoreactive processes arching across the dorsal caudoputamen and showing the spatial relationships between these processes and the transient calbindin-positive cells of the dorsal caudoputamen. The montage is oriented dorsal up, medial left and follows the curve of the processes. The upper and lower parts of the montage overlap as indicated by fiducial marks (line segments). Many calbindin-positive processes stretch from the ventricular zone (VZ), which is intensely immunoreactive for calbindin, across the subventricular zone (SVZ) and dorsal striatum (St) to the external capsule (just beyond the right edge of the montage). Note that some of the processes are packed together closely to form fasciculi (example at double straight arrows). Many of the calbindin-positive processes can be followed continuously from the germinal epithelium into the caudoputamen proper. Two examples are indicated by fascicles a/a' and b/b'. Many transient calbindin-immunoreactive cells are scattered among this array of elongated calbindin-immunoreactive processes. Some of them appear to be directly on the calbindin-positive processes. Examples are illustrated at high magnification in the inset (double curved arrows). Scale bars: montage, 100 μm ; inset, 20 μm .

Figure 6. Confocal images showing double immunostaining of radial processes by calbindin and Rat.401 antisera. *A* and *B* illustrate the same field in the dorsal caudoputamen of a P0 brain. *A*, Laser-Nomarski image showing calbindin-positive radial processes. The immunostaining was carried out by the avidin-biotin-peroxidase method with DAB as substrate. *B*, Immunofluorescence image showing radial processes immunostained for Rat.401. The staining was done with Texas Red as fluorochrome. *Straight arrows* in *A* and *B* point to examples of colocalization of calbindin and Rat.401 antigens in the same processes. Note that a few Rat.401-positive cells (example at the *curved arrow* in *B*) are not immunoreactive for calbindin. *C* and *D*, Confocal images showing double immunostaining for calbindin (*C*) and Rat.401 (*D*) in the dorsal caudoputamen of a P3 rat brain. Examples of correspondence between calbindin-positive processes and Rat.401-positive processes are indicated by *straight arrows*. The *curved arrow* in *C* points to a calbindin-positive cell that is not immunoreactive for Rat.401. Scale bar, *D* (for *A–D*), 25 μ m.

continued alignment of the calbindin-positive patches and dorsal islands was confirmed. By P15, however, TH-like immunoreactivity in the extra-islandic matrix had greatly increased so much that only rarely could matches be attempted between the nearly faded calbindin-immunoreactive patches and the island system.

Postnatal decline of the transient calbindin-positive systems of the striatum

The scattered calbindin-immunoreactive cells and patches lingered slightly longer than the calbindin-immunoreactive GE cells and radial processes. A few calbindin-immunoreactive cells

and weak calbindin-immunoreactive patches were still present in the rostradorsal caudoputamen and the dorsal and lateral parts of the middle and caudal caudoputamen at P7 (Fig. 7C) and P15, but they were not detected in the mature caudoputamen. By P7, only a few calbindin-immunoreactive processes remained in the most rostral part of the caudoputamen. Calbindin-like immunostaining was also dramatically reduced in the ventricular epithelium by P7, even far rostrally. No calbindin-immunoreactive processes could be detected in any part of the caudoputamen by P15. Some of the cells of the ependyma were still stained, however, in regions adjoining calbindin-positive parts of the striatum.

Figure 7. *A* and *B*, Serial sections through the P3 striatum showing the immunostaining patterns for calbindin (*A*) and TH (*B*). The transient calbindin-positive patches visible in *A* are aligned with TH-positive dopamine islands in the adjoining section (*B*) (e.g., see the *corresponding asterisks*). Note that the strong calbindin-positive patchwork in the ventrolateral striatum (*double asterisks* in *A*) is part of the developing permanent calbindin-positive mosaic system (see Liu and Graybiel, 1991). *C*, In the P7 brain, a few transient calbindin-positive processes, cells, and patches (e.g., *asterisk*) are only faintly stained. A typical calbindin-poor zone in this mosaic is indicated by the *star*. *D*, A section through the P3 caudoputamen demonstrating the specificity of the calbindin immunostaining obtained with the calbindin antiserum provided by Dr. P. C. Emson. The section was incubated with 1:4000 calbindin antiserum preabsorbed with calbindin protein purified from the rat kidney (Dr. S. Christakos). The preabsorption procedure almost completely eliminates the immunostaining in the striatum and the neocortex. AC, anterior commissure. Scale bars: *B* (for *A* and *B*), 500 μ m; *C*, 500 μ m; *D*, 500 μ m.

Figure 8. A series of photomicrographs of the developing neocortex, taken at the same magnification to illustrate the sequential division of a single calbindin-positive cortical anlage into calbindin-positive cortical subplate and marginal zones. The zones illustrated are from the regions indicated by arrows in Figures 1*A,C'*, 3*A,B*, 4*A*. *A*, At E13, the dorsal and dorsolateral part of the ventricular zone (VZ) is immunoreactive for calbindin and many calbindin-positive cells are stacked up in vertical arrays within the ventricular zone. The cortical preplate (PP) consists of a layer of one or two tangentially oriented calbindin-positive cells. *B*, By E15, the ventricular zone has lost calbindin-like immunoreactivity except that a few calbindin-positive fibers remain (examples at *double arrows*). The calbindin-positive preplate contains many calbindin-positive cells, and these are intermingled with highly calbindin-immunoreactive fibers. *C*, By E16, an intermediate zone (IZ) containing calbindin-positive fibers appears beneath the cortical plate (CP). A few calbindin-immunoreactive fibers (*double arrows*) are still present in the ventricular zone. *D* and *E*, Views of the dorsomedial (*D*) and dorsolateral (*E*) parts of the cortical anlage at E18. The division of a single calbindin-positive cortical preplate into the calbindin-positive subplate (SP) and calbindin-positive marginal zones (MZ) has just started in the dorsomedial part of cortical anlage (photograph shows a region medial to position of the arrow in Fig. 3*B* and was rotated 90° clockwise to permit comparison with other panels). A few calbindin-positive cells (example at the *curved arrow* in *D*) are present in the ventricular zone. In the same section, a clear separation of the calbindin-positive subplate and calbindin-positive marginal zones has occurred in the dorsolateral part of the cortical anlage, and many fusiform calbindin-positive cells are scattered between. A few calbindin-positive cells (example at the *curved arrow* in *E*) appear below the subplate. *F*, By E20, the separation between the calbindin-positive subplate and calbindin-positive marginal zones is even greater and many calbindin-positive cells with radially oriented processes are visible. Scale bar: *A* (for *A–F*), 100 μ m.

Developmental segregation of the calbindin-positive cortical subplate and calbindin-positive marginal zones from a single calbindin-positive cortical primordium

The wave of calbindin expression documented here for the ventricular zone of the striatum began as an earlier wave of calbindin expression in the ventricular zone of the cortex subsided (see above and Fig. 1). During the E13–E20 period, however, there was intense calbindin-like immunoreactivity in the developing cortical anlage, as documented in Figure 8, and this calbindin expression appeared in transient patterns.

The expression of calbindin-like immunoreactivity in the cortical anlage started at E13 with a single-cell thick plexiform plate containing tangentially orientated calbindin-immunoreactive cells (Fig. 8A). As this plexiform plate gradually expanded, more calbindin-immunoreactive cells appeared, and by E15 (Fig. 8B) most if not all of the cells in this plate appeared immunostained. At E16, the calbindin-positive plexiform plate remained as a single layer dorsally (Fig. 8C), but in the ventrolateral cortical anlage it began to split into two layers packed with numerous calbindin-immunoreactive cells and separated by a layer with far fewer calbindin-positive cells (Fig. 3A).

This separation process followed a ventrolateral to dorso-medial gradient that was very obvious at E18. In fact, the only region lacking the split was the dorsomedial cortical anlage, where the two layers were still fused (Figs. 3B, 8D). The outer and inner layers of calbindin-immunoreactive cells appeared to correspond to the marginal zone and the subplate, respectively (Fig. 8E). Many fusiform calbindin-immunoreactive cells were distributed between these two layers. They did not seem to have a single (e.g., radial) orientation. The distance between the two calbindin-positive layers increased as the cortical anlage became increased in size, but by E20 the intensity of calbindin immunostaining in the subplate and marginal zones had decreased (Fig. 8F), and it continued to decrease with age. A few calbindin-immunoreactive cells were present in these two zones in the neocortex of adult rats, along with other calbindin-positive cortical neurons.

Changing patterns of calbindin-like immunoreactivity in the fiber bundles of the caudoputamen

During the time that the patterns of calbindin-like immunoreactivity changed in the developing cortex and striatum, a marked change in calbindin expression was apparent also in the fiber fascicles in the cortical white matter, many of which penetrated the striatum from its lateral side and then organized to form the dispersed fiber bundles characteristic of the internal capsule of the mature rodent (see Figs. 3A,B; 4A,D). Intensely calbindin-immunoreactive fibers sweeping between the developing cortical plate and the deepest part of the corona radiata became incorporated in the lateral striatum by E20. These calbindin-immunoreactive fascicles aggregated to form calbindin-immunoreactive bundles squeezed into the medial and ventral part of developing striatum (Fig. 4A). Curiously, the most dorsal of these calbindin-immunoreactive bundles, seen in cross section, consisted of calbindin-immunoreactive rings around calbindin-poor centers. From P0 (Fig. 4D) to P7, the calbindin-positive internal capsule remained visible and was mainly confined to the ventral part of the caudoputamen, but the calbindin-like immunostaining had disappeared by P15. By contrast, at P15 many weakly stained small calbindin-immunoreactive bundles

began to appear for the first time in the dorsal and lateral caudoputamen. They were not found in the mature caudoputamen.

Immunohistochemical controls

Controls for the specificity of immunostaining suggested that calbindin-like immunoreactivity observed in the GE and in the developing striatum and cortical plate reflected authentic calbindin-D_{28K} or a closely related antigen. Sections incubated without primary antiserum had no immunostaining. The preabsorption control sections, taken from E20, P0, and P3 brains to test the effects on each of the four transient calbindin-positive systems (Fig. 7D), showed that immunostaining in the GE, striatum, and cortex was virtually abolished by the addition of calbindin-D_{28K} protein, as was all staining in the white matter (see also below). Interestingly, calbindin-immunoreactive cells were still present in the basal forebrain, though compared to basal forebrain neurons in noncontrol sections processed at the same time, these neurons were weakly stained. Finally, the intensity of calbindin immunostaining in all regions decreased as the concentration of primary antiserum decreased.

Controls for double immunostaining for calbindin and Rat.401 showed no staining for Rat.401 in sections in which the secondary antibody, goat anti-mouse conjugated with Texas Red, was omitted during sequential procedure of double staining. Similarly, no calbindin immunostaining was present in sections in which the secondary antibody, biotinylated goat anti-rabbit, was omitted.

Discussion

Transient calbindin-like immunoreactivity in regionally specialized zones of GE

Our findings establish that a wave of expression of calbindin-like immunoreactivity occurs in the ventricular zone of the ganglionic eminence in the perinatal period. During the entire time that calbindin-positive cells appear in this ventricular zone (~E18–P7), they are spatially concentrated in its dorsal and rostral parts. The specificity of the immunostaining for calbindin-D_{28K} is suggested by the immunohistochemical controls, and the pronounced regional pattern of immunostaining was confirmed in all brains analyzed.

This differential distribution bears emphasis because it suggests that the differential antigen expression may be related to the division of the GE into medial and lateral eminences (striatal ridges, ventricular ridges). These ridges are thought to contribute to different structures in the mammalian forebrain (Smart and Sturrock, 1979; Sidman and Rakic, 1982; Bayer and Altman, 1987; Müller and O'Rahilly, 1988, 1990). They thus might represent lineage restriction compartments analogous to those observed in the hindbrain (Lumsden and Keynes, 1989; Wilkinson and Krumlauf, 1990; see also McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Interpreted in this light, our results would point to one pattern of gene expression that might be selective for the lateral (dorsal and lateral) compartment.

We could not directly test this possibility in the present study because the cleft visible between the medial and lateral ridges, although striking at E14 and E15, becomes obscured before calbindin is strongly expressed in the GE. Nevertheless, the consistent dorsolateral location of the calbindin-positive zone of the GE in the immediately following developmental period suggests that the calbindin-rich part of this germinal zone may correspond to the lateral ganglionic ridge.

The entire region of the striatum as well as other structures of the basal ganglia (the pallidum and the amygdala) and certain other structures of the forebrain are thought to derive from the GE. The striatum itself is thought to arise predominantly from the lateral ridge (Müller and O'Rahilly, 1990). Morphological observations further suggest that the GE of later embryonic stages originates by fusion of earlier-appearing medial and lateral ridges, and that these may have separate origins (Lammers et al., 1980; but see Källén, 1951). The more medial ridge, according to this view, derives from the basal part of the telencephalic wall, probably near the junction of the diencephalon and telencephalon, whereas the lateral eminence derives from the lateral wall of the telencephalon. Interestingly, calbindin was expressed in the ventricular zone of the cerebral cortex as well as in the lateral part of the GE. The joint expression of calbindin could reflect their common derivation. Yet the waves of calbindin expression that we observed in these two regions were temporally and spatially distinct, and they were marked by a clear boundary at the sulcus subpallii dorsalis separating the two telencephalic germinal zones.

The idea that the restricted expression of calbindin in the GE could reflect gene-specific compartmentation of this germinal zone finds some indirect support in two other sets of findings. First, the homeobox gene, *Distal-less*, has been shown to be expressed in the GE (including both medial and lateral parts), but not in adjoining ventricular epithelia, during development in the rodent (Price et al., 1991). This specificity demonstrates unequivocally that there is gene-specific compartmentation in the germinal epithelia of the forebrain. Second, molecular specialization of the dorsal (dorsolateral) part of the GE has been noted in previous developmental studies. Both the JONES and the D1.1 monoclonal anti-ganglioside antibodies stain the dorsal, but apparently not the ventral, part of the GE of the E18 rat brain (Mendez-Otero et al., 1988). These antibodies are thought to mark ganglioside molecules important in cell adhesion patterns during development, possibly in relation to migrations guided by radial glia. Our evidence that a transient subset of calbindin-positive radial glia is associated with the dorsolateral GE suggests that the calbindin-positive radial glia could be related to specific migratory paths of cells derived from this zone, including those of the striatum.

In the regions of densest calbindin expression, the entire ventricular zone was intensely immunostained, and no unstained cells could be detected. It is unlikely, therefore, that the transient calbindin expression was restricted to radial glia or their precursors. Very early during the onset of calbindin expression, however, did we see isolated columns of calbindin-positive cells in the ventricular layer of the GE. These recall the small foci of proto-oncogene expression reported by Johnston and van der Kooy (1989).

The question of what functions such intense but transient calbindin expression serves in the germinal epithelium clearly is not settled. One intriguing possibility is that during ontogeny, calbindin may participate in differential regulation of calcium ions in subdistricts of the GE and, according to our observations on the developing cortex, the germinal epithelium of the cortical plate as well. As calcium can modulate the mitotic process (Rasmussen and Means, 1989; Silver, 1990; Whitaker and Patel, 1990), calbindin, with its calcium-binding ability, may in turn differentially regulate cell proliferation in subregions of the GE. Interestingly, S-100 β protein, another member of the superfamily of calcium-binding proteins, has been shown to be a mitogen for glial cells (Selinfreund et al., 1991).

Transient population of calbindin-positive radial glia in the developing striatum

The concurrent appearance and disappearance of calbindin-positive cells in the GE and radial processes in the striatal anlage, and their matching dorsorostral distributions, suggested that the epithelial cells and radial elements might be related. The fact that these calbindin-positive processes expressed Rat.401 strongly suggests that they are radial glia. The Rat.401 antigen nestin is an intermediate-filament protein expressed by radial glia as well as by neuronal and glial precursor cells (Hockfield and McKay, 1985; Frederiksen and McKay, 1988; Lendahl et al., 1990). Thus, our results strongly suggest that the radial glia associated with the GE are heterogeneous, falling into at least calbindin-positive and calbindin-negative populations. The physiological function of expression of calbindin in radial glia is unknown. It would be of great interest to compare the migratory patterns of cells along calbindin-positive and calbindin-negative radial glia (e.g., Gasser and Hatten, 1990). Radial glia in the embryonic brainstem and spinal cord have been shown to be positive for S-100 protein as mentioned above (Gomez et al., 1990).

There was a clear complementarity between the dorsal and rostral distribution of the transient calbindin-immunoreactive radial processes and the ventral and caudal location of the growing mosaic of calbindin-positive medium-sized cells. The only exception was that some calbindin-immunoreactive processes did pass through a calbindin-positive "lateral band system" (Liu and Graybiel, 1991) that lay in the ventrolateral part of the rostral striatum. By contrast, the distribution of the calbindin-positive radial processes matched that of the scattered transient calbindin-positive cells in the dorsal part of the developing caudoputamen. It was not possible to determine whether the instances of close contact between the processes and cells actually represent a functional association (Rakic, 1971, 1972, 1988). However, the identification of the calbindin-positive processes as Rat.401 positive is consistent with a guidance function for these radial processes.

A transient population of calbindin-immunoreactive cells is present in the developing striatum

Our findings demonstrate that there is a transient population of calbindin-positive cells in the developing striatum visible from E20–P15. These appeared to have neuronal morphology, but we did not succeed in obtaining double-staining evidence for this phenotype. Some cells had quite large perikarya with ramified processes, and in general they appeared to be aspiny and otherwise phenotypically distinct from the calbindin-positive neurons in the calbindin-positive mosaic developing in the ventral parts of the caudoputamen (Liu and Graybiel, 1991). The dorsal location of these transient calbindin-positive cells included the region in which little calbindin-like immunoreactivity is expressed in the mature striatum and, as noted above, largely corresponds to the region penetrated by transient calbindin-immunoreactive radial processes. Calbindin-positive cells with similar phenotypes were only very rarely identified farther ventrally, but it is conceivable that the increasing immunoreactivity of the medium-cell mosaic hid others from view.

What accounts for the disappearance of the transient calbindin-immunoreactive cells remains an open question. They may be selectively removed during the period of neuronal death in the first postnatal week (Fentress et al., 1981; Fishell et al., 1987) or undergo a transition of neurochemical phenotype, or they

may be cells migrating through the striatum during development. This last possibility is plausible, because the phenotype of the larger of these calbindin-immunoreactive cells was similar to that of calbindin-positive cells found in the adjoining developing neocortex, and in some sections the transient calbindin-positive cells seemed to be piled up along the lateral edge of the caudoputamen.

Transient calbindin-immunoreactive patches aligned with dopamine islands in the developing striatum

A surprising finding in the present study is that there is a transient system of calbindin-positive patches in the striatum corresponding to dopamine islands. The calbindin-positive patches were primarily located in the dorsal and lateral caudoputamen and were visible from P0 to P15. The close correspondence between these transient calbindin-immunoreactive patches and dopamine islands was clear from serial-section comparisons with all four of the immunocytochemical markers of the islandic system that we used (antisera to TH, DARPP-32, SV48, and CCPK II) (Foster et al., 1987; Newman-Gage and Graybiel, 1988).

Dopamine islands mark the sites of developing striosomes (Graybiel, 1984; Moon Edley and Herkenham, 1984; Murrin and Ferrer, 1984; van der Kooy, 1984). Calbindin-like immunoreactivity is selectively expressed in neurons and neuropil of the matrix compartment of the mature rat's striatum except for a dorsolateral zone in which very little calbindin is present (Gerfen et al., 1985). Thus, on two counts the developmental-regulated calbindin-positive patchwork is inconsistent with the later pattern of expression of calbindin: the transient calbindin-positive patches are at the sites of future striosomes, not future matrix, and they are best developed dorsolaterally, where calbindin expression will be lowest. Moreover, at the same time that the transient system of calbindin-immunoreactive patches is developing dorsally, calbindin expression develops ventrally in a steadily increasing proportion of medium-sized neurons of the matrix (Liu and Graybiel, 1991). Thus, the calbindin-immunoreactive elements of the developing caudoputamen can be divided into two systems with respect to the neurochemical compartmentation of the striatum. One is a transient calbindin-positive system located in developing striosomes, and the other one is a permanent calbindin-positive system located in the matrix.

Two possible origins of the calbindin-immunoreactive fibers in the dopamine islands were suggested by our immunohistochemical findings. First, some of the transient calbindin-immunoreactive cells scattered through the dorsolateral caudoputamen were associated with these patches and so could contribute at least part of the neuropil of the patches. The coordinate time of disappearance of the calbindin-immunoreactive cells and patches (around P15) is consistent with this idea. The slightly earlier appearance of the transient calbindin-positive cells (E20 as opposed to P0) would also fit if the cells, once in dopamine island locations, then generated processes. They might, however, simply have more antigen in the cell body than in coexisting processes.

A second possibility is that the source of the calbindin-immunoreactive neuropil in the patches is extrinsic to the striatum; for example, they could derive from calbindin-immunoreactive cells of the developing neocortex. We observed calbindin-immunoreactive cells in the developing cortical plate and the immediately adjoining subplate. In the mature rat, neurons in the deep layers of neocortex project predominantly to the striosomal

(patch) compartment of the striatum (Gerfen, 1989). Moreover, in the ferret, subplate neurons have been shown to project to subcortical regions earlier than do neurons in the deep cortical layers (McConnell et al., 1989). Later in development, neurons of the subplate are thought to undergo programmed cell death (Luskin and Shatz, 1985; Chun and Shatz, 1989). If the calbindin-immunoreactive neuropil in the dopamine islands is derived from axons of calbindin-immunoreactive subplate neurons, a disappearance of the calbindin-positive patches would be predicted: as calbindin-immunoreactive subplate neurons die during development, so would the calbindin-immunoreactive patches disappear. Calbindin-immunoreactive subplate neurons apparently are not projection neurons in the ferret (Antonini and Shatz, 1990); given our findings, it would be interesting to look for this connectivity in the rodent.

Cortical subplate and marginal zones share the property of expressing intense calbindin immunostaining following an early wave of calbindin expression in the ventricular zone

Our findings demonstrate that a single-cell layer of calbindin-immunoreactive cells appears in the cortical plexiform plate as early as E13 and that, at about E16, this single calbindin-positive plexiform plate begins to split into two calbindin-positive zones that become the subplate and marginal zones. This developmental separation is in good accord with the developmental sequence observed by Luskin and Shatz (1985) with ³H-thymidine neuronography in the cat's visual cortex. These authors proposed that cells in the cortical subplate and marginal zones are transient cells first cogenerated in a single zone, that they appear earlier than other cells of the cortex, and that the original single zone is subsequently separated into two bands by the insertion of later-born cells constituting the cortical plate. Our results suggest that the early-forming cells share the property of calbindin immunoreactivity and that this chemospecificity is retained during an extended period of cortical development that culminates finally in a major wave of cell death in the calbindin-positive cell populations in the marginal and subplate zones. Moreover, we have shown that this developmental sequence of calbindin expression in the cortical anlage is preceded by a period of intense calbindin expression in the underlying ventricular zone. Thus, our observations collectively suggest that in the developing cortex, as in the developing striatum, there are consecutive waves of transient calbindin expression in precursor cells of their germinal epithelia and in a restricted set of cells derived from these epithelia.

References

- Antonini A, Shatz CJ (1990) Relation between putative transmitter phenotypes and connectivity of subplate neurons during cerebral cortical development. *Eur J Neurosci* 2:744-761.
- Bayer SA, Altman J (1987) Directions in neurogenetic gradients and patterns of anatomical connections in the telencephalon. *Prog Neurobiol* 29:57-106.
- Choi DW (1988) Calcium mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci* 11:465-469.
- Chun JJM, Shatz CJ (1989) Interstitial cells of the adult neocortical white matter are the remnant of the early generated subplate neuron population. *J Comp Neurol* 282:555-569.
- DiFiglia M, Christakos S, Aronin N (1989) Ultrastructural localization of immunoreactive calbindin-D28K in the rat and monkey basal ganglia, including subcellular distribution with colloidal gold labeling. *J Comp Neurol* 279:653-665.
- Evered D, Whelan J (1986) Calcium and the cell. Chichester: Wiley.
- Fentress JC, Stanfield BB, Cowan WM (1981) Observations on the

- development of the striatum in mice and rats. *Anat Embryol* 163: 275–298.
- Fishell G, van der Kooy D (1987) Patch striatonigral neurons preferentially survive the period of striatal cell death. *Soc Neurosci Abstr* 13:1575.
- Foster GA, Schultzberg M, Hökfelt T, Goldstein M, Hemmings HC Jr, Ouimet CC, Walaas SI, Greengard P (1987) Development of a dopamine- and cyclic adenosine 3':5'-monophosphate-regulated phosphoprotein (DARPP-32) in the prenatal rat central nervous system, and its relationship to the arrival of presumptive dopaminergic innervation. *J Neurosci* 7:1994–2018.
- Frederiksen K, McKay RDG (1988) Proliferation and differentiation of rat neuroepithelial precursor cells *in vivo*. *J Neurosci* 8:1144–1151.
- Gasser UE, Hatten ME (1990) CNS neurons migrate on astroglial fibers from heterotypic brain regions *in vitro*. *Proc Natl Acad Sci USA* 87:4543–4547.
- Gerfen CR (1989) The neostriatal mosaic: striatal patch-matrix organization is related to cortical lamination. *Science* 246:385–388.
- Gerfen CR, Baimbridge KG, Miller JJ (1985) The neostriatal mosaic: compartmental distribution of calcium-binding protein and parvalbumin in the basal ganglia of the rat and monkey. *Proc Natl Acad Sci USA* 82:8780–8784.
- Gomez LA, Brusco A, Saavedra JP (1990) Immunocytochemical study of S-100 positive glial cells in the brainstem and spinal cord of the rat embryo. *Int J Dev Neurosci* 8:55–64.
- Graybiel AM (1984) Correspondence between the dopamine islands and striosomes of the mammalian striatum. *Neuroscience* 13:1157–1187.
- Graybiel AM, Ragsdale CW (1983) Biochemical anatomy of the striatum. In: *Chemical neuroanatomy* (Emson PC, ed), pp 427–504. New York: Raven.
- Graybiel AM, Moratalla R, Robertson HA (1990) Amphetamine and cocaine induce drug-specific activation of the *c-fos* gene in striosome-matrix and limbic subdivisions of the striatum. *Proc Natl Acad Sci USA* 87:6912–6916.
- Hidaka H, Carafoli E, Means AR, Tanaka T (1988) Calcium protein signaling. New York: Plenum.
- Hockfield S, McKay RDG (1985) Identification of major cell classes in the developing mammalian nervous system. *J Neurosci* 5:3310–3328.
- Hsu SM, Raine L, Fanger H (1981) The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (peroxidase) procedure. *J Histochem Cytochem* 29:577–590.
- Johnston JG, van der Kooy D (1989) Protooncogene expression identifies a transient columnar organization of the forebrain within the late embryonic ventricular zone. *Proc Natl Acad Sci USA* 86:1066–1070.
- Källén B (1951) The nuclear development in the mammalian forebrain with special regard to the subpallium. *Kgl Fysiogr Sällsk Lund Handl N F* 61:1–43.
- Kater SB, Mills LR (1991) Regulation of growth cone behavior by calcium. *J Neurosci* 11:891–899.
- Lammers GJ, Gribnau AAM, ten Donkelaar HJ (1980) Neurogenesis in the basal forebrain in the Chinese hamster (*Cricetulus griseus*). II. Site of neuron origin: morphogenesis of the ventricular ridges. *Anat Embryol* 158:193–211.
- Lendahl U, Zimmerman LB, McKay RDG (1990) CNS stem cells express a new class of intermediate filament protein. *Cell* 60:585–595.
- Liu F-C, Graybiel AM (1991) Heterogeneous development of calbindin-D_{28k} expression in the developing striatum. *J Comp Neurol*, submitted.
- Lumsden A, Keynes R (1989) Segmental patterns of neuronal development in the chick hindbrain. *Nature* 337:424–428.
- Luskin MB, Shatz CJ (1985) Studies of the earliest generated cells of the cat's visual cortex: cogeneration of subplate and marginal zones. *J Neurosci* 5:1062–1075.
- Mayer ML, Westbrook GL (1987) Cellular mechanisms underlying excitotoxicity. *Trends Neurosci* 10:59–61.
- McConnell SK, Ghosh A, Shatz CJ (1989) Subplate neurons pioneer the first axon pathway from the cerebral cortex. *Science* 245:978–982.
- McMahon AP, Bradley A (1990) The *wnt-1* (*int-1*) proto-oncogene is required for development of a large region of the mouse brain. *Cell* 62:1073–1085.
- Mendez-Otero R, Schlosshauer B, Barnstable CJ, Constantine-Paton M (1988) A developmentally regulated antigen associated with neural cell and process migration. *J Neurosci* 8:564–579.
- Moon Edley S, Herkenham M (1984) Comparative development of striatal opiate receptors and dopamine revealed by autoradiography and histofluorescence. *Brain Res* 305:27–42.
- Müller F, O'Rahilly R (1988) The development of the human brain, including the longitudinal zoning in the diencephalon at stage 15. *Anat Embryol* 179:55–71.
- Müller F, O'Rahilly R (1990) The human brain at stages 21–23, with particular reference to the cerebral cortical plate and to the development of the cerebellum. *Anat Embryol* 182:375–400.
- Murrin LC, Ferrer JR (1984) Ontogeny of the rat striatum: correspondence of dopamine terminals, opiate receptors and acetylcholinesterase. *Neurosci Lett* 47:155–160.
- Newman-Gage H, Graybiel AM (1988) Expression of calcium/calmodulin dependent protein kinase in relation to dopamine islands and synaptic maturation of cat striatum. *J Neurosci* 8:3360–3375.
- Price M, Lemaistre M, Pischetola M, Di Lauro R, Duboule D (1991) A mouse gene related to *Distal-less* shows a restricted expression in the developing forebrain. *Nature* 351:748–751.
- Rakic P (1971) Guidance of neurons migrating to the fetal monkey neocortex. *Brain Res* 33:471–476.
- Rakic P (1972) Mode of cell migration to the superficial layers of fetal monkey neocortex. *J Comp Neurol* 145:61–84.
- Rakic P (1988) Specification of cerebral cortical areas. *Science* 241:170–176.
- Rasmussen CD, Means AR (1989) Calmodulin, cell growth and gene expression. *Trends Neurosci* 12:433–438.
- Rogers JH (1989) Calcium binding proteins: the search for functions. *Nature* 339:661–662.
- Rubin RP, Weiss GB, Putney JW (1985) Calcium in biological systems. New York: Plenum.
- Selinfreund RH, Barger SW, Pledger WJ, Van Eldik LJ (1991) Neurotrophic protein S100 β stimulates glial cell proliferation. *Proc Natl Acad Sci USA* 88:3554–3558.
- Sidman RL, Rakic P (1982) Development of the human central nervous system. In: *Histology and histopathology of the nervous system* (Haymaker W, Adams RD, eds), pp 3–145. Springfield, IL: Thomas.
- Silver RB (1990) Calcium and cellular clocks orchestrate cell division. *Ann NY Acad Sci* 582:207–221.
- Smart IHM, Sturrock RR (1979) Ontogeny of the neostriatum. In: *The neostriatum* (Divac I, Oberg R, Guanila E, eds), pp 127–146. New York: Pergamon.
- Sternberger LA (1979) Immunocytochemistry. New York: Wiley.
- Takeichi M (1988) The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development* 102:639–655.
- Thomas KR, Capecchi MR (1990) Targeted disruption of the murine *int-1* proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* 346:847–850.
- Uylings HB, Van Eden CG, Parnavelas JG, Kalsbeek A (1990) The prenatal and postnatal development of rat cerebral cortex. In: *The cerebral cortex of the rat* (Kolb B, Tees RC, eds), pp 35–76. Cambridge, MA: MIT Press.
- van der Kooy D (1984) Developmental relationships between opiate receptors and dopamine in the formation of caudate-putamen patches. *Dev Brain Res* 14:300–303.
- Walaas SI, Nairn AC (1985) Calcium-regulated protein phosphorylation in mammalian brain. In: *Calcium and cell physiology* (Marmé D, ed), pp 239–264. Heidelberg: Springer.
- Welsh MJ (1988) Localization of intracellular calcium-binding proteins. In: *Calcium-binding proteins, Vol II, Biological functions* (Thompson MP, ed), pp 1–19. Boca Raton, FL: CRC.
- Whitaker M, Patel R (1990) Calcium and cell cycle control. *Development* 108:525–542.
- Wilkinson DG, Krumlauf R (1990) Molecular approaches to the segmentation of the hindbrain. *Trends Neurosci* 13:335–339.