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Development of Local Connections in Ferret Somatosensory Cortex

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ABSTRACT

Ferrets have become recognized as a useful and interesting model for study of neocortical development. Because of their immaturity at birth, it is possible to study very early events in the ontogeny of the brain. We used living slices of ferret somatosensory cortex to study the formation and development of intrinsic elements within the neocortex. A small number of fixed, hemisected brains injected with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were also used. The slices were obtained from ferret kits aged postnatal day (P)1 to P62 and maintained in a chamber; each slice received injections of fluorescent-labeled dextrans. The injections were made at different ages in several distinct sites, which included the proliferative ventricular zone, the intervening white matter (or intermediate zone), and different sites of developing cortex, including the deeper cortical plate, which incorporated the subplate in young animals, and more superficial cortical sites, depending on the age of the animal. Several animals also received injections into the ventrobasal thalamus. Injections into young animals (P1–7) produced a dominant radial pattern that extended from the ventricular zone into the cortex. Injections into the ventricular zone labeled many cells that appeared morphologically like radial glia as well as presumptive neurons. Although the predominant pattern was radial, injections in the ventricular zone often produced tangentially oriented cells and horizontally arranged fibers at the outer edge of the proliferative zone. These cells and fibers may provide a substrate for tangential dispersion of neurons within the neocortex. More superficial injections within the slice labeled lines of cells that appeared to be stacked upon one another in a radial pile in the cortex; the cortical plate received very few lateral projections. Data obtained from more mature slices indicated that, although the overall pattern of staining remained radial, the precise character of the pattern changed to include more lateral spread into surrounding cortex, which eventually refined and developed into distinct patches by P28, when the overall cortical architecture appeared adult like. The data involving thalamocortical connections were more limited, but they indicated that the thalamus projects precisely to the somatosensory cortex in a point-to-point fashion from the earliest date studied (P0) and that the ventrobasal nucleus terminates upon the somatosensory cortex in a patchy manner during the early postnatal days of development. This study of the development of the somatosensory cortex confirms the ubiquitous nature of column-like connections throughout the neocortex and provides a novel view of the radial nature of early neocortical maturation.

Indexing terms: radial glia, ventricular zone, neuronal migration, cortical column, cortical slice

Because the cortical mantle is immature at birth in ferrets, early stages of connectional patterns and cellular morphology can be studied in postnatal animals. One of the ubiquitous features of cortical organization is the presence of columnar units. These units are integrated structurally and connected precisely within the vertical dimension of the cortex and extend in the lateral dimension through intermittent, patchy terminations to selected clusters of cells (see, e.g., Mountcastle, 1978; Gilbert, 1992; Katz and Callaway, 1992). The visual cortex has a clear column-like arrangement in many species, including ferrets (Hubel and Wiesel,

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1977; Chapman et al., 1991). Although many response properties of visual cortex are organized in a columnar fashion (such as orientation specificity), an *unequivocal* link between structure and function has been identified only for ocular dominance columns.

Recent studies in cats and ferrets have made substantial gains in understanding the development of the connections that form columns in visual cortex (Katz and Callaway, 1992; Antonini and Stryker, 1993a,b; Chapman and Stryker, 1993). From these studies and from earlier work on primates, we know that intrinsic vertical connections within and between cortical layers occur by directed growth, so that neurons do not send nonspecific axons into inappropriate loci but guide axons expressly into appropriate layers (Lund et al., 1977; Lund, 1988; Katz and Callaway, 1992). Tangential connections, on the other hand, extend initially as a relatively diffuse or crude distribution, which refines to discrete clusters (Callaway and Katz, 1990; Katz and Callaway, 1992). Axons from the thalamus that form the ocular dominance columns appear diffuse initially and eventually form discrete terminal patches by eliminating nonspecific collaterals and by directing growth into appropriate loci (Antonini and Stryker, 1993b).

The function and structure of the somatosensory system are distinctly different from those of the visual system.

TABLE 1. Numbers of Injections Into Slices of Ferret Somatosensory Cortex

Age	VZ	WM/IZ	SP/Deep Ctx	Cortical Plate
P1–2	24	58	42	18
P5–7	10	23	42	23
P14–16		15	18	31
≥ P28		34 (Layer 5/6)	30 (Layer 4)	28 (Layer 2/3)
With halothane/ octanol				
P1–2	5	13	5	1
P5–7		2	5	
Dil injections				
P1–2	2 (VB thalamus)			9
P5–7	3 (VB thalamus)	1		2
P18				2

A total of 29 ferrets were used; multiple slices were obtained from each ferret and multiple injections were made in each slice. Indicated above are different locations that received injections; they differed depending on the age of the animal.
VZ, ventricular zone; WM, white matter; IZ, intermediate zone; SP, subplate; Ctx, cortex; VB, ventral basal.

Researchers have identified patchy terminations from the thalamus or from other cortical regions into the somatosensory cortex (Jones et al., 1978, 1979, 1982; Schwark and Jones, 1989). In addition, patterns of columnar activation have been observed electrophysiologically or with 2-deoxyglucose uptake (Sur et al., 1984; Favorov and Whitsel, 1988;

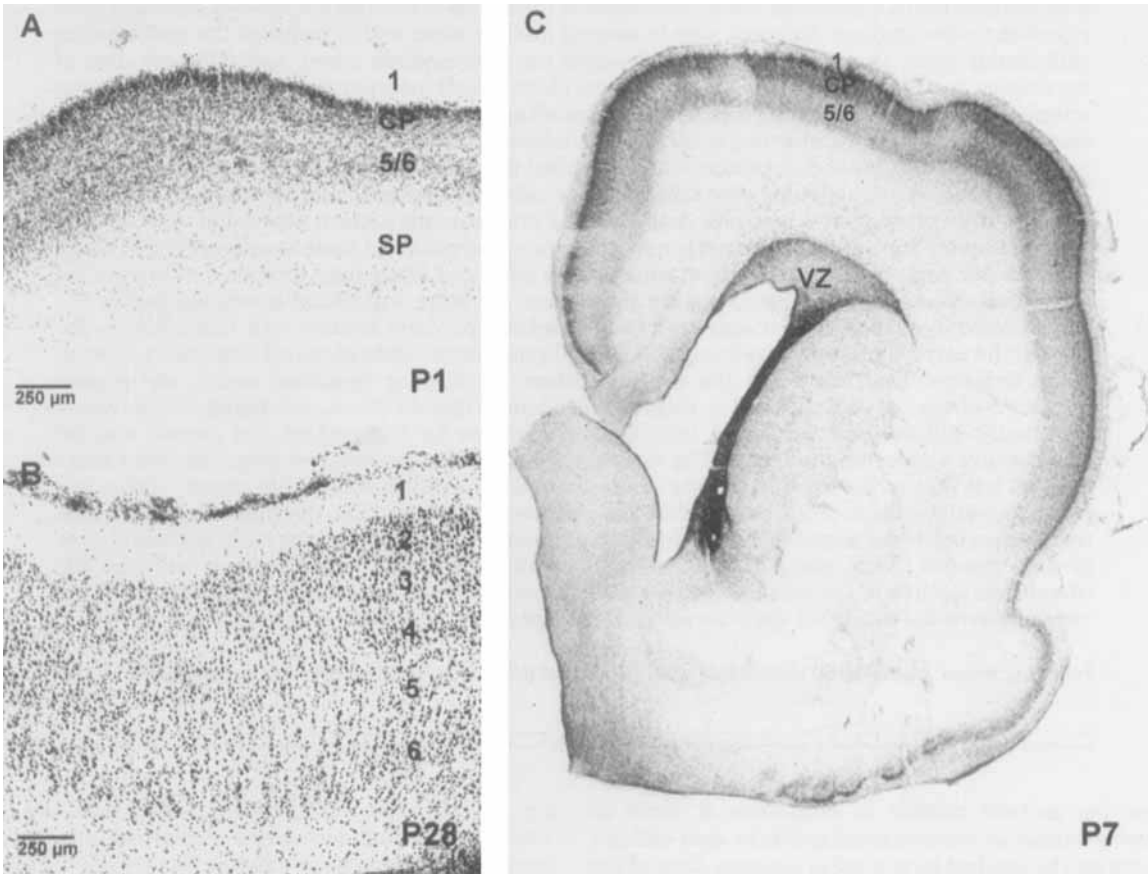

Fig. 1. Nissl-stained sections of somatosensory cortex in the coronal plane at postnatal day (P)1 (**A**) and at P28 (**B**), when the cytoarchitecture is relatively mature. **C** is a low-power view of a coronal section taken through the somatosensory cortex of a P7 ferret stained with neutral red. At P1, the cortical layers are not distinct; a dense cortical plate (CP) can be seen as well as a substantial subplate (SP). At P28, cortical layers are distinct; layers are indicated by numbers. At younger ages (e.g., at P7 in C), the ventricular zone (VZ) is substantial and diminishes as the animal matures. Cortical layers (when they are distinguishable) are indicated by numbers.

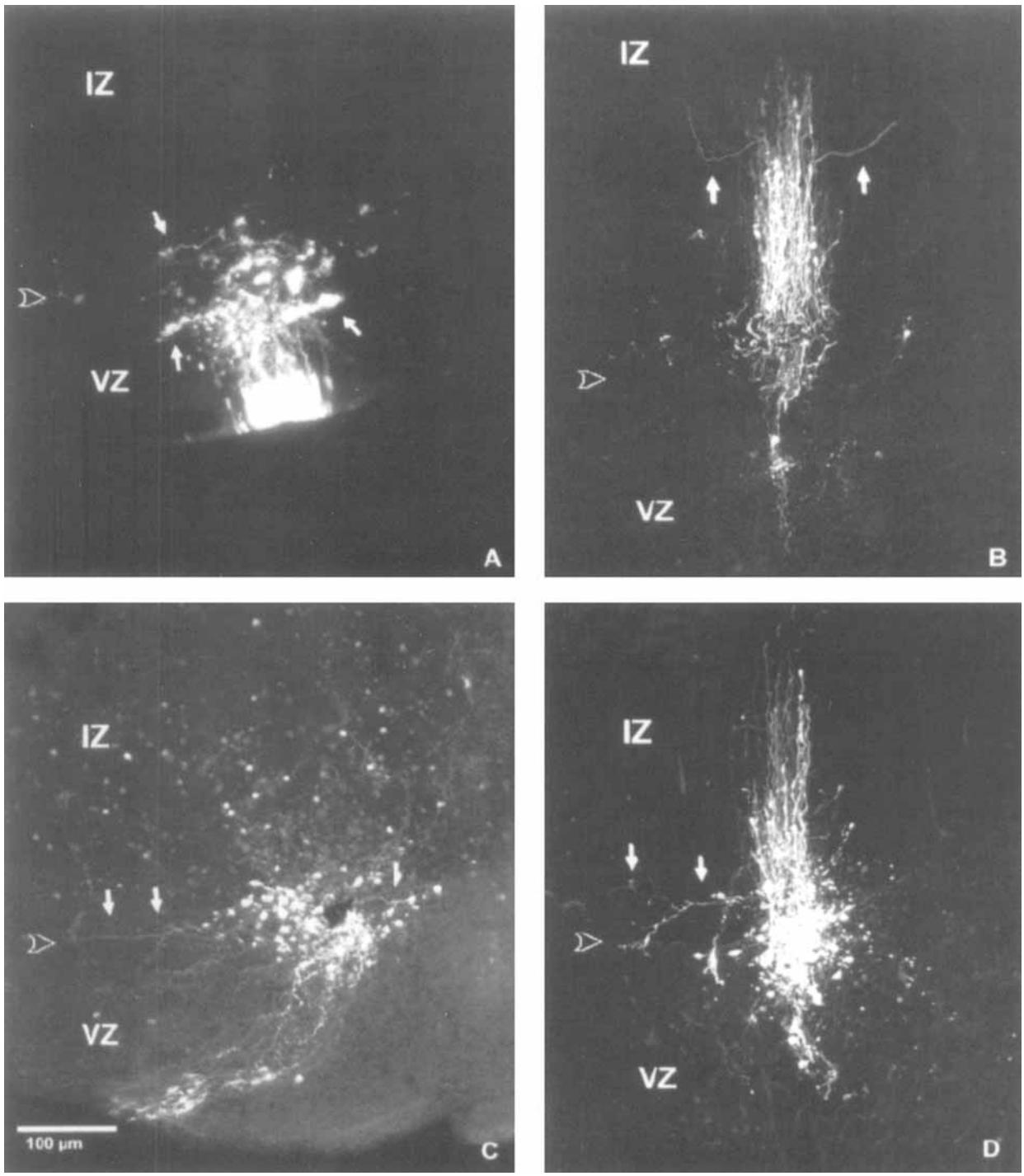

Fig. 2. **A–D:** Photomicrographs of dextran injections in or just outside the VZ in slices of ferret somatosensory cortex. After injections in the VZ, horizontally oriented cells can be seen (A) that are not following a radial path (arrows). Horizontally oriented fibers (arrows) can also be observed at the superficial portion of the VZ (C,D). In other instances (B), individual fibers peel off from a radially oriented bundle of fibers, head horizontally for a short distance, and then turn radially (arrows). Closely packed cells can be seen at the ventricular border in (A and C). Open arrowheads indicate the border between the VZ and the intermediate zone (IZ).

Juliano et al., 1990). Although the somatosensory cortex in ferrets and in other species displays a columnar organization, there is no feature comparable to ocular dominance columns in the somatic system of higher mammals, in which a structural unit relates precisely to a specific function. It is not obvious, therefore, that the developing somatosensory cortex will follow the same rules as the developing visual cortex.

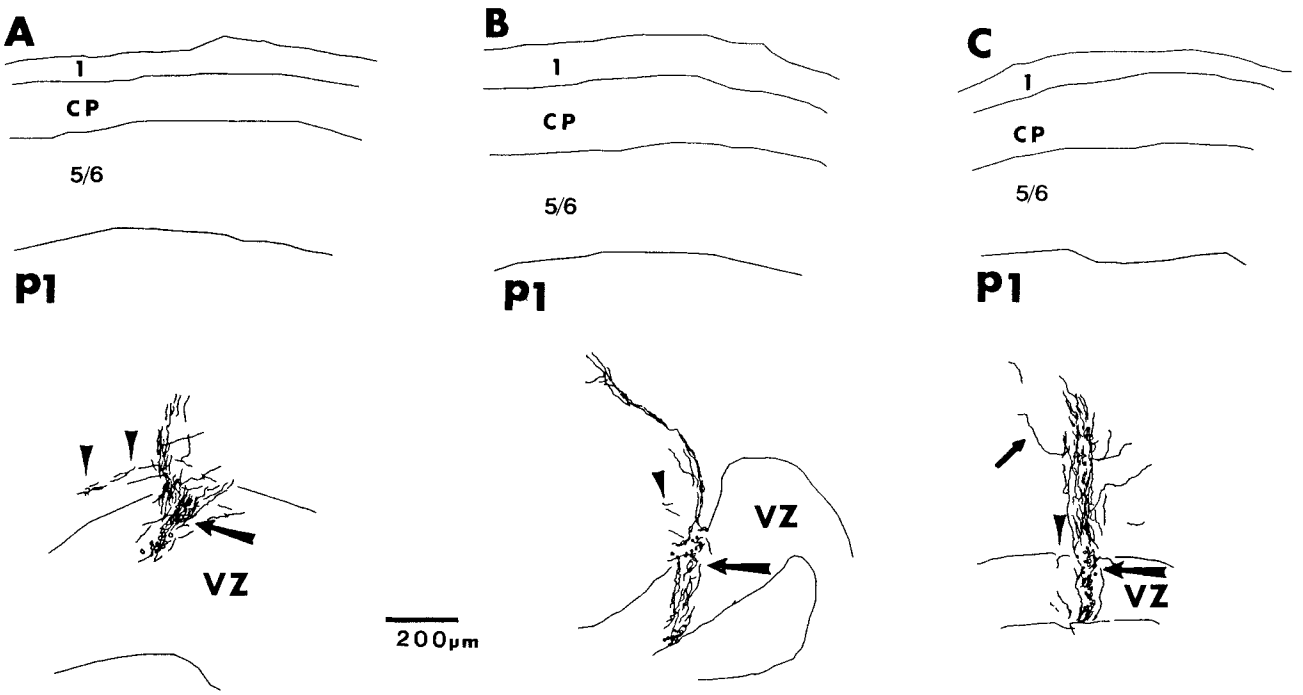

Fig. 3. **A–C:** Neurolucida drawings of label resulting from dextran injections into the VZ of P1 ferrets. The injection sites are indicated by the large arrows. In these examples, horizontally oriented fibers can be seen at the superficial portion of the VZ (arrowheads). In B, radially oriented fibers can be seen headed toward the cerebral cortex. In C, individual fibers diverge horizontally from the main bundle of fibers for a short distance and then head radially (small arrow). For abbreviations, see Figure 1; cortical layers are indicated by numbers.

On the other hand, a substantial amount of information is available regarding the development of rodent somatosensory cortex. In this system, there is a precise projection from the peripheral organ (i.e., the mystacial whisker) to discrete barreloid structures in the pathway from whisker to cortex, culminating in the barrel (see, e.g., Jones and Diamond, 1995). Recent experiments suggest that, in contrast to visual thalamocortical development, a substantial degree of topographic precision is present in the initial growth of axons into the barrel cortex (Agmon et al., 1993). This type of isomorphic connection pattern is not found in the higher mammals, and, as a result, rules governing development of the projection through the rodent somatosensory barrel system may not transfer to higher mammals either.

New technical developments allow analysis of immature connections in substantially more detail and at earlier time points than were previously available. Because ferrets have a relatively short gestational period, many neurons that populate the neocortex migrate to their final positions after birth; as a result, we were able to study very early aspects of developing cortex as neurons made their way from the ventricular zone (VZ) to the neocortex and established connections vertically and laterally. Such early developmental events regarding intrinsic connectivity have rarely been studied in detail in any species and specifically have not been studied in somatosensory cortex. Because our experiments conducted on maturing brains allowed us to visualize very immature connectional patterns, we were able to follow cells as they left the VZ and headed for the neocortex. The data presented here describe the development of radial units in the somatosensory cortex of the ferret, including their progression from the VZ to the cortex. In addition, we followed the development of the tangential growth of axons within the cortex to determine when lateral projections emerged, in which layers, and when a patchy distribution of intrinsic connections could be observed. The data described here can serve as a basis for understanding the development of the somatosensory cortex in relation to the birth of cortical neurons in this region and their migration to the cortex. A preliminary account of these data was presented in abstract form (Juliano et al., 1994).

MATERIALS AND METHODS

Twenty-nine ferrets of either sex ranging in age from P1 (day of birth = P0) to P62 were used in these experiments. Most animals were used to prepare slices of neocortex that were kept alive in an oxygenated tissue chamber perfused with artificial cerebrospinal fluid (aCSF). Injections of anatomic tracers were made into the slices, which were subsequently left in the chamber for 5–8 hours. The distribution of label was analyzed later either in the thick slice or after further cutting into sections. A smaller number of perfused, fixed brains were used for placement of the carbocyanine dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) either in the form of crystals or in solution (see Table 1).

Slice preparation and tracer injection

After delivering i.p. doses of pentobarbital Na (50 mg/kg) to the ferrets, each brain was removed and blocked, and

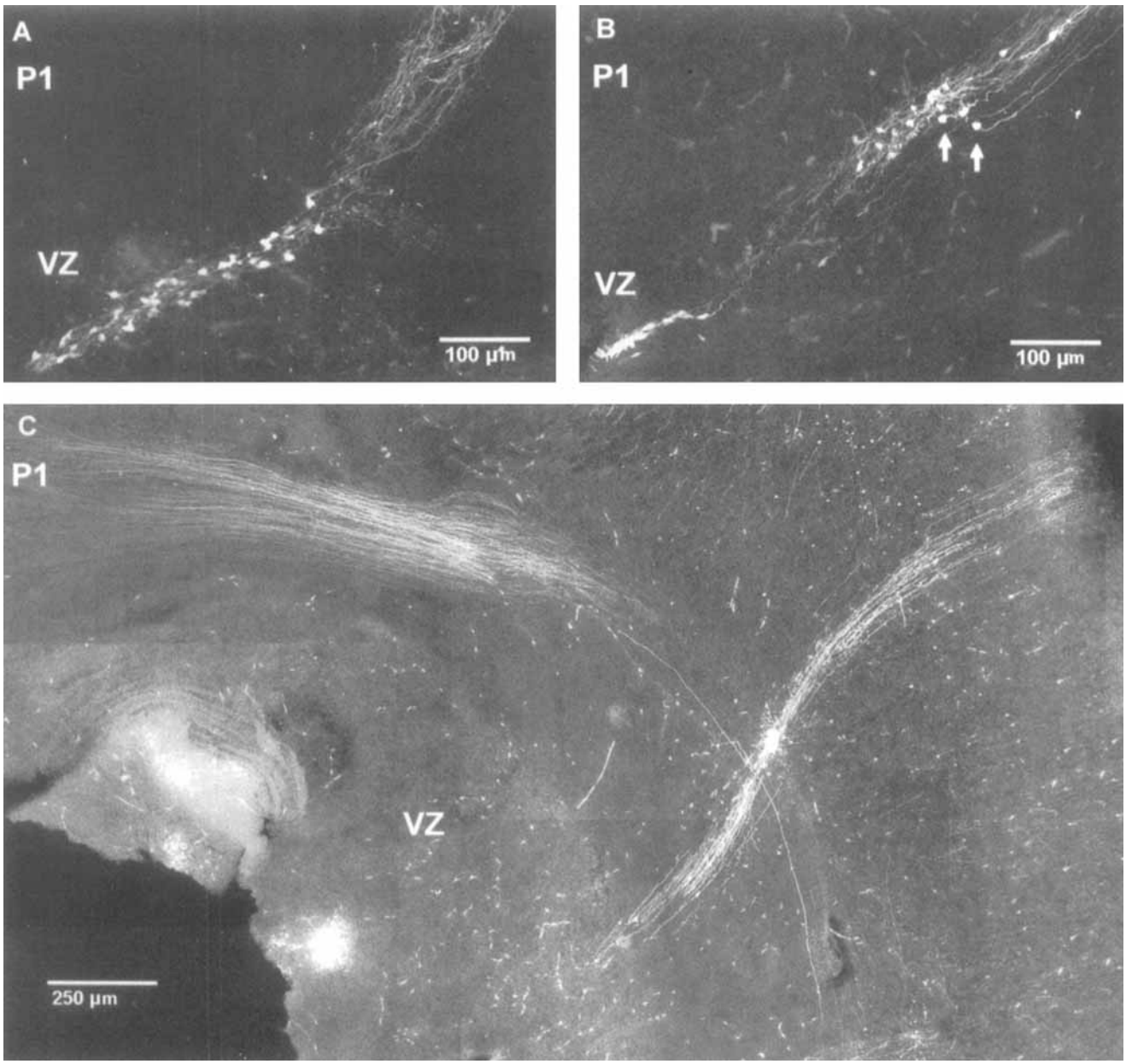

Fig. 4. Digitized images obtained from the confocal microscope resulting from dextran injections made into slices of ferret somatosensory cortex. Injections were made into the white matter at P1. The distinct radial pattern after such injections is evident. Presumptive radial glia can be seen piled up in the VZ, as seen in **A**. In a different injection, cells can be seen removed from the VZ presumably traveling toward the cerebral cortex (**B**, arrows). In **C**, fibers emanate from the injection site, likely toward distant targets. A fiber from a different injection in the same slice can be seen running through the label.

selected regions were cut in the coronal plane on a tissue chopper at 400 µm thickness under chilled conditions. The slices were collected, briefly placed in ice-cold aCSF, and then put in a tissue chamber and perfused continuously with oxygenated aCSF (O_2:CO_2;95:5). After an equilibration period of 30–40 minutes, injections were made under microscopic guidance into specific sites of each slice. Most of the injections delivered fluorescent-tagged dextrans (either Fluorescein dextran or "Fluororuby"; Molecular Probes, Eugene, OR) through pipettes that were 10–15 µm in diameter using iontophoresis for 3–4 minutes (3 µ Amp, positive alternating current). In a limited number of slices (n = 4), biocytin injections (Fluorescein biocytin; Molecular Probes) were made in a similar fashion. After the injections, the slices remained in the chamber for 5–8 hours. At this point, they were placed in fixative (4% buffered paraformaldehyde with 10% sucrose) overnight and transferred to the same fixative with 20% sucrose for 1-2 additional days. The slices were then examined either at full thickness or after cutting at 40 µm on a freezing microtome. Most

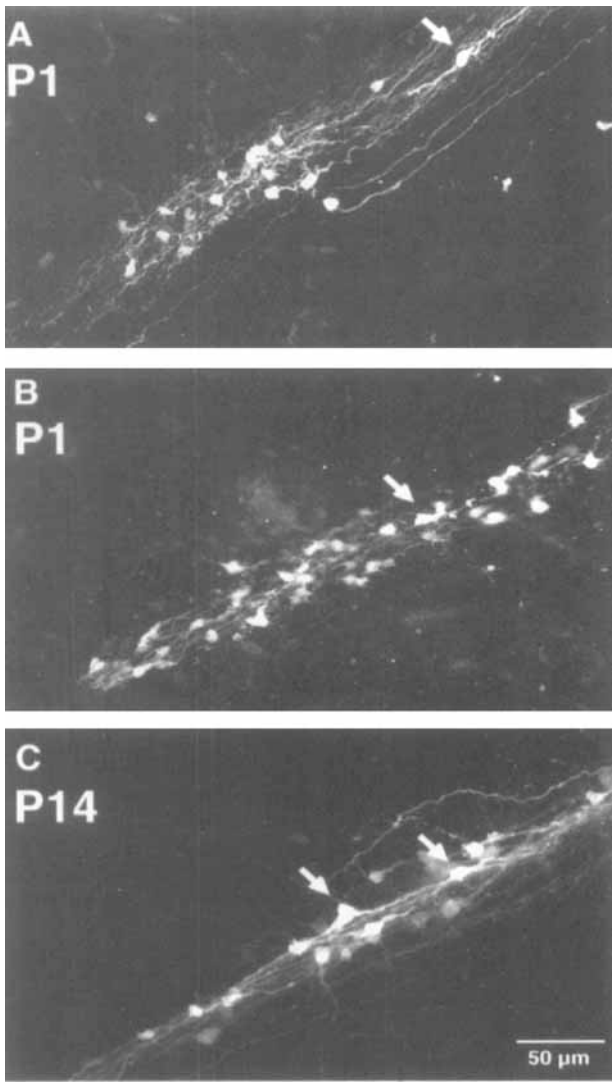

Fig. 5. **A–C:** Higher power views of presumptive neurons (indicated by arrows) that are more bipolar or multipolar than radial glial cells. Some of these cells are opposed to radial glial fibers. These images were obtained from animals at P1 or at P14.

slices were also stained with bisbenzimide (0.0025% in 0.1 M phosphate buffer) for identification of cytoarchitecture.

Immunohistochemistry

Representative slices were studied for immunoreactivity to antibodies directed against vimentin. This intermediate filament protein has been shown to be useful for identifying radial glial cells in forebrain of developing ferrets (Voigt, 1989; Cameron and Rakic, 1991). For this procedure, slices were removed from the chamber after the 5–8 hour incubation and placed in fixative (4% paraformaldehyde in 0.1 M phosphate buffer) for 1-2 hours. After this, the slices sank in phosphate buffer with 10% followed by 20% sucrose. They then cut at 40 μm thickness on a freezing microtome and placed immediately in blocking solution [10% normal horse serum, with 0.3% Triton X-100 in 0.1 M phosphate-buffered saline (PBS)]. They were then incubated in the primary antibody (1:1,000, antivimentin, clone V9; Boehringer Manheim) overnight, rinsed, and the secondary antibody added (1:50, Texas red, anti-mouse; Vector). Sections were visualized by using conventional fluorescent optics.

DiI injections

Eight animals of different ages (P1–18) received injections of the carbocyanine dye DiI. These were made into fixed brains by one of two methods (each hemisphere was used independently, allowing for use of 12 hemispheres): 1) by placing a small crystal of DiI on a pipette and inserting it into the somatosensory cortex or the ventral basal thalamus or 2) by pressure injections of solutions of DiI in ethanol (4%) using a Picospritzer. After this, the brain pieces were kept in fixative (4% paraformaldehyde in 0.1 M phosphate buffer) for 1–3 months. At this point, the brain blocks were embedded in agar and sectioned on a Vibratome at 50–200 μm thickness; most sections were also stained with bisbenzimide.

Gap-junction blockers

Several studies have shown that, early during the development of neocortex, cells communicate via gap junctions; as the cortex matures, the prevalence of gap-junction communication declines (see, e.g., Yuste et al., 1995). To determine the possibility that the tracers we used were transported through gap junctions, we conducted several experiments (12 slices, ages between P1–7) in which drugs known to block transmission through gap junctions were used in the slice preparation. These included halothane (2.0–2.5%, bubbled through the oxygenated tissue chamber) and octanol (1 mM) added to the perfusion solution (Peinado et al., 1993; Yuste et al., 1995). In these instances, slices were treated as described above, with either halothane or octanol added to the perfusion bath. Biocytin injections as well as dextran injections were made in the slices that were treated with gap-junction blockers. Biocytin is a tracer with a small molecular weight that is known to pass through gap junctions; we used this tracer for comparison with the dextran injections, which have a larger molecular weight. For several slices, both biocytin and Fluororuby were added to a single pipette, allowing us to evaluate the effect of both tracers in a single injection.

Analysis of dextran and DiI injections

Sections were analyzed by using conventional fluorescent microscopy or a confocal microscope (MRC-600; Bio-Rad). The label resulting from each injection was digitized and saved in files by using Comos software (developed by Bio-Rad). Many of the digitized images used in this paper were enhanced by increasing the contrast in order to visualize labeling details. The distribution of staining from each injection was drawn from the digitized files by using Neurolucida software (Microbrightfield, Inc.), which allowed us to visualize the details of each injection site. In cases where the thick slices were further sectioned into thinner sections, the Neurolucida program allowed us to combine information from several sections into a single drawing.

RESULTS

Injections were placed in several distinct sites in slices of somatosensory cortex of all ages. These included 1) the VZ (at earlier ages only), 2) the white matter or intermediate

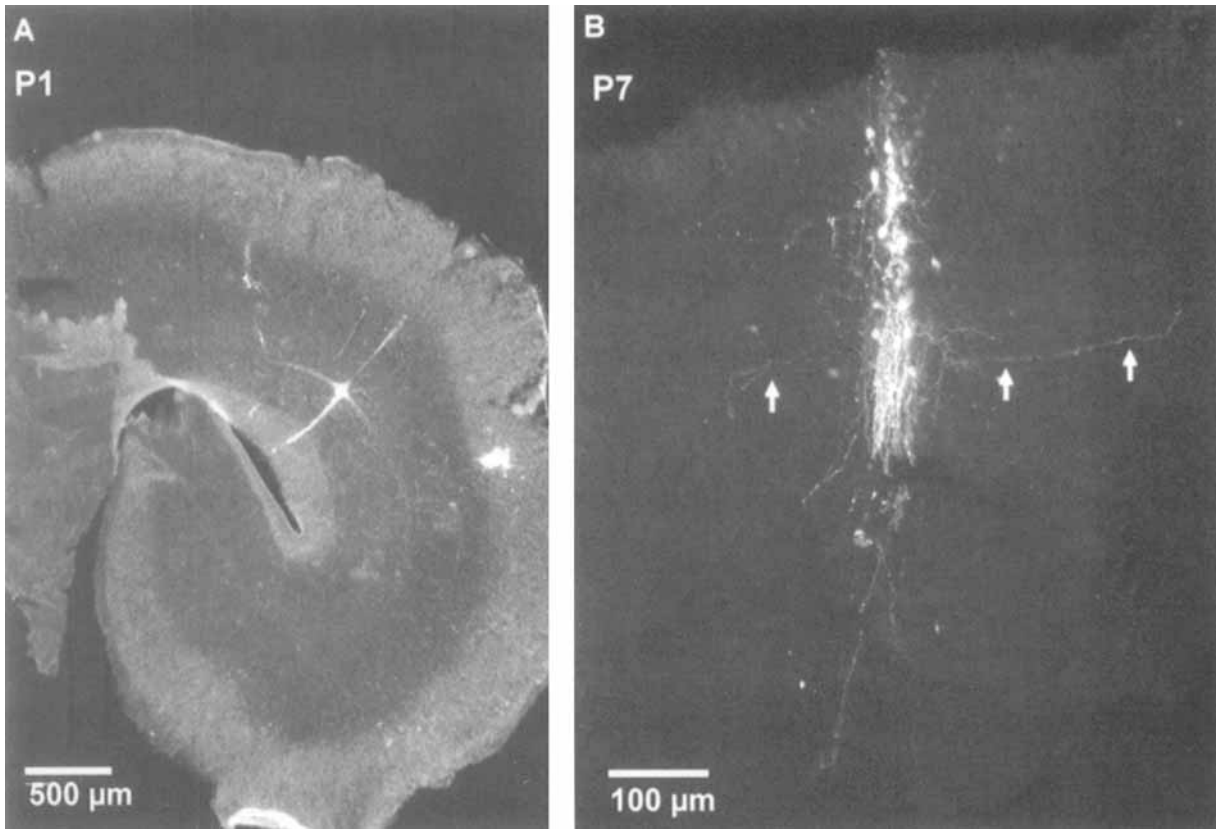

Fig. 6. Digitized images of slices showing dextran injections. **A:** Low-power view of the radial distribution of label after an injection into the white matter on P1. **B:** A linear array of cells resulting from an injection on P7 into the deep cortical plate; a few tangential fibers can be observed running in the deep cortex (arrows).

zone between the ventricle and the cortex, 3) deep cortical locations that included the subplate region or layers 5 and 6, and 4) superficial cortical locations, which included either the immature cortical plate or specific layer 2, 3, or 4, depending on the age of the animal. Injections were made into the center of each slice (i.e., at a depth of 200 μm). Because most slices were further sectioned at 40 μm thickness before analysis, the diameter of each injection site could be measured accurately. We found that the injections ranged from 13 to 35 μm in diameter (mean = 22 μm). These ranges were distributed equally for all ages studied; therefore, slices at each age contained injection sites of comparable size. Ferrets were examined at several specific times during postnatal development. The animals were placed into groups according to ages that showed similar patterns of development: P1-2, P5–7, P14, and P28 or older. A few animals at different ages received DiI crystals placed either directly into the somatosensory cortex or into the ventrobasal nucleus of the thalamus (see Table 1).

P1–2

At this age, the ferret somatosensory cortex is immature. Neogenesis of cortical neurons is largely, but not entirely, complete by this age; many neurons are still migrating to the cortex (Noctor et al., 1994). The sulci defining this cortical region are not fully formed and can be seen only as slight depressions in the cortical mantle. We were able to judge the position of the somatosensory cortex by using depressions made by the developing sulci and by the known relationships of somatosensory cortex to underlying deeper structures (such as the anterior commissure, caudate nucleus, globus pallidus, and thalamus). We conducted an extensive study of the cytoarchitecture and response properties of the adult somatosensory cortex, which is part of a different study (McLaughlin et al., 1995). Although features of the cytoarchitecture are not clearly distinguishable at this age, according to our observations of adult cytoarchitecture, electrophysiology, and correlation with morphologic features, the injections made in this study were located predominantly to areas 3b and 1 of somatosensory cortex. This was also confirmed by our finding that DiI injections into the targeted regions labeled clusters of cells in the ventrobasal nucleus of the thalamus (see below). The VZ is substantial, containing many densely packed cells. The cortex is quite thin, demonstrating poorly formed layers 5 and 6 and a thin cortical plate with no distinguishable layers. Layer 1 is present (Fig. 1A).

Injections into the VZ. The VZ appeared to have two divisions: one immediately adjacent to the ventricle and one in more superficial portions of the zone. After injections that labeled cells in the portion immediately lining the ventricle, the only clearly visible labeled cells were apparent radial glia attached to the ventricular border of the VZ.

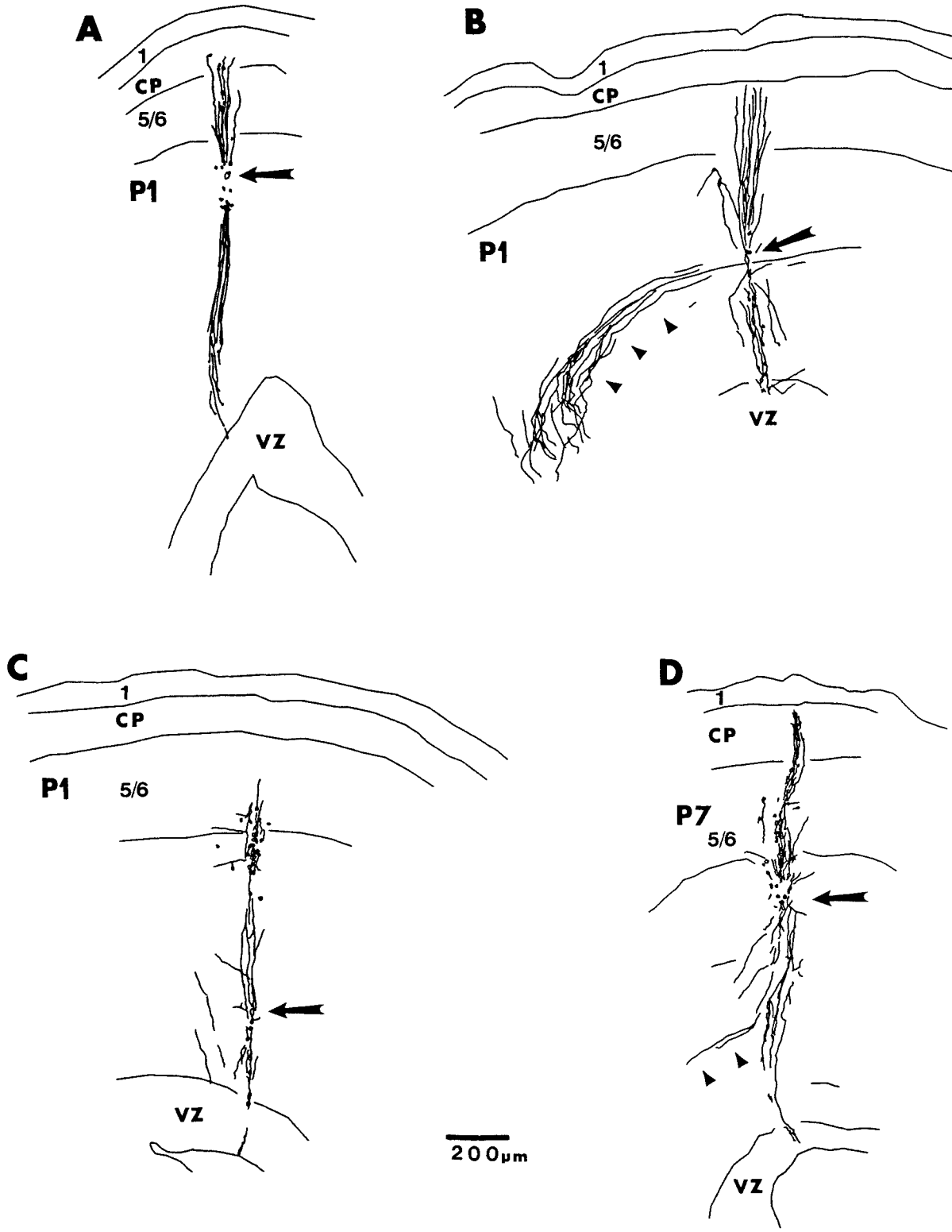

Fig. 7. Neurolucida drawings of label resulting from dextran injections into the intermediate zone of ferret slices taken at P1 (**A–C**) or at P7 (**D**). Injection sites are indicated by arrows. The radial nature of the label can be observed as well as fibers emanating from injection sites and traveling toward more distant targets (B and D, arrowheads). Cortical layers, when they are present, are indicated by numbers.

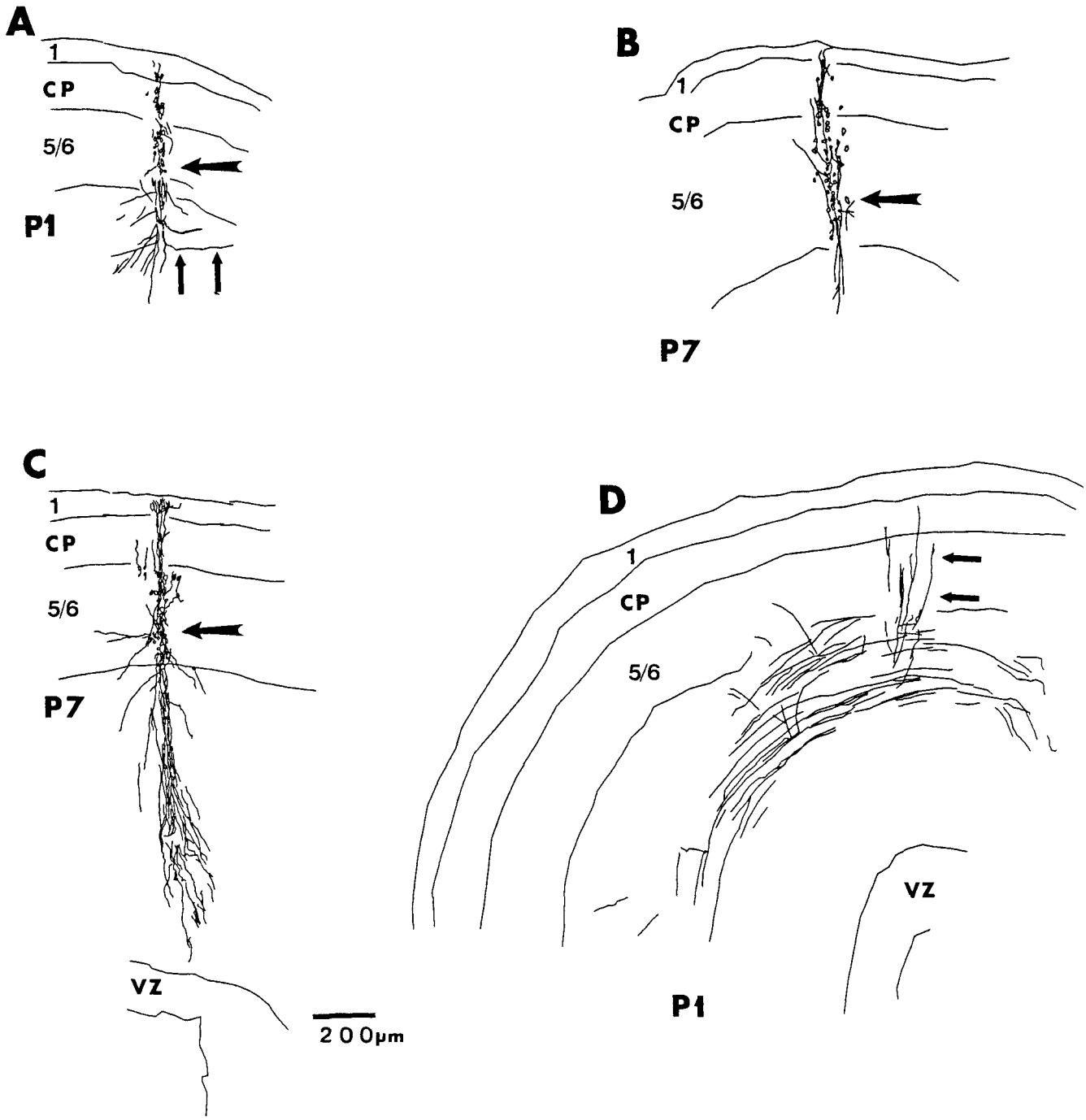

Fig. 8. Neurolucida drawings of label resulting from dextran injections into slices of ferret somatosensory cortex at P1 and at P7. The injection shown in **A** (large arrow) is in the deep cortical plate at P1. The injections shown in **B** and **C** (arrows) are also into the deep cortical plate, but at P7. Linear arrays of cells result from these injections, and occasional fibers travel laterally in the deep cortical plate and deep to the cortex in the underlying white matter (A, small arrows). Injection sites are indicated with large arrows. **D** represents label resulting from an injection into the intermediate zone at some distance from the distribution shown here. The fibers heading into the cortex are presumptive axons originating in the thalamus that terminate in discrete clusters within the somatosensory cortex (small arrows). The large bundle of fibers deep to the cortex travels in a location known to be occupied by thalamocortical axons, as determined by 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) injections into the somatosensory thalamus in similarly aged ferrets; an example is shown in Figure 7. Cortical layers, when distinguishable, are indicated by numbers.

These labeled cells were very close together and were not distributed tangentially (Figs. 2A,C, 3B,C). They extended processes toward the pia and could often be traced headed toward layer 1 (Fig. 3B). For the most part, these extending processes headed directly and radially toward the overlying cortex, but, occasionally, a single fiber would turn laterally

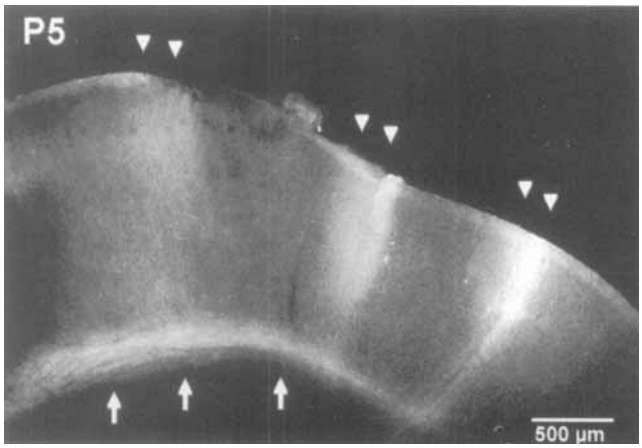

Fig. 9. Photomicrograph of a coronal section taken through ferret somatosensory cortex after a DiI injection into the ventrobasal thalamus at P5. The label can be seen distributing in well-formed, intermittent clusters within the cortex (arrowheads). A band of afferent fibers (arrows) runs in a position similar to the fiber bundle seen in Figure 6D.

for a short distance and then aim radially toward the cortex (Figs. 2B, 3C, 11D).

In the more superficial portion of the VZ, which corresponds to the subventricular zone, labeled cells were often dispersed in the tangential dimension, and they appeared generally to be less organized and "spread out" compared with the distribution near the ventricular edge (Fig. 2A,C,D). Horizontally oriented cells could be seen in this zone, and fibers ran laterally either near the superficial border or just outside it in the white matter (i.e., the intermediate zone; Figs. 2A,C,D, 3A).

Injections into white matter. The most obvious feature after injections of dextran into the white matter (i.e., intermediate zone) was the filling of many radial glial cells that extended from the VZ into the cortex. The radial glia appeared similar in morphology to previous descriptions and included features such as round cell bodies, long processes, and fine filopodial expansions (Figs. 4A,B, 7, 11C; Rakic, 1972, 1990; Cameron and Rakic, 1991; Voigt, 1989). Often, the cell bodies were close to the VZ and were attached to the border of the VZ; equally frequently, the cell bodies had moved from the ventricular border but continued to have long process that extended superficially (Fig. 4A,B). At this age and also at slightly older ages, studded on the processes of the radial glia, there were cells that may have been neurons migrating toward the cortex. Morphologically, the somata of these cells were more elongated or multipolar than the presumptive glial cells, and shorter processes often could be observed extending from the cell body (Fig. 5A–C). In several instances, we double labeled the dextran-injected sections with antibodies directed against vimentin. By using these data, we found many vimentin-positive cells with long processes, similar to those reported in other studies (see, e.g., Misson et al., 1988; Voigt, 1989). Along the vimentin-positive fibers, there were dextran-labeled cells that were not double labeled with vimentin immunoreactivity but that were aligned along the vimentin-positive fibers, similar to the migrating neurons described by Misson et al. (1991; see Fig. 11E). Occasional double-labeled fibers were also observed.

In addition, within the white matter or intermediate zone, axons could be seen leaving the injection site and were followed clearly toward the corpus callosum or the thalamus (Figs. 4C, 7B). It was not always clear whether these axons were collaterals from the labeled vertical array or whether they were labeled fibers of passage. Obviously, the label could be followed only for limited distances in a slice. In the DiI labeled brains, however, similar injections could easily be followed to the thalamus or headed toward the opposite hemisphere. We occasionally observed fibers that appeared to be thalamic axons terminating in the cortex after dextran injections in slices. Although these could not be unequivocally confirmed as originating from the thalamus, this seems the most likely source because of the position that the fibers occupied in the intermediate zone (Fig. 8D). In these instances, the fibers appeared to be loosely organized into a cluster of terminations in the cortex. The terminal clusters contained presumptive axons that were oriented radially on route to the somatosensory cortex.

Labeled cells observed in the cortex or just deep to the cortex usually followed strictly the processes of the radial glia (Figs. 4B, 7C). They were located along the radial "stream" of processes. In the subplate region, an occasional labeled cell was removed from the radial pattern and occurred lateral to the column-like stream of label (Fig. 7B).

Injections into deep cortical layers or subplate. These injections also predominantly labeled a stream of cells. Many cells were stacked in a linear fashion from the deepest point of the injection to the pia (Figs. 6B, 8A,B). The cells that were stacked in a stream varied in their morphology to some degree, but they tended to have either round or elongated somata.

Although the most obvious feature of the labeling pattern at this age (P1-2) was the column-like pile of cells, many fibers could be observed running laterally. These fibers were located predominantly just deep to the cortex, but they also occurred within layers 5 and 6 (Fig. 8A); the labeled fibers could often be followed for long distances. The cortical plate (i.e., immature layers 2–4) was not invaded by lateral growth. We observed the same finding after DiI injections into the cortex. Although these injections tended to be substantially larger than the dextran injections, after cortical plate injections, no fibers extended laterally into the cortical plate, although many presumptive axons invaded the deep layers. In several experiments, a small crystal of DiI was placed into the somatosensory cortical plate of ferrets at P1, and retrogradely labeled neurons were found in the thalamus, suggesting that thalamic axons reached the cortex by P1 (Fig. 11A,B).

Injections into cortical plate. The results from cortical plate injections were not as consistent in character as those into deeper regions. In most instances, a radial column of labeled cells resulted from a cortical plate injection. In a few cases, however, injections led to a small number of labeled cells that were distributed horizontally through the cortical plate.

P5–7

At this age, the architecture of the cortex and VZ is similar to the structure at P1. The VZ is still quite large, and the cortex remains immature. The deeper cortical layers 5 and 6 can be identified more clearly, and the cortical plate is thicker (Figs. 1C, 6A). A prominent subplate remains.

Injections into the VZ and white matter. These injections were similar in character to the results in P1-2

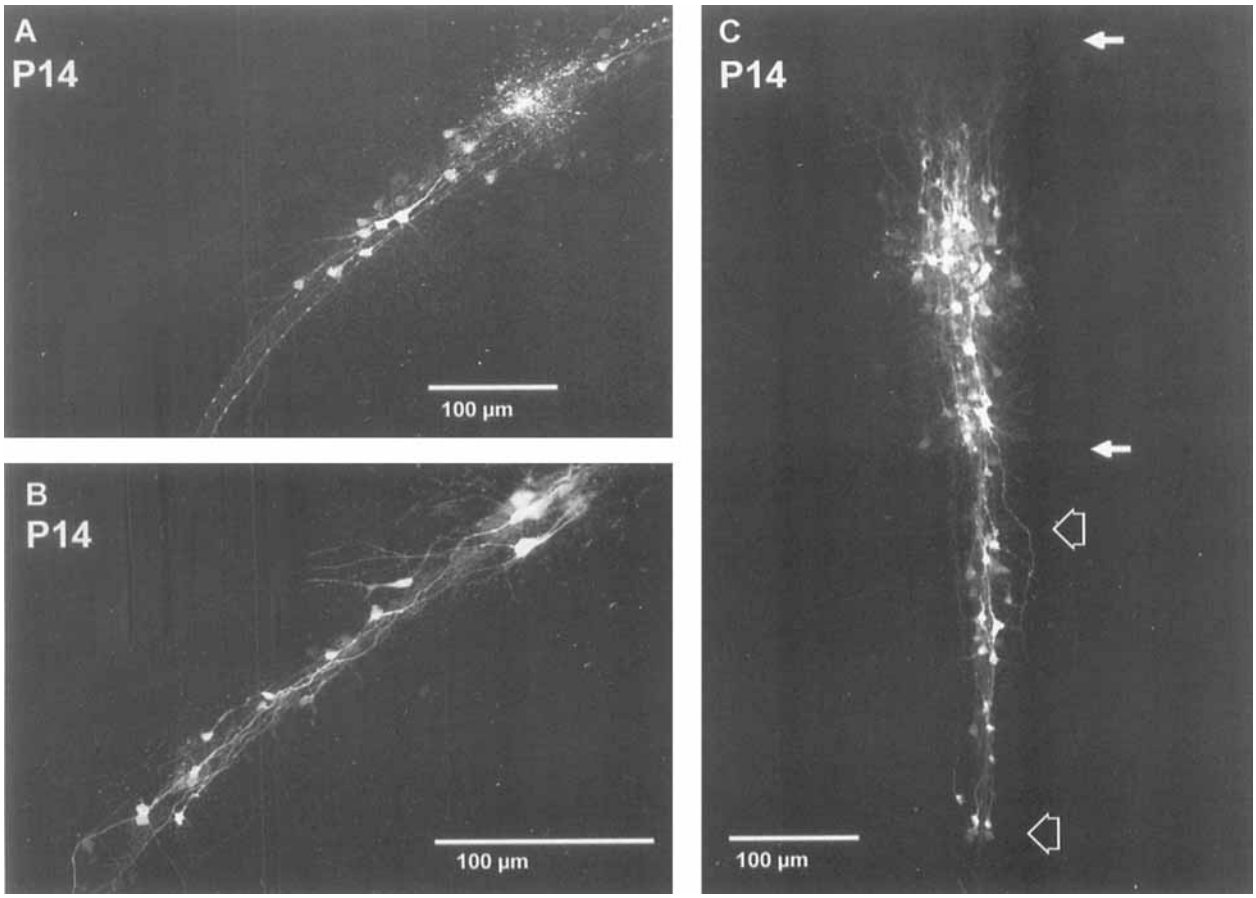

Fig. 10. **A–C:** Digitized images obtained from the confocal microscope after dextran injections into the deep cortical plate (B,C) or white matter (A) in slices of ferret somatosensory cortex at P14. At this age, most of the label is moved from the VZ and white matter and is located within, and immediately beneath, the cortex. In B and C, the increased complexity of cellular morphology can be seen with many processes extending into surrounding regions and a more complex dendritic morphology. The arrows in C indicate the pial border (top solid arrow) and the junction between layer 6 and the white matter (bottom solid arrow). The open arrows in C delimit the approximate region shown in A and B of different injections.

animals. Injection sites in the VZ or white matter labeled thin radial streams of processes that extended toward more superficial sites (Fig. 7D).

Injections into subplate and deep cortex. Injections into the subplate also tended to label radial columns of cells. Linear arrays extended from the injection site to superficial cortex. Processes that more distinctly resembled dendrites were also seen. Injections directly into layer 5 or 6 led to more lateral extensions of axons into surrounding cortex than similarly placed injections at P1-2 (Fig. 8C).

Injections into cortical plate. At this age, injections into the not-yet-distinct superficial cortical layers often resulted in a number of well-filled cells in the vicinity of the injection, with processes extending into surrounding cortex. Retrogradely labeled cells were rarely observed distant from the immediate vicinity of the injection site. Injections into the cortical plate also occasionally led to a vertical array of cells confined to the upper layers.

Projections from the thalamus to the somatosensory cortex. Although we did not study thalamocortical projections in detail, we injected DiI into the thalamus of three kits on P5; these injections filled most of the ventrobasal thalamus. The resulting cortical label was clearly organized into clusters that occurred at a periodicity of about 750 μm from the center of one patch to the next. Although individual fibers could not be distinguished, the patchy nature of the overall distribution was easily apparent (Fig. 9). The example illustrated in Figure 9 was taken from rostral portions of somatosensory cortex; the injection could be traced clearly to the thalamus.

P14–16

The cortex is substantially thicker at this age than at earlier ages; layers 5 and 6 were more easily distinguished. Although the cortical plate was still immature, layers 2–4 began to be differentiated. The VZ, although still present, was considerably smaller than at P7.

Injections into white matter. Injections into the white matter also labeled radial distributions, and a thin line of cells could often be traced from the injection site to the cortex (Fig. 10). Within the cortex itself, the labeled cells had processes that appeared to be more mature than those seen at younger ages.

Injections into deep cortex or subplate. Although injections into layer 5 and 6 also produced a radial distribution

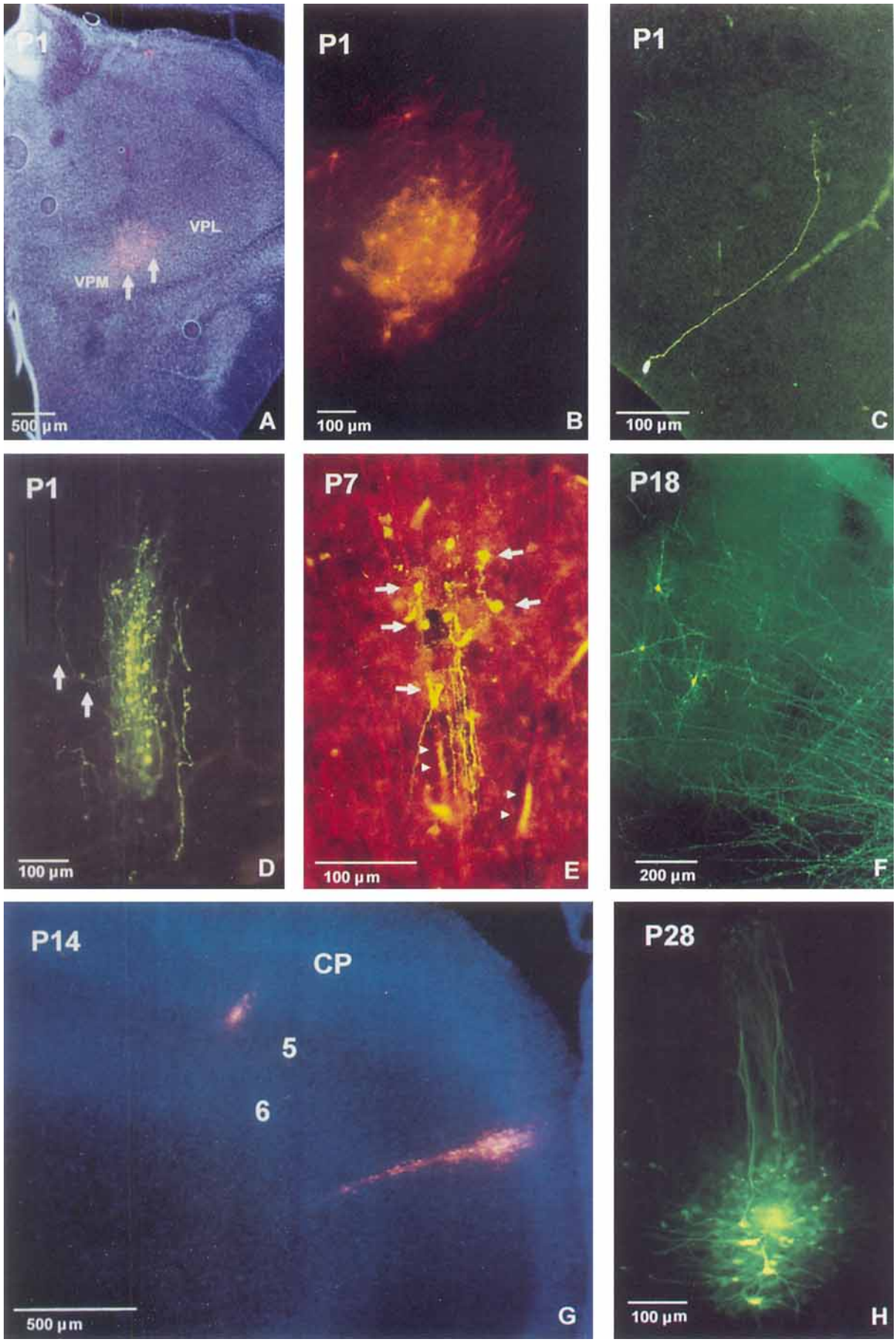
P1
VPL
VPM
500 µm
A
P1
100 µm
B
P1
100 µm
C
P1
100 µm
D
P7
100 µm
E
P18
200 µm
F
P14
CP
5
6
500 µm
G
P28
100 µm
H

Figure 11

of label, this result was primarily due to labeled dendrites from cells filled with the dye that extended toward the cortical surface rather than cells piled on top of one another. Injections into layer 5 labeled pyramidal cells that sent their axons deeply. A thin line of cells often trailed into the subplate and subjacent white matter (Figs. 10A–C, 11G, 12A,B). Many fibers also extended into surrounding cortex in all layers, but they did not appear to be organized in a precise pattern.

Injections into cortical plate. Labeled cells appeared up to several hundred microns from the injection site. Fibers extended into neighboring regions, but they appeared to be restricted more to the upper layers than the pattern after deeper injections, without a particular stratification or pattern; labeled fibers were present in all cortical layers (Fig. 12A–D). Injections into central cortex led to a vertical array of cells (Fig. 12E)

P28 or older

At this age, the architecture of the brain looked mature. The proliferative zone shrank to a thin layer of cells lining the ventricle. The cortical layers were distinct and could be identified clearly (Fig. 1B). Because the VZ was nonexistent, there were no injections in that site.

Injections into deep cortex. At this age, the character of the label changed. Linear arrays of cells did not occur. Clusters of large cells were labeled with typical apical dendrites that extended toward the cortical surface (Fig. 11H). Numerous processes ran laterally within layers 5 and 6 (most densely within layer 6; Fig. 13A,B). Labeled cells could be seen in layers 2 and 3 within the column of the injection (Fig. 13B). The complex morphology of individual cells was evident, with processes extending from the soma; many multipolar cells were labeled.

Injections into layer 4. Injections into this layer caused a vertical distribution of label. A two-tiered distribution of labeled cells were seen, with retrogradely labeled cells observed in layers 3 and 5. A scattering of labeled cells were observed in layer 4, immediately adjacent to the injection site.

Injections into layers 2–3. These injections were distinct. Axons traveled laterally and often terminated in small clusters of retrogradely labeled cells in layer 3 (Fig. 13D,E). A few labeled cells were present in all layers within the vertical column of the injection, but they occurred more prominently in layer 5, resulting in a bilaminar pattern from the distributions in layer 3 and layer 5. Fibers could be seen in all layers, but more were more heavily distributed in layers 2-3 and, to some extent, in layers 5-6 (Fig. 13D). DiI injections at this age also led to distinct clusters in layer 2-3. Again, although these injections were substantially larger than the dextran injections, clear clusters were found (Fig. 13E). Because the DiI injections were large, substantially more labeled axons were observed in the deeper layers. A cluster of cells from the reconstruction in Figure 13E can be seen in Figure 11F.

Experiments using gap-junction blockers. In slices taken either at P1 or at P7 with either halothane or octanol in the perfusion, the pattern of label after dextran injections did not change from normal. In each experiment in this series, selected slices were injected with biocytin and dextran; in most cases, the pipette contained both biocytin and dextran, which were delivered together by iontophoresis. The distribution of fluorescent dextran was in a distinct radial pattern and, otherwise, was similar to the configuration in a normal ferret. The occurrence of label resulting from the biocytin injections, on the other hand, was distinctly different in character from that resulting from the dextran injections. Biocytin injections resulted in a small spot that was highly localized, with no radial spread (Fig. 14). In other "control" experiments in which no halothane or octanol was delivered, the biocytin label was much more widespread and was not constrained to a single, small spot.

DISCUSSION

Development of vertical organization and patchy intrinsic connections

During the development of ferret somatosensory cortex, cells travel from the VZ on scaffolding provided by radial glia; the scaffold extends from the cortex to the VZ. During the time when the brain matures and the cortical layers form, individual cells extend processes into surrounding cortex, first into the originally established deeper cortical layers and, later, into newly arriving, more superficial layers. When distinct layers become firmly established, individual neurons form patchy connections between clusters of cells: a type of connectional pattern that appears to be ubiquitous in neocortex. The connectional pattern evolves from a strongly radial distribution, which is dominated by radial glia and associated cells, to a combined radial and tangential pattern, including vertically linked cells and tangentially running fibers that distribute in patches. In addition, the horizontal projection of axons into surrounding cortex initially occurs without precise stratification of axons into specific layers or clustering of neurons in distinct aggregates, but develops into a more specific distribution in mature somatosensory cortex. The observation that ferret somatosensory cortex develops into precisely linked "columns" and patches is additional evidence suggesting that a columnar organization is highly significant in the function of mammalian cerebral cortex. Although distinct patches seem to be present in the ferret somatosensory

Fig. 11. **A:** Photomicrograph of a coronal section taken through the ventrobasal thalamus of a ferret after a DiI injection into the somatosensory cortex at P1. The section is stained with bisbenzimide to visualize cytoarchitecture; labeled neurons can be seen at the juncture between the ventral posterior medial (VPM) and the ventral posterior lateral (VPL) nuclei (arrows). **B:** Photomicrograph of a higher power view of the labeled neurons seen in A; the photomicrograph in B was photographed with a green filter only; thus, the bisbenzimide stain is not visualized. **C:** Photomicrograph of a presumptive radial glial cell labeled after a dextran injection into the white matter of a slice taken at P1. **D:** Photomicrograph of fibers labeled after an injection of fluorescein dextran into the VZ of a slice obtained from a P1 ferret. An occasional fiber can be seen traveling tangentially for a short distance (arrows) and then turning radially. **E:** Photomicrograph of a section double labeled for immunoreactivity of antibodies directed against vimentin (red) and cells labeled after dextran injection into the white matter (yellow green). A number of cells not positively stained for vimentin are studded along the immunoreactive fibers (arrows), suggesting that the dextran-labeled cells are neurons. An occasional double-labeled fiber can be seen (arrowheads). **F:** Photomicrograph of a cluster of labeled cells observed after an injection of DiI into the somatosensory cortex of a P18 ferret. The injection site cannot be seen, but it is to the right of the labeled cluster. **G:** Low-power photomicrograph of a slice taken from a P14 ferret. The slice is stained with bisbenzimide to visualize cytoarchitecture, and the label resulting from two injections of dextran can be seen in red. Numbers indicate cortical layers. **H:** Photomicrograph of a dextran injection into a slice taken from a P28 ferret. Many large cells with complex dendritic morphology are labeled. Apical dendrites extend radially toward the pia.

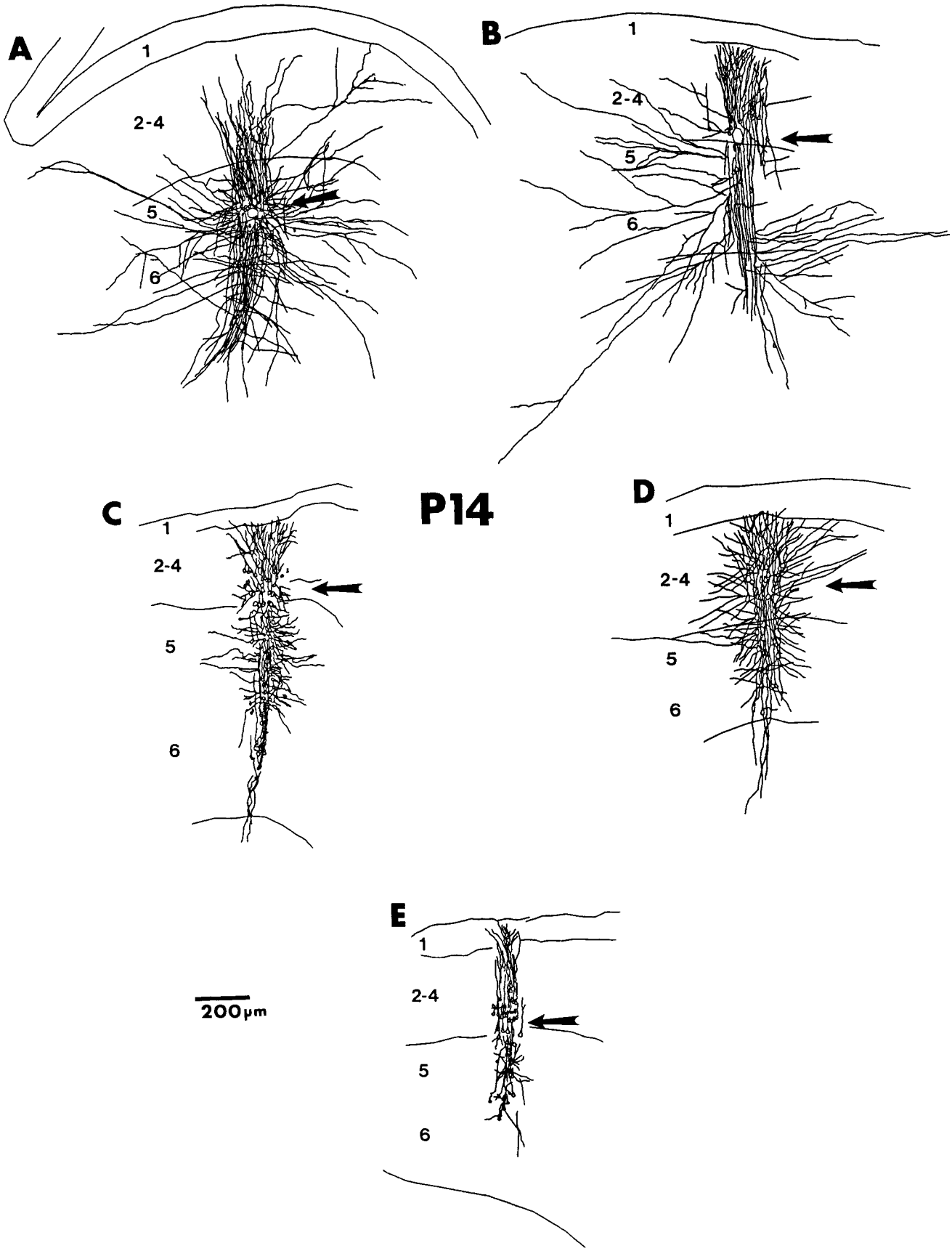

Fig. 12. Neurolucida drawings of label resulting from dextran injections into the deep cortex (**A**), superficial cortex (**B–D**), and central region of cortex (**E**) all in slices of P14 ferret somatosensory cortex. Many fibers extend into surrounding cortex after deep (A) or superficial (B–D) injections. Injections into central cortical regions lead to a vertical array of labeled cells confined to the upper half of the cortex (E). Cortical layers are indicated by numbers, and injection sites are indicated by arrows.

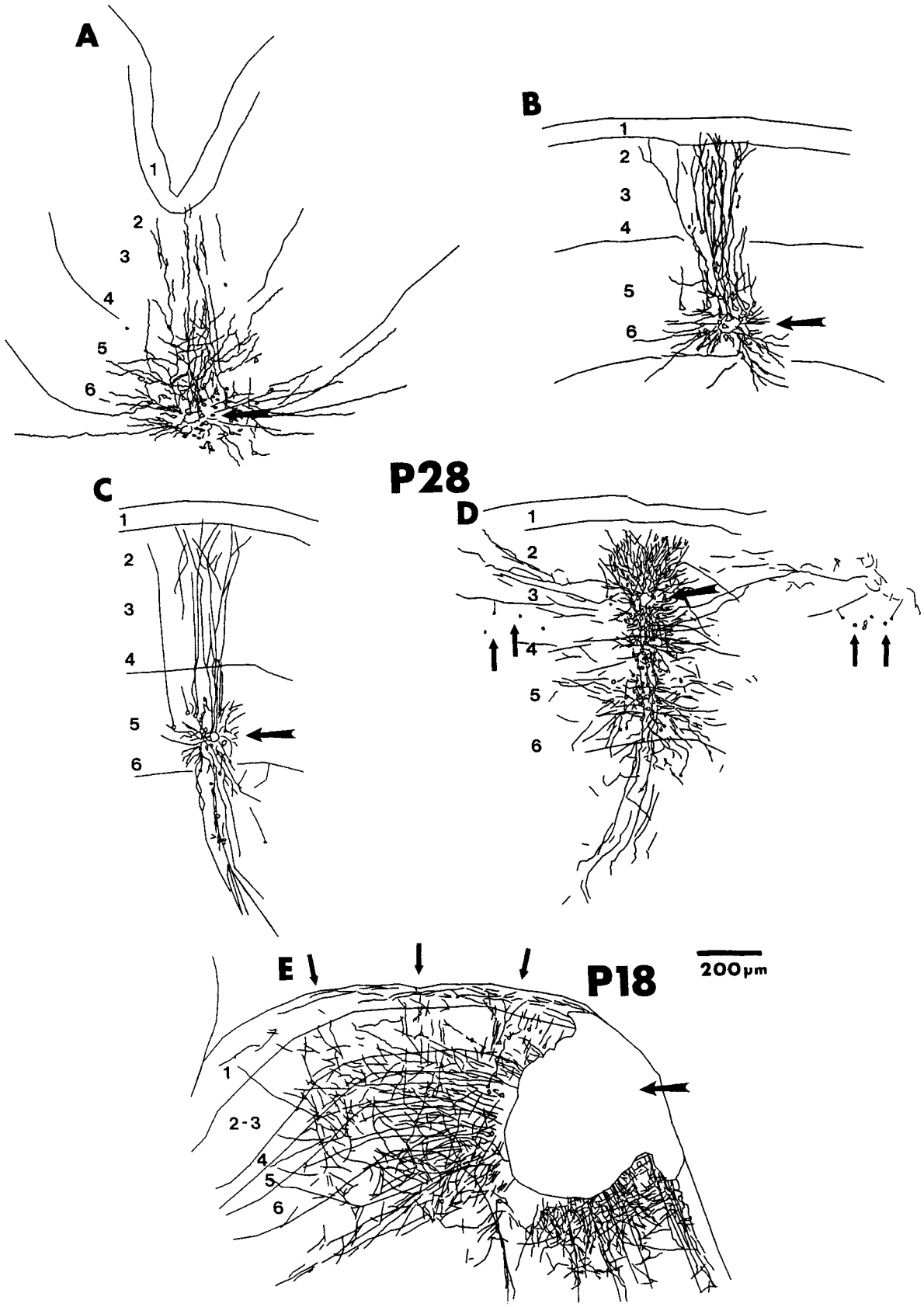

Fig. 13. Neurolucida drawings of label resulting from dextran injections into slices taken from P28 ferrets. Injections were made into deep cortex (layers 5 and 6; **A–C**), or superficial layers of the cortex (**D**). Injections into layers 5 or 6 lead to a number of well-filled cells that extend apical dendrites toward the pia. Injections into the superficial layers also lead to clusters of cells a few hundred microns from the injection site (D, arrows). **E** shows a much larger DiI injection into all cortical layers. Because of the injection size, many fibers can be seen running laterally in deeper layers. Clear clustering can be seen in the upper layers (small arrows). Injection sites are indicated by large arrows.

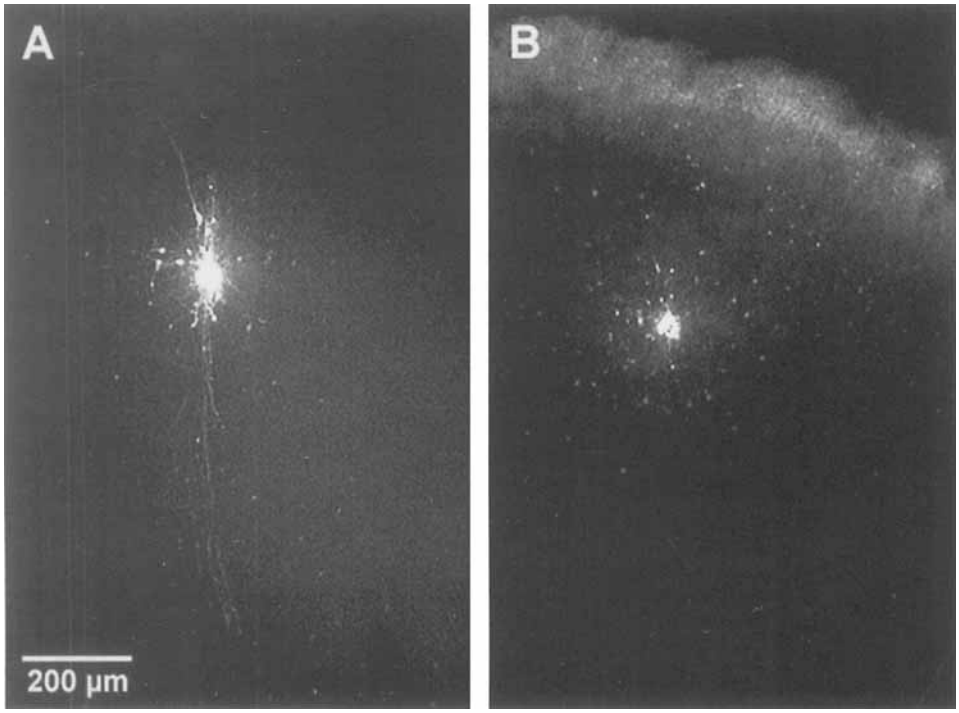

Fig. 14. Photomicrographs of the same section taken with either a green (**A**) or a red (**B**) filter. This is a slice obtained from a P1 animal that was injected simultaneously with biocytin (B) and fluorescein dextran (A), while the oxygenated slice chamber was bubbled with halothane. The label resulting from the dextran injection appears normal, with a distinct radial pattern (A), whereas the label resulting from the biocytin injection has no distinct pattern and seems to be localized to the injection site (B).

cortex structurally and functionally, their precise operation is not clear in ferret or in the somatosensory cortex of other higher mammals. One possibility suggested by work both in the visual cortex and in the somatosensory cortex is that the patchy connections link columns of cells that possess a response feature in common with the cells of origin (Ts'o and Gilbert, 1988; Gilbert and Wiesel, 1989; Juliano et al., 1990; Gilbert, 1992).

We also observed that thalamic terminations seemed to be highly organized into distinct clusters by P5. Earlier studies indicate that thalamocortical terminations in adult somatosensory cortex in a number of species are also organized into patches (Kosar and Hand, 1981; Jones et al., 1982). The projections from the ventrobasal thalamus synapse in layer 4 or in layers 4 and 3. We found it surprising that, after a large DiI injection in the thalamus, the label in the somatosensory cortex formed patches extending throughout the thickness of the cortex rather than terminating distinctly in layer 4. This may be partly due to the immaturity of the cortex; that is, layer 4 is not yet clearly formed. It may also result from the ability of DiI to diffuse transneuronally across synapses or other connections between cells. These data support a number of recent findings suggesting that thalamic afferents in the somatosensory system of rodents arrive earlier, and in a more precisely organized form, than was thought originally (Catalano et al., 1991; Agmon et al., 1993; O'Leary et al., 1994a; Schlaggar and O'Leary, 1994). If this proves to be a universal finding in somatosensory regions of cortex, then it will be an interesting contrast to developing thalamocortical afferent fibers in visual cortex. In the visual system of young mammals, projections from each eye share a high degree of overlap from thalamic afferents into neighboring ocular dominance columns. These overlapping patches undergo refinement during normal development, leading to precisely segregated patches of thalamic terminals from each eye (for review, see Goodman and Shatz, 1995). The columnar terminations in somatosensory regions, however, appear to require comparatively little "pruning" and are relatively focused on arrival, whereas, in the visual cortex, afferents from the lateral geniculate require a combination of elimination of nonspecific collaterals and directed outgrowth into appropriate sites to produce normal, adult-like, discrete patches (Antonini and Stryker, 1993b). This may be an interesting dichotomy between cortical regions.

What contributes toward forming the radial pattern?

The distribution of radial glia and their extension from the VZ to cerebral cortex is a feature of cortical development that has been known for many years (for review, see Rakic, 1995). The finding that small injections of tracers link a column-like collection of cells during development is a more recent finding. Katz and colleagues established that small cortical injections of biocytin in rat somatosensory cortex lead to radial columns of labeled cells, similar to the findings presented here (Peinado et al., 1993). They provide convincing evidence that, in young rat cortex, radial columns result from gap-junction communication between neighboring cells. In our experiments, the distribution of label is almost certainly not due to gap-junction communica-

tion, because the vast majority of injections used dextrans tagged with fluorescein or rhodamine. The dextrans we used are too large (10,000–30,000 MW) to pass through gap junctions, because these cellular specializations appear to accommodate passage of small molecules and ions (Saez et al., 1989). To definitively exclude the possibility that the dextran injections in our study might have passed through gap junctions, we exposed the slices to halothane or octanol (both blockers of gap junctions); neither of these drugs blocked the radial distribution pattern. In the same injection site, transport of biocytin fluorescein (814 MW) was blocked completely. These findings strongly suggest that the column-like pattern seen in young ferrets does not result from communication between gap junctions.

Another possible contribution to the radial pattern is the presence of radially arranged synapses. In young ferrets (14 days or less), synaptic contacts are not likely to be very numerous or strong. Voigt et al. (1993), by using antibodies directed against synaptophysin, demonstrated that synaptogenesis in ferret cortex develops according to the arrival of neurons into the cortex, i.e., development of synapses increases in deeper layers before more superficial layers. In young animals, synapse density is quite low; however, even at P0, synaptophysin immunoreactivity is strong in the subplate of the occipital cortex. Denser synaptophysin reactivity, therefore, is likely to extend into deep cortical plate in more rostral regions of cortex, because rostral regions develop earlier.

A recent study by Dalva and Katz (1994) demonstrated that synapses are functional in slightly older ferrets. In young animals (i.e., P14–17), local synaptic activity and connections are strong, leading to a high density of functional contacts within a column of cells. While the ferret matures, axons refine their lateral projections, and functional contacts to clusters of cells at a greater distance become stronger. In relation to the experiments presented here, a similar developmental pattern might contribute to the distribution that we observed. Local synaptic activity is arranged in a radial column, resulting in the distribution of label observed in newborn animals in the current study. Synapses are more likely to be found within a radial column at a young age and at greater distances when horizontal connections extend to the newly formed cortical layers.

Burkhalter et al. (1993) reported a similar overall anatomical pattern using DiI injections in developing human visual cortex. They described an early dominant pattern of vertical development followed by lateral extensions into surrounding cortex. They also reported that functional circuits are first established vertically and that interlaminar horizontal functional circuits develop later.

It is likely that the pattern of dextran label visualized in our slices is a result of a combination of factors, including complete filling of cells at the injection site as well as a small amount of both retrograde and anterograde transport. We assume that fibers of passage were filled in the young animals (7 days old or less), which was demonstrated by the labeling of presumptive axons after injections into the intermediate zone. The capacity of dextrans to be taken up by fibers of passage seems to reduce as the animals get older. From the age of P14 and older, we did not see label easily construed as filling of axons of passage. Indeed, in a number of slices obtained from older animals, we attempted specifically to inject "fibers of passage" in the hope of injecting thalamic afferents beneath the cortex, but we were unable to do so despite successful injections in the cortex of the same slice. It is not clear why fibers lose their ability to take up dyes at older ages, but similar observations have been noted in a number of other studies. Injections into older animals, however, were quite effective in completely filling cells at the site of an injection (see, e.g., Fig. 9H).

A series of studies by O'Leary and colleagues (for review, see O'Leary et al., 1994b) found that thalamocortical afferents avoid growing into an immature cortical plate and preferentially select more mature cortical preparations. Gotz et al. (1992) also demonstrated that afferents from the thalamus will not grow into cortical explant cultures until the usual target layer, i.e., layer 4, is formed. Although our study of thalamic afferents was limited in this area, the data from O'Leary and colleagues and from Bolz and colleagues (Bolz, 1994) imply that the immature cortical plate is not a permissive substrate for axonal growth and that a certain level of maturity, presumably accompanied by the presence of permissive substrates, must be reached before axons can extend into surrounding cortex. The data reported here support this notion, in that intrinsic connections within the cortex also did not extend axons into surrounding regions until their target layers were clearly formed.

Migration

In young animals, the most obvious characteristic after injection into the VZ, the white matter, or the cortical plate is a precise radial stream to or within the cortex. When label extends from the VZ to the cortical plate, it occurs predominantly in a point-to-point fashion. This strengthens the notion that clones of cells migrate in a modular, radial fashion from VZ to cortical plate and arrive in a column of cells that may operate as a functional unit. A number of studies have provided evidence that, although clones of cells migrate from the VZ to the neocortex in a predominantly radial direction, members of a specific clone can travel tangentially as well, leading to a lateral dispersion of cells during migration (Walsh and Cepko, 1992). The precise mechanism that contributes to tangential migration and the methods by which cells move nonradially are not entirely clear. In our study, injections into the VZ of young animals, although dominated by a radial pattern, often resulted in tangentially oriented cells specifically located in the subventricular zone that appeared to be aligned in a horizontal, rather than a radial, direction. Menezes and Luskin (1994) identified a population of cells immunoreactive for class III tubulin, a neuron-specific tubulin (TuJ1), that appeared morphologically and positionally to be very similar to the cells labeled in this study. Menezes and Luskin have suggested several functions that may be associated with these cells. One idea is that the TuJ1-positive cells contribute to horizontal migration. In many of the slices we examined, cells that were labeled after dextran injections at the superficial border of the VZ (i.e., the subventricular zone) had "broken" with the dominant radial distribution and were located with a strong lateral orientation some several hundred microns from the injection site. It seems likely, as suggested by Menezes and Luskin (1994), that these horizontally oriented cells are not traveling on radial glia. Although migration on radial glia

seems to be a predominant route for neurons to travel to the cortex, other routes have been suggested, such as using neuronal guides (Rakic, 1972). This may be one way for neurons to accomplish the widespread tangential migration into the neocortex that has been reported by a number of groups (Austin and Cepko, 1990; O'Rourke et al., 1992; Walsh and Cepko, 1992, 1993; Fishell et al., 1993). On the other hand, we also observed deviations from the radial pattern in many elongated fibers more clearly in the intermediate zone that were presumed to be radial glia. If this is so, then they may provide another substrate contributing to tangential migration.

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