Development of Local Circuits in Human Visual Cortex

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How we see the world largely depends on the organization of neuronal circuits in visual cortex. Physiological recordings in mammals indicate that circuits develop over a period that extends well into early postnatal ages (LeVay et al., 1980; Albus and Wolf, 1984). Our understanding of how these circuits are assembled during development is still fragmentary (Katz and Callaway, 1992). Here we describe the development of local connections within visual cortex, using the fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate to trace axonal projections in post-mortem human brains.

Vertical (intracolumnar) connections between layers 2/3 and 5, which link neurons representing the same point in the visual field, develop prenatally at 26–29 weeks gestation. In contrast, horizontal (intercolumnar) connections between different points in the visual field develop later. They first emerge prenatally at ≈ 37 weeks gestation within layers 4B and 5. After birth (>40 weeks gestation) the fiber density increases rapidly, showing a uniform plexus of connections at 7 weeks postnatal. The more adult-like patchiness of the projection, however, emerges after 8 weeks postnatal. Long-range horizontal connections within layer 2/3 develop after the connections within layers 4B, 5, and 6. These connections emerge after 16 weeks postnatal, long after cytochrome oxidase blobs have developed, and reach mature form sometime before 15 months of age. Unlike the patchy horizontal projections within layers 4B and 5, which seem to develop through a process of collateral elimination, long-range projections within layer 2/3 are patchy from the outset and seem to develop with greater topographical precision.

The finding that intracolumnar connections develop before intercolumnar projections suggests that circuits that process local features of a visual scene develop before circuits necessary to integrate these features into a continuous and coherent neural representation of an image. In addition, the sequential development of horizontal connections within layer 4B before those within layer 2/3 suggests that circuits that may be related to the processing channel for visual motion develop in advance of those that may be more inti-

mately related to the processing of form, color, and precise stereoscopic depth.

[Key words: human brain, visual cortex, intracortical connections, magnocellular pathway, parvocellular pathway, prenatal development, postnatal development]

Behavioral and electrophysiological testing of visual functions in newborn infants indicates that the human visual system is immature at birth (Atkinson, 1984; Boothe et al., 1985; Van Sluyters et al., 1990). This is likely due to structural immaturities at all levels of the visual pathway, but most especially the visual cortex.

The most direct evidence that the visual cortex in newborn infants is immature derives from the absence of visual functions that are attributed to selective response properties of cortical neurons (Hubel and Wiesel, 1977). Newborns lack binocular vision, and stereoscopic depth perception is not present until 4 months of age (Birch et al., 1982). Similarly, orientation-selective mechanisms are not present at birth and can only be detected after the second postnatal month (Braddick et al., 1986; Atkinson et al., 1989). The same is true for directionally selective mechanisms that do not begin to emerge until 2 months of age (Wattham-Bell, 1991, 1992). It is generally assumed that all of these functions are generated within distinct cortical circuits (Gilbert, 1983), dependent upon inter- and intralaminar connections that develop postnatally.

Support for this notion derives from studies of synaptogenesis in human visual cortex. Synapses begin to form in the cortical plate (CP) at 23 weeks gestation (Molliver et al., 1973), but prenatally synaptic contacts proliferate slowly (Huttenlocher and de Courten, 1987). Postnatally, synaptic density rapidly rises to a peak at 8 months of age. This phase is followed by a prolonged period of synapse elimination until the adult density is reached at about 10 years of age. Thus, the initial phase of postnatal synaptogenesis proceeds in remarkable synchrony with the maturation of cortical functions (Wilson, 1988). However, the exact relationship between synaptogenesis and the emergence of circuits remains unknown.

Although the onset of all basic cortical functions is between the second and fourth postnatal months, some functions develop more rapidly than others: direction selectivity reaches relative maturity at 3 months of age (Wattham-Bell, 1991), whereas stereoacuity does not reach adult levels until 6 months of age or older (Birch et al., 1982). A similarly slow maturation was found for orientation-selective mechanisms, which are relatively immature until 6–8 months of age (Morrone and Burr, 1986). All of these data suggest that different cortical circuits develop at different rates but that 8 months after birth basic circuits are in place.

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The purpose of our study was to determine the organization of local connections at different developmental ages, in order to provide more detailed knowledge of the way in which neuroanatomic connections correlate with infant psychophysics. The motivation for our work was that different stages in the development of vision must correlate with emergence of specific connections, which should be evident as distinct patterns of local axonal projections within striate cortex. Because the neuronal circuits for cortical functions are controversial (Bolz and Gilbert, 1986; Chapman et al., 1991; Grieve and Sillito, 1991; Wörgötter and Eysel, 1991), we focused on two basic types of connections: (1) vertical connections between layers that provide for local interactions at a single point of the visuotopic map, and (2) horizontal connections within layers that provide for interactions between distant points of the map. To link structure and function more closely, we were particularly interested in comparing the development of horizontal connections within layers 4B and 2/3. Layer 4B is part of the magnocellular (M) processing stream, which conveys information that is utilized mainly for the analysis of stimulus motion (Schiller et al., 1990; Merigan et al., 1991b). Layer 2/3, on the other hand, is dominated by inputs from the parvocellular (P) processing stream (Nealey and Maunsell, 1991) and might be expected to be more concerned with the analysis of color and form (Schiller et al., 1990; Merigan et al., 1991a). To reveal the formation of connections within these streams, we injected primary visual cortex (V1) in post-mortem, aldehyde-fixed human brains, ranging from 24 weeks gestation to 5 years postnatal, using DiI as a label of local axonal connections (Godement et al., 1987; Burkhalter and Bernardo, 1989a).

Preliminary reports on the development of local connections within human visual cortex have been published in abstract form (Burkhalter and Bernardo, 1989b; Burkhalter et al., 1990).

Materials and Methods

Brains without neurological disease were obtained at autopsy. After aldehyde fixation in 2.6% phosphate-buffered paraformaldehyde containing 0.1 M lysine-HCl, 0.8% NaIO₄, and 0.8% iodoacetic acid for 24–48 hr at 4°C, brains were blocked into ≈5 mm slabs with horizontal cuts along the fundus of the calcarine sulcus and coronal cuts posterior to the parieto-occipital sulcus. In poorly myelinated young infant brains in which the stria of Gennari is not readily visible, primary visual cortex (V1) was identified by its characteristic cytoarchitecture. For this purpose, prior to DiI labeling, a section was cut from each block and was subsequently stained with bisbenzimidazole (0.001% in 0.1 M phosphate buffer, pH 7.4) and viewed under UV fluorescence optics. In fetal brains with underdeveloped cytoarchitecture, sections from each block were stained for choline acetyltransferase (Tago et al., 1986), whose distribution served as a marker of the V1/V2 border (Kostovic and Rakic, 1984). For placing DiI (1,1'-diiododecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes) injections, counterstained sections were aligned with the parent tissue block and small crystals were inserted with a fine glass needle. Injections were made into gray matter, which was approached parallel to the pial surface. This enabled large injections throughout the thickness of cortex and more restricted injections of deep layers without involvement of upper layers. The tissue was stored in 0.1 M phosphate buffer at 21°C for 1–6 months to allow for the dye to diffuse along axons (Godement et al., 1987; Burkhalter and Bernardo, 1989a). Sections were then cut on a Vibratome at 75 μm, coverslipped with 0.1 M phosphate buffer, and viewed under a fluorescence microscope equipped with rhodamine optics (excitation filter, 546 nm; barrier filter, 580 nm). For visualizing layers and areal borders, sections were counterstained with bisbenzimidazole and viewed under UV optics (excitation filter, 330–380 nm; barrier filter, 420 nm). Layers were identified in fetuses (24–29 weeks gestation) using Chatel's (1976) scheme for human isocortex. In perinatal and postnatal brains, layers were identified according to the description of Braak (1984). Results were

obtained from brains at different gestational (G, weeks from ovulation) and postnatal (P) ages and in each case from a large number of injections (I): G24 weeks ($n = 2$, $I = 24$), G26 weeks ($n = 1$, $I = 5$), G29 weeks ($n = 1$, $I = 8$), G37 weeks ($n = 1$, $I = 10$), G40 weeks ($n = 1$, $I = 5$), P9 days ($n = 1$, $I = 8$), P7 weeks ($n = 1$, $I = 8$), P4 months ($n = 1$, $I = 10$), P15 months ($n = 1$, $I = 8$), P2 years ($n = 1$, $I = 11$), P5 years ($n = 1$, $I = 6$). After DiI-labeled axonal patterns were graphically reconstructed using a camera lucida, or photographed, sections were taken off the slide and stored in phosphate buffer to preserve labeling for further examination.

In some cases, at the end of the incubation period for DiI, alternate sections were stained for cytochrome oxidase (CO) activity (Tootell et al., 1988). In a single newborn (1 d postnatal) and a single infant (4 months postnatal), the occipital cortex was flattened (Olavarria and Van Sluyters, 1985) and equilibrated in 30% sucrose, and frozen sections were stained for CO.

Results

Vertical connections in 24-week-old fetus

To determine the sequence in which vertical and horizontal connections within striate cortex develop, we examined the projections of neurons in superficial layers. Superficial layers are known to send descending axon collaterals to layer 5 and to make long-ranging lateral projections within layer 2/3 (Gilbert and Wiesel, 1983; Rockland and Lund, 1983; Burkhalter and Bernardo, 1989a). In two 24-week-old fetuses, DiI was placed in superficial parts of the CP (Fig. 1*A,B*). In each case, DiI labeled a narrow band of radial glial fibers that provide the pathway for neuronal migration from the ventricular zone (VZ) to what will be the most superficial layers of cortex (Rakic, 1978). In the inner half of the marginal zone (MZ), labeling spread horizontally for a considerable distance. At higher power, it was apparent that this labeling was associated with fibers originating from Cajal-Retzius cells (Fig. 2). Just as in Golgi-stained material (Marin-Padilla and Marin-Padilla, 1982), these cells typically showed two thick horizontal dendrites from which short vertical branches extended toward the pial surface. A single axon exited from the cell body and traveled horizontally in parallel with the dendrites. The bulk of labeling in the CP was in vertical register with the injection site, and very few fibers and cells were seen beyond the lateral margins of the injection (Fig. 1*A*). Directly below the injection site, labeling consisted not only of radial glial fibers and migrating neurons (Fig. 2), but also of vertically oriented axons and dendrites that arose from ovoidal-shaped neurons that presumably were labeled through the distal tips of apical dendrites (Fig. 1*C*). Many of these CP neurons gave rise to single descending axons that were always unbranched as they passed through the CP. Typically, these efferent fibers entered the underlying intermediate zone (IZ) at an acute angle. At this developmental stage, the CP was also innervated by ascending fibers. Some of these inputs were collaterals of retrogradely labeled subplate cells (Fig. 1*D*; Kostovic and Rakic, 1980). Other afferents were labeled in a 26-week-old fetus after injection of the thalamic radiation, which is easily recognized in the unstained tissue as a relatively opaque band below the IZ (Fig. 3*A*). Such injections labeled radial glial fibers and presumed thalamocortical afferents (Fig. 3*B*). As in Figure 1, the brightest labeling was associated with radial glial fibers. Compared to this prominent bundle, the putative thalamocortical input was less conspicuous. However, the afferents could easily be distinguished from radial glial fibers by their less coordinated trajectories within the IZ. At higher power, many of these input fibers could be followed deep into the CP (Fig. 3*C*). They often looked varicose, were of small caliber, lacked the lamellate ex-

Figure 1. Development of interlaminar connections in human primary visual cortex. *A*, DiI injection (center is marked with asterisk) into superficial half of CP in 24-week-old fetus: fluorescence photomicrograph of coronal section showing band of radial glial fibers (*g*) extending from VZ to MZ. Cortical input and output axons traverse IZ obliquely. In CP most fibers and cell bodies are immediately below the injection site and horizontal fibers are extremely rare. Labeling in inner half of MZ on both sides of the injection site represents horizontal fibers. *B*, Section shown in *A*: fluorescent counterstain with bisbenzimidide. CP is not yet differentiated into different layers. *C*, DiI-labeled cell bodies in CP of 24-week-old fetus after superficial CP injection. Section was taken 0.1 mm from margin of injection site. Descending fibers enter IZ obliquely. *D*, DiI-labeled subplate cells (arrows) in 24-week-old fetus. Center of injection is marked with asterisk. Scale bars, 0.5 mm.

pansions of radial glial fibers (Rakic, 1972), and many terminated in growth cones. Interestingly, these injections never labeled subplate cells. This rules out the possibility that the labeled intracortical fibers represent collaterals of subcortically projecting subplate cells (McConnell et al. 1989) and favors the interpretation that they represent thalamocortical input fibers.

Vertical connections in 29-week-old fetus

The projection from embryonic layer 2/3 to layer 5 was first evident in a 29-week-old fetus. At this time the bisbenzimidide

counterstain revealed the six layers (Fig. 4*B*), but provided no evidence for sublaminae within layer 4. Thus, it was not surprising that bundles of radial glial fibers could still be followed deep into the IZ (Fig. 4*A*), suggesting that neuronal migration was still in progress (for review, see Sidman and Rakic, 1973) and thus the cortical lamination pattern is still immature.

Similar to the case shown in Figure 1, the injection site was restricted to the supragranular layers. The vast majority of fibers that emerged from this injection were directed vertically, and few if any horizontal axons were seen in supragranular layers.

Figure 2. DiI-labeled Cajal-Retzius neuron in inner half of MZ of primary visual cortex of 24-week-old human fetus. Radial glial fibers (*g*) ascend through the CP and terminate in the inner half of the MZ. Dye transfer from radial glial fibers (*g*) occasionally gave rise to labeling of migrating neurons (*n*). Notice that radial glial fiber runs across the weakly labeled neuron. Scale bar, 0.1 mm.

The descending fibers traversed embryonic layer 4 with little branching. By contrast, upon entering layer 5 the fibers produced a dense tangle of short horizontal collaterals that was seen at low power as a prominent band of labeling (Fig. 4*A*). This projection zone always contained fibers and cell bodies, suggesting that layers 2/3 and 5 have established reciprocal connections. In marked contrast to the adult cortex (Burkhalter and Bernardo, 1989a), at 29 weeks gestation the band of labeled axon arborizations in embryonic layer 5 was much narrower and had about the same width as the injection site.

Horizontal connections in 24-, 26-, and 29-week-old fetus

In the adult primate visual cortex, columns in distant parts of the topographic map are linked through long connections in layers 2/3, 4B, 5, and 6 that run parallel to the pial surface (Rockland and Lund, 1983; Livingstone and Hubel, 1984b; Burkhalter and Bernardo, 1989a). To determine the sequence in which these long-range connections develop, DiI injections were made throughout the depth of V1 to label horizontal axon collaterals simultaneously in all layers. At 24 weeks gestation, such cortex-penetrating injections produced labeling patterns (Fig. 5*A,B*) that were similar to those seen after injections restricted to superficial parts of the CP (Fig. 1*A*): a vertical bundle of radial glial fibers connected the VZ with the pial surface, horizontal fibers extended only within the MZ, and there were no horizontal fibers within the CP. (Notice that in Fig. 5*A*, the labeling within the CP on both sides of the injection was not associated specifically with fibers or cell bodies and represents background staining due to local diffusion of DiI.) Well-defined, but short (<0.5 mm), horizontal fibers within the CP were first seen at 26 weeks gestation. This was in rough synchrony with the beginning of laminar differentiation (Figs. 3*B*, 6*B*) and the appearance of interlaminar connections (Fig. 4). However, these short horizontal fibers did not emerge simultaneously in every layer. Instead, they appeared at very specific locations: the top of layer 4 and the layer 5/6 border (Fig. 6*A*). The presence of retrogradely labeled neurons in layers 4 (Fig. 6*A*), 5, and 6 indicated that at least some of the short horizontal fibers may represent local axon collaterals that originated from within the CP. However, because layers 4 and 6 receive thalamocortical afferents (Hubel and Wiesel, 1972) that invade the cortex at about this time (Kostovic and Rakic, 1984), we cannot rule out that some of the fibers may represent thalamocortical afferents.

Figure 3. DiI injection into thalamic radiation (*TR*) below primary visual cortex of 26-week-old human fetus. *A*, Bright-field photomicrograph of unstained coronal section through calcarine sulcus showing injection site (*asterisk*) and emerging layers of CP. *B*, Section shown in *A* viewed under fluorescence illumination. DiI labeled radial glial fibers (*g*) spanning between VZ and layer 1. Radially and obliquely oriented putative thalamocortical afferents (*tca*) enter CP. Center of injection in thalamic radiation is marked with *asterisk*. *C*, High-power fluorescence photomicrograph of section shown in *B*. Presumed thalamocortical afferents enter all layers of CP. Scale bars: *A* and *B*, 1 mm; *C*, 0.5 mm.

Figure 4. *A*, DiI injection (asterisk) into layer 2/3 of primary visual cortex of 29-week-old human fetus. Labeling to the left of bright spot, representing injection site, is due to DiI diffusion from the injection site after section was cut. Unbranched fibers traverse layer 4. The small-caliber fibers are descending axons of neurons in superficial layers. The larger-caliber fibers represent radial glial processes. Descending axons issue short collaterals in layer 5, forming a dense plexus of terminal arborization (arrow). Bundle of radial glial fibers (*g*) traverses IZ. *B*, Section in *A* counterstained with bisbenzimidazole, showing emerging cortical layers. Scale bars, 0.5 mm.

Interestingly, we have never seen horizontal fibers in layer 2/3 in prenatal brains.

Horizontal connections in 37-week-old fetus

At 37 weeks gestation, shortly before birth, the lateral projections were much longer (up to 2 mm) and denser (Fig. 7*A*). At this age the cortex has matured to a stage where layer 4 showed distinct sublaminae (Fig. 7*B,D*), the IZ has become subcortical white matter, and there was no trace of radial glial labeling (Fig. 7*A*). Nevertheless, horizontal fibers were still confined mainly to the middle of the CP and the layer 5/6 border. Compared with layer 4, tangential projections in layer 2/3 were extremely sparse and labeled fibers and cell bodies were rare. At this stage

the layers were well differentiated and we are confident that the mid-cortical horizontal fibers occupied layer 4B immediately above the largely unlabeled thalamocortical input layer, 4C. The presence of retrogradely labeled cells within layer 4B indicates that these fibers originated within the cortex. This is further supported by the absence of labeled fibers within layer 4C. In this layer, dense arborizations were only seen when the injection site included white matter and presumably labeled thalamocortical input fibers (Fig. 7*C,D*). The reason for this preferential labeling of thalamocortical fibers may lie in the profuse branching of thalamocortical afferents within gray matter compared to the fasciculation of unbranched axon trunks within white matter (Blasdel and Lund, 1983).

Figure 5. Development of intralaminar connections in human primary visual cortex. *A*, Fluorescence photomicrograph of coronal section through V1. DiI injection (asterisk) marks center through depth of CP in 24-week-old fetus. Weak labeling on both sides of injection represents dye diffusion from the injection site after section was cut. IZ is traversed by bundle of radial glial fibers (*g*) and obliquely running axons (arrows). No horizontal fibers are seen in CP. *B*, Section shown in *A* counterstained with bisbenzimidazole. Scale bars, 0.5 mm.

Figure 6. *A*, DiI injection into layers 2/3, 4, and 5 of primary visual cortex of 26-week-old human fetus. Margin of the injection site is at left border of photomicrograph. Short horizontal fibers travel along superficial border of layer 4 and along layer 5/6 border. Labeled neurons at top of layer 4 (arrows) indicate that some horizontal fibers are local collaterals of cortical neurons and do not represent afferents from sources outside of V1. *B*, Fluorescence photomicrograph of bisbenzamide-stained section represented in *A*, showing emerging cortical layers. Scale bars, 0.5 mm.

Horizontal connections in 4-month-old infant

The postnatal development of long-range projections within the superficial layers was surprisingly protracted. Seven weeks after birth, and even at 4 months of age (see Fig. 10*A*), the pattern of horizontal connections in every part of V1 was remarkably similar to that seen at around birth and essentially confined to layers 4B, 5, and 6 (Fig. 7*A*). This is astonishing considering that the CO pattern in superficial layers is present at birth and appears mature at 4 months postnatal. One day after birth, the CO staining was already nonuniform and showed an organized pattern of rows of CO-rich blobs and CO-poor interblobs (Fig. 8*B–D*). However, unlike in mature brains, the staining was weak, which made it difficult to measure the size of blobs accurately. Although the size range (0.2–0.5 mm) of blobs in newborns was broader than in 4 month olds, the center-to-center spacing (0.6–0.7 mm) was similar, suggesting that the modular pattern does not obviously change after birth. At 4 months postnatal the CO staining pattern appeared mature and virtually identical to that of adults (Fig. 9; Horton and Hedley-Whyte, 1984; Burkhalter and Bernardo, 1989a). In V1, supragranular layers showed darkly stained blobs (diameter, 0.25–0.35 mm; center-to-center spacing, 0.6–0.75 mm) that were often connected through thin bridges with high enzymatic activity. In V2, CO staining revealed a regular pattern of dark (1–1.5 mm wide) and pale (2–2.5 mm wide) stripes in layer 2/3 that ran perpendicular to the V1/V2 border. The staining within the stripes was nonuniform, indicating the presence of distinct subdivisions. Thus, even though the development of visual cortex had progressed to a stage where different functional compartments (DeYoe and Van Essen, 1988) could be distinguished anatomically, layer 2/3 contained very few labeled fibers and tangentially spreading projections were confined to layers 4B, 5, and 6 (Fig. 10*A*). To ascertain that this result was real, we repeated the experiment ($n = 10$) by making much larger injections into layer 2/3 (Fig. 10*C*). In each case the outcome was the same, and we are con-

fident that the labeling pattern is not due to the size and exact sublaminar location of the injection site.

Horizontal connections in children aged 15 months to 5 years

Although the laminar distribution of horizontal fibers remained unchanged during the first 4 months after birth, the fiber density in layers 4B, 5, and 6 increased dramatically (Figs. 7*A*; 10*A,C*; 11*A*). Similar to newborns, however, the fibers were uniformly distributed and the intralaminar termination pattern differed from the patchy pattern seen in layer 4B of a 5 year old (Fig. 11*C*). This suggests that patchy projections may form from initially more widespread projections that are subsequently pruned. However, the logical possibility exists that the uniform labeling at early ages is the result of injecting initially more closely spaced patches. The patchy layer 4B projections shown in Figure 12*A* indicated that this elimination was complete at 15 months of age. Because the elimination of inappropriate axon collaterals occurred after 4 months of age, it appears that the developmental signals that induce CO compartments in superficial layers are different from those that trigger the segregation of fibers into patchy projections within layer 4B.

Puzzled by the absence of local upper layer collaterals of layer 2/3 cells at 4 months of age, a time when these cells have already developed long projections to layer 5 (Fig. 4), we wondered whether at this point other collateral projection systems of layer 2/3 could be labeled by injections into superficial layers. For this purpose, we sought to label output and input fibers to and from V2. We therefore made DiI injections throughout the depth of V1 near the V1/V2 border. Figure 13 shows a case with large injections in layers 2/3 and 4 and a smaller injection in lower layer 6 that included subcortical white matter. The result was unambiguous: a large number of labeled fibers passed through upper layers of V1 and entered V2, but very few horizontal fibers traveled in the opposite direction and penetrated deeper into the territory of V1. As expected, most fibers in the territory between the injection site and the V1/V2 border were un-

Figure 7. *A*, Five DiI injections (centers indicated with asterisks) distributed throughout the thickness of primary visual cortex in 37-week-old human fetus. Although all layers were injected (adjacent sections show that the injection reaches through the depth of layer 2/3), the vast majority of horizontal fibers extend within layer 4B and at the layer 5/6 border. Layers superficial to layer 4B contain extremely few labeled fibers. Labeled cell bodies (arrows) indicate that at least some of the horizontal fibers represent local collaterals of cortical neurons. *B*, Section shown in *A*, counterstained with bisbenzimidazole. Notice sublamination within layer 4. *C*, DiI labeling in primary visual cortex of 1-week-old neonate: multiple injections (asterisks) involving layers 4–6 and subcortical white matter (WM). Unlike “clean” cortical injections (*A*), white matter injections produce terminal labeling in layer 4C. *D*, Section shown in *C*, counterstained with bisbenzimidazole to reveal layers. Scale bars, 0.5 mm.

branched. This abruptly changed in V2, where vertical collateral branches were abundant. Such branches were often found in projection targets, which suggests that we have labeled mainly fibers that go from V1 to V2. This result strongly indicates that extrinsic collaterals to V2 develop in advance of intrinsic collaterals within V1, and that it is unlikely that methodological problems account for the absence of horizontal projections within superficial layers of V1.

We are unable to tell exactly when upper layer long-range projections within V1 develop, because we have not had access to brains between the ages of 4 and 15 months. We do know, however, that these projections are present in a 15 month old. As in the 4-month-old case (Fig. 10), the injection that led to the labeling shown in Figure 12*A* included all layers of the cortex. Unlike the pattern shown in Figure 10, *A* and *C*, where labeling in upper layers was absent, Figure 12*A* shows distinct projection clusters in layer 2/3, 4B, and 5. Although the projections within layer 2/3 were clearly present, they were much sparser than in a 2 year old (Fig. 14*A*). This is interesting and supports the notion that at 15 months the projection is still immature.

By 2 years of age, for the first time, long-range projections within layer 2/3 are robust and the density and patchiness of

the projections resemble those seen in adults (Burkhalter and Bernardo, 1989a). Comparing the development of projections within layer 2/3 and layer 4B, one can see similarities in that both projections were sparse initially (Figs. 7*A*, 12) and denser later in development (Figs. 10*A,C*; 11*A*; 14*A*). However, if one looks at patchiness, a striking difference becomes apparent: patches in layer 4B appear to develop from initially more widespread projections (Figs. 7*A*; 11*A,C*; 12*A*), whereas in layer 2/3 patches were already present when the projections were still extremely weak (Fig. 12*A*). Thus, it appears that the patchy projections become increasingly refined by the elaboration of additional terminal branches (Fig. 14*A*).

At 5 years of age, the laminar pattern of long-ranging projections within V1 was similar to that at 2 years of age: clustered projections extended in layers 2/3, 4B, 5, and 6. Because the CO activity was well preserved in this case, we were able to test whether upper layer projections displayed the compartmental specificity known to exist in adults. For this purpose, attempts were made to target DiI injections into blobs or interblobs. The result of an interblob injection is shown in Figure 14, *B* and *C*. (Notice that the spot labeled as the injection site is dark because of the purple color of DiI crystals and does not represent CO

Figure 8. Distribution of CO activity in striate cortex of human neonate (1 d postnatal). *A*, Transverse section through V1 showing nonuniform staining in layer 2/3 that resembles blobs (arrows). Dense staining is also seen in layer 4C. There is no systematic difference in CO activity in superficial and deep parts of 4C. Although V1 contains a layer 4A, it does not express CO activity and can therefore not be distinguished from 4B. *B–D*, Horizontal section through V1 showing regular array of blobs (arrows). Scale bars, 1 mm.

activity.) Although labeled fibers radiated from the injection site and arborized preferentially in nearby interblob regions, it is obvious that the segregation of inputs was incomplete since many fibers seemed to arborize at the boundaries between blobs and interblobs. Thus, it seems that this pattern reflects a developmental transition to the mature state where blobs are more exclusively connected to blobs and interblobs are linked to interblobs (Burkhalter and Bernardo, 1989a).

Discussion

Our work is the first description of development of local axonal connections within the visual cortex of humans. We have shown that local circuits within striate cortex are assembled in a sequence in which vertical connections between layers develop at the beginning of the third trimester of gestation, whereas long horizontal connections within layers do not emerge until shortly before birth and continue to develop throughout the first postnatal year and possibly beyond.

This pattern of cortical development is different from that

deduced from a survey of the density of synaptic contacts in human visual cortex (Huttenlocher and de Courten, 1987). One of the important findings of this study was that the synaptic density in newborn human infants was extremely low. This gave rise to the idea that the intracortical circuitry is poorly developed at birth. Our findings indicate that this view is only partially correct and that the development of specific intracortical circuits (i.e., intracolumnar connections) is in fact remarkably advanced. In addition, we have shown that the development of intralaminar connections within superficial layers overlaps with the period during which synaptic density decreases (Huttenlocher and de Courten, 1987). Thus, our results indicate that the period of synapse elimination should not simply be regarded as a phase in development in which previously existing connections are lost. Rather, our evidence supports the interpretation that it is also a time that allows for the construction of new neuronal circuits. The important lesson from this is that it is necessary to determine the development of different subsets of projections to gain an understanding of the developmental status of the cortex.

Figure 9. Reverse-contrast photomicrograph of horizontal section through flattened visual cortex of 4 month post-natal human stained for CO activity. V1 shows a mature pattern of blobs (light dots). V2 shows CO-rich stripes (arrows) that run perpendicular to the V1/V2 border. Staining within stripes is irregular, indicating compartmentalization of stripes. Scale bar, 5 mm.

Technical considerations

Our work is the first effort aimed at determining local neuronal connections in developing human cortex. It is important here to explore several technical considerations upon which hinge the validity of our methods and results.

Working on the human brain imposes constraints that are experimentally more limiting than when working with experimental animals. Perhaps the least obvious of these is the availability of suitable material, which left us with significant gaps in the chronology of the development of intracortical connections.

An issue more pertinent for the interpretation of the results is whether the DiI labeling pattern was influenced by the post-mortem interval before tissue fixation. It is well established that inappropriate fixation changes the integrity of cell membranes (Palay et al., 1962), which conceivably affects the intramembranous migration of DiI. Comparison of the DiI labeling patterns seen in immersion-fixed human striate cortex (Burkhalter and Bernardo, 1989a) with the labeling pattern produced by WGA-HRP observed in perfusion-fixed monkey visual cortex (Livingstone and Hubel, 1984a,b), however, indicates no apparent differences. Thus, it seems that axonal tracing with DiI provides an accurate picture of the organization of connections in the human brain.

Because DiI dissolves in cell membranes (Honig and Hume, 1986), it is likely that it is taken up by fibers of passage. This is a potential problem for identifying labeled fibers as local collaterals of cortical neurons and to discriminate these from extrinsic input fibers. The specific case we have to consider here is whether the labeled fibers in embryonic layer 4 (Figs. 6A, 7A) originate from layer 4 neurons, whether they could be recurrent axon collaterals of layer 6 pyramidal neurons (Lund and Boothe, 1975), or whether they represent geniculocortical afferents. The

most direct way to answer the question would be to label the geniculocortical input with a second tracer and to look for overlap of labeling. We have not explored this possibility because of the length of the geniculocortical pathway and the prohibitively long transport time for the tracer. We are therefore forced to make an argument based on three sets of observations: (1) the presence of labeled neurons in layer 4B, (2) the laminar position of labeled fibers at the border between the granular layer and layer 2/3, and (3) the presence of dense layer 4 labeling, only after white matter is included in the injection site (Fig. 7C). Each of these observations is consistent with the interpretation that the labeling in Figures 6A and 7A represents mainly intralaminar collateral projections of layer 4B neurons.

Finally, a word to the sensitivity of the DiI tracing technique. How can we be sure that if we do not see labeled fibers in layer 2/3 as late as 4 months after birth (Fig. 10), they are not there? To prove a negative result is a well-recognized problem. The important control here is that we were able to label fibers that link V1 and V2 in the very layer in which we failed to label local collaterals (Fig. 13). This rules out most technical explanations related to the duration of incubation, fixation, size, and location of injection site. Although the mean diameter of local collaterals is slightly smaller (0.5–1 μm) than the caliber of interareal fibers (0.75–1.5 μm), it seems unlikely that this could account for the difference. However, the logical possibility remains that for some unknown reasons immature local axon collaterals did not transport DiI. This seems unlikely because numerous examples indicate that unmyelinated or poorly myelinated axons, such as those in developing visual cortex, are labeled more readily than myelinated fibers (Honig and Hume, 1989). Nevertheless, it is important to interpret DiI tracing data with caution because similar transport times may yield different labeling patterns. For this reason, we have avoided conclusions based on the extent and density of projections.

Figure 10. Postnatal development of horizontal connections in human primary visual cortex. *A*, Fluorescence photomicrograph of transverse section through V1 of 4 month postnatal human. Six DiI injections (asterisks mark centers) involving layers 2/3 and 4B–6. Horizontal projections extend for more than 1.5 mm within layers 4B, 5, and 6. Within layer 4C, very few fibers travel horizontally and labeling is restricted to the immediate vicinity of the injection site. Within layer 2/3, labeling is extremely sparse. The few fibers and cell bodies that are labeled are seen within 0.5 mm of the injection. *B*, Section shown in *A*, counterstained with bis-benzimide to reveal layers. Notice that the staining does not reveal layer 4A. *C*, Large DiI injections into layers 2/3 and 4 of primary visual cortex of 4-month-old human. Although injection in layer 2/3 is larger than in *A*, labeling in layer 2/3 is extremely sparse and resembles that seen in *A*. *D*, Section shown in *C*, counterstained with bis-benzimide to reveal layers. Notice that staining does not reveal layer 4A. Scale bars, 0.5 mm.

Sequential development of vertical and horizontal connections: functional implications

Our results provide evidence that at the time when neurons of the CP first develop local connections (26–29 weeks gestation) the CP is already innervated by afferents to striate cortex. Some of these afferents originate from subplate cells (Kostovic and Rakic, 1980), which we have always found directly below the injection site (Fig. 1*D*). This is consistent with laterally restricted projections to the CP and indicates that subplate cells in humans may resemble subplate cells in kitten visual cortex, which were found to project to middle and superficial layers of a column of cortex $\approx 400 \mu\text{m}$ wide (Friauf et al., 1990). In addition to this input, we obtained evidence for afferents from the optic radi-

ation (Fig. 3), which represent in all likelihood thalamocortical fibers. Kostovic and Rakic (1984), using AChE histochemistry, have postulated that these afferents originate from the pulvinar. However, recordings of visually evoked potentials in occipital cortex of a (25-week-old) preterm infant suggest that inputs also arise from the LGN (Purpura, 1975). The simultaneous presence of geniculocortical and subplate inputs is believed to be critical for the development of normal intracortical circuits (Friauf et al., 1990). In this view, it seems plausible that CP cells receive input from both small groups of spontaneously active LGN neurons and subplate cells (Gosh and Shatz, 1992) and that the interactions of these inputs may play a role in the columnar restriction of the interlaminar connections.

The most obvious difference in the local labeling pattern pro-

Figure 11. *A*, DiI injection (asterisk) into layer 4B of human primary visual cortex at 7 week postnatal. Most fibers are confined to layer 4B and terminate in uniform fashion. Many fibers descend into subcortical white matter. Some fibers terminate in layer 6 (arrow). Projections to superficial layers ascend vertically, but laterally spreading fibers are extremely rare. *B*, Section shown in *A*, counterstained with bisbenzimidide to reveal layers. *C*, Distribution of DiI-labeled axon arborizations in primary visual cortex after injection into layer 4B of 5-year-old human. Layer 4B contains clusters of terminal arborizations. Above layer 4B, labeling is extremely sparse. Below layer 4B, patches of axon arborizations are seen, although less dense in layer 6. Scale bars, 0.5 mm.

duced by upper layer injections in fetal and adult visual cortex is that fibers in layers 2/3 and 5 of the adult brain extend far beyond the margins of the injection (Burkhalter and Bernardo, 1989a). In contrast, in the fetal brain extremely few collaterals extend laterally and the projection to layer 5 is in perfect vertical

register with the injection site (Fig. 4). This indicates that connections that link similar points of the visuotopic map (Hubel and Wiesel, 1974) develop before lateral connections that link distant points of the topographic representation. Thus, it appears that connections within columns form before connections between columns. It is interesting that the establishment of intracolumnar connections in kitten visual cortex (Katz, 1991; Callaway and Katz, 1992) coincides with the time cells in all layers become visually responsive (Albus and Wolf, 1984). This indicates that the functional specification of columns through appropriate intracolumnar circuits precedes the linkage of functionally similar columns through horizontal connections (Livingstone and Hubel, 1984b; Burkhalter and Bernardo, 1989a; Gilbert and Wiesel, 1989). Since many of the basic response properties of cortical neurons are generated within columns of cortex $\approx 600 \mu\text{m}$ wide, this could mean that the circuits for analyzing local features develop in advance of the circuits that are responsible for the integration of local features into a coherent image of the world.

Although based on the presence of terminal labeling in layer 5 this seems the most likely interpretation, we cannot rule out that part of the labeling is due to axon collaterals of layer 5 cells, rather than terminals of layer 2/3 neurons. Such local collaterals may have accumulated DiI either through retrograde labeling of layer 5 neurons via projections to layer 2/3 or through direct labeling via distal dendritic arbors. Unequivocal evidence against this scenario awaits reconstruction of the axonal projections of individual layer 2/3 neurons. However, the absence of retrogradely labeled neurons in layers 4 and 6, which in fetal monkey send axons to layer 3B and whose dendrites do not rise above layer 4 (Lund et al., 1977), strongly suggests that the labeling pattern shown in Figure 4 is evidence that the reciprocal intracolumnar circuit between layers 2/3 and 5 is mature at 29 weeks of gestation.

Sequential development of M- and P-horizontal circuits

Association of layers 4B and 2/3 with M- and P-processing stream. Studies in macaque monkey have shown that the main input to layer 4B derives from the magnocellular LGN and is relayed through layer 4Ca (Hubel and Wiesel, 1972; Lund and Boothe, 1975). Within layer 4B the input from a single point in space is distributed by an extensive network of lateral connections made by spiny pyramidal and stellate neurons (Rockland and Lund, 1983). It is likely that these intralaminar connections represent the horizontal fibers we have observed in layer 4B of human striate cortex. Although this intralaminar network processes mainly signals from the M-pathway, layers 4Ca and 4Cb are interconnected through putative inhibitory interneurons (Lund, 1987), suggesting the possibility that layer 4B receives a mixture of M- and P-inputs. In spite of this convergence, layer 4B shows a high incidence of directionally selective cells with high contrast and velocity sensitivities (Orban et al., 1986; Hawken et al., 1988), indicating a clear domination by inputs from the M-pathway.

In contrast to layer 4B, it is more ambiguous to relate the local projections within layer 2/3 to a single processing stream. Recent evidence has shown that layer 2/3 receives input from the M- and the P-pathway: parvocellular LGN input is conveyed through layers 4Cb and 4A (Lund and Boothe, 1975; Fitzpatrick et al., 1985), and M-input is relayed via layer 4Ca (Lachica et al., 1992) and layer 4B (Blasdel et al., 1985; Yoshioka and Lund, 1990). Although interblobs receive pure P-input and blobs are

Figure 12. *A*, Fluorescence photomicrograph of DiI-labeled fibers in striate cortex of 15-month-old human. A radial row of DiI injections were made into layers 2/3–6. Dense, clustered, horizontal projections are seen in layer 4B. Fibers and terminal arborizations are also seen in layers 2/3 and 5. Notice that although the projections in superficial layers are sparse, they are organized in distinct clusters. *B*, Section shown in *A*, counterstained with bis-benzimide to reveal layers. Scale bar, 0.5 mm.

supplied by M- and P-inputs (Lachica et al., 1992), because we have not stained every case for CO we are uncertain whether we have labeled connections between blobs or interblobs. However, for the purpose of this discussion it is important to stress that this shortcoming does not alter the interpretation that the M-dominated circuits within layer 4B develop in advance of the M- and P-related circuits in superficial layers (see below).

Different mechanisms for the formation of patchy projections within layers 4B and 2/3. Our results show that horizontal projections develop shortly before birth. Within layer 4B, collaterals proliferate rapidly during the first 2 postnatal months and soon blanket a large area with projections (Fig. 11*A*). These are subsequently remodeled into regular arrays of patches, presumably by a process of collateral elimination (Fig. 11*C*). Exactly when these patches are formed remains uncertain. All we know is that they appear after cortex has assumed the modular organization revealed by the blob pattern in striate cortex of newborns (Fig. 8), but it seems likely that the formation of patchy intracortical projections succeeds the segregation of geniculocortical afferents into ocular dominance columns, which is thought to be complete at 4–6 months postnatal (Hickey and Peduzzi, 1987). Similarly, there is uncertainty about the time of emergence of superficial layer projections. It is clear, however, that they develop after the projections within layer 4B. In addition, because layer 2/3 projections appear to be patchy from the outset, it seems likely that they emerge after layer 4B projections assume their patchy appearance.

We do not know for certain if the development of the circuitry within layer 4B (and layers 5 and 6) differs from that within layer 2/3, nor do we understand why this may be so. However, it is interesting to note that the development of projections within layer 4B resembles more closely that of geniculocortical afferents in layer 4C than that of intracortical projections. Intracortical projections typically develop from widely spread groups of neurons, but their terminations emerge with a fair

amount of topographic precision (Innocenti, 1981; Price and Zumbroich, 1989; Callaway and Katz, 1990; Fish et al., 1991). By contrast, in layer 4C overlapping inputs from the two eyes are gradually segregated over a period that in monkeys extends from late gestation to the second postnatal month (Rakic, 1977b; LeVay et al., 1980). This formation of ocular dominance columns is correlated with the acquisition of stereopsis (LeVay et al., 1978; Timney, 1981). Thus, it is possible that the formation of patchy projections within layer 4B may be synchronous with the emergence of disparity-selective mechanisms that are essential for stereoscopic vision. These disparity-selective binocular interactions may be important for the maturation of direction-selective mechanisms in layer 4B. Specifically, it is possible that they contribute to the loss of the initial bias to nasally directed motion, recorded in striate cortex (Hamer et al., 1990) and observed in humans with arrested binocular development (Tychsen and Lisberger, 1986). As a matter of fact, Tychsen (1992a) has argued that the acquisition of symmetrical motion processing is dependent upon the development of normal connections within layer 4B. In contrast, connections within layer 2/3 develop long after ocular dominance columns have formed and binocular connections have been established. Layer 2/3 neurons may therefore need to receive correlated binocular inputs from topographically corresponding points before they can develop horizontal collaterals. Indeed, recent findings in the cat have shown that normal binocular experience is important for the development of topographically precise connections within upper layers of cat striate cortex (Löwel and Singer, 1992). Preliminary results in naturally strabismic monkey suggest that similar mechanisms may play a role in establishing appropriate connections for binocular vision within primate primary visual cortex (Tychsen and Burkhalter, 1992).

Functional implications. The finding that horizontal intralaminar connections within layer 4B develop before similar connections emerge in layer 2/3 suggests that intracortical circuits

Figure 13. *A*, Montage of photomicrographs showing DiI injections into primary visual cortex of 4-month-old human. Injections (asterisks) are located near the V1/V2 border (arrowhead). Within V1, fiber labeling is dense in layers 4B and 6. Labeling in layer 2/3 is asymmetrical: extremely few fibers are seen within V1 on the left of the injection site, whereas numerous fibers are seen on the right, many of which cross the V1/V2 border and penetrate deep into V2. Within V2 fibers give off vertical collaterals, indicating terminal projections. *B*, Montage shown in *A*, counterstained with bisbenzimidate to reveal layers and areal boundaries. V1/V2 border is indicated by broken line. Scale bars, 0.5 mm.

related to the M-pathway mature before circuits associated with the P-pathway. To the extent that the M-pathway contributes to motion processing (Schiller et al., 1990; Merigan et al., 1991b) and the P-pathway (Schiller et al., 1990; Merigan et al., 1991a) contributes to form and color vision, this scheme agrees with the general notion that object recognition in infants is more dependent on motion information than on static information (Kellman and Spelke, 1983; Kellman, 1984; Dannemiller and Freedland, 1989) and emerges before precise form and color vision (for review see Tychsen, 1992b).

Support for an early development of the M-pathway derives from birth dating of LGN neurons in monkey, which shows

that M-layers are generated before P-layers (Rakic, 1977a). In addition, Garey and Saini (1981) have shown that in monkey, M-neurons assume the mature morphology in advance of P-neurons. Surprisingly, despite the developmental lead of M-neurons, geniculocortical fibers arrive simultaneously in layers 4Ca and 4Cb of monkey striate cortex (Rakic, 1977b). Whether this indicates that synapses in 4Ca and 4Cb become functional at the same time is not known, although the synchronous development of geniculocortical M- and P-axons in *Galago* (Florence and Casagrande, 1990) suggest that they may do so. What is known, however, is that the synaptic density in layer 4B is maximal at 4 months of age, which is 4 months earlier than

Figure 14. *A*, DiI-labeled projections within primary visual cortex after injection through depth of gray matter in 2-year-old human. Coronal section taken near the injection site (lowermost extension marked with asterisk) shows long-ranging axonal projections within layers 2/3, 4B, 5, and 6. Terminal arborizations are distributed in nonuniform fashion and appear clustered (arrows). *B*, Horizontal section through primary visual cortex of 5-year-old human: DiI injection into interblob (asterisk). Local projections are widespread and tend to arborize in interblob regions. Most prominent interblob projection site is marked by arrow. Blobs are outlined by white dots. *C*, CO staining pattern in section adjacent to that shown in *B* (compare blood vessels). Injection site (asterisk) is dark because of purple color of DiI crystals and does not represent increased CO activity. Notice that fibers preferentially arborize within interblob regions. Scale bars, 0.5 mm.

layers 2/3, 4C, and 5 reach the same state (Huttenlocher and de Courten, 1987). Interestingly, the laminar sequence in which layers become myelinated follows a similar pattern (Conel, 1939–1967). These results strongly suggest that functional circuits within layer 4B mature before those in other layers. Furthermore, Flechsig's (1904) finding that area 12, the putative human homologue of area MT (Clarke and Miklossy, 1990; Corbetta et al., 1991) and likely projection target of layer 4B (Maunsell and Van Essen, 1983), becomes myelinated in advance of other extrastriate visual areas suggests that the precocious developmental schedule of layer 4B may extend throughout the entire M-pathway.

It is interesting to ask whether this sequential maturation of intracortical circuits is consistent with the development of visual perception in infants. A large number of behavioral and electrophysiological studies strongly indicate that this is the case. One of the most compelling findings for the sequential development of M- and P-related mechanisms derives from visual evoked potential studies of the development of contrast sensitivity. They show that adult contrast sensitivity is reached at 9 weeks of age and that the increase in acuity between 2 and 8 months of age is determined purely by an improvement in spatial resolution (Norcia and Tyler, 1985; Norcia et al., 1990). Directional selectivity develops shortly after contrast sensitivity has reached maturity (Wattham-Bell, 1991). Since directionally selective mechanisms originate in the cortex (Hubel and Wiesel, 1968), this suggests that the ability to discriminate the direction of local motion at 8 weeks postnatal (Wattham-Bell, 1992) is correlated with the emergence of circuits within layer 4B (Figs. 7A, 11A,C).

The P-channel mediates information that is necessary for the perception of color, texture, shape, pattern, and stereopsis (DeYoe and Van Essen, 1988; Schiller et al., 1990). All of these functions are encoded by neurons in the cortex that acquire their selective receptive field properties through specific intracortical connections. Among these are horizontal connections within layer 2/3 that form distinct networks between color-selective blobs and between orientation-selective interblobs (Livingstone and Hubel, 1984b; Burkhalter and Bernardo, 1989a). These connections form after 4 months postnatal and may assume maturity at 15 months of age. Thus, outgrowth of horizontal collaterals from upper layer cells of the color and form channel are delayed until at least 4 months of age, when orderly maps of blobs and interblobs have fully matured (Fig. 9; Horton and Hedley-White, 1984). At this time, infants have good color (Brown, 1990) and stereoscopic vision (Birch et al., 1982) and are able to discriminate orientations (Braddick et al., 1986; Atkinson et al., 1989). Interestingly, the ability to perform figure-ground discriminations, a task that requires the comparison of orientation-selective responses in distant points of the visual field, matures over an extended period and does not reach adult levels until after 1 year of age (Atkinson and Braddick, 1992; Sireteanu and Rieth, 1992). Since it appears that texture segregation may be processed preferentially in primary visual cortex (Lamme et al., 1992), it is possible that the neural basis for this may lie in the late formation of horizontal connections within superficial layers that we have described in this report.

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