Development of Connections Within and Between Areas V1 and V2 of Macaque Monkeys

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ABSTRACT

We have investigated the development of intrinsic and interareal connections in areas V1 and V2 of the macaque monkey using postmortem transport of the lipophilic fluorescent tracer diI, applied to brains fixed at different prenatal and postnatal ages. Intrinsic connections in the deep layers of V1 are evident on embryonic day 108 (E108), but are not robust in the superficial layers until around E118, when migration is largely complete. Both intrinsic horizontal projections and extrinsic projections to V2 initially have a continuous distribution. Patchy projections are first evident in V1 around E145, the same age at which cytochrome oxidase blobs appear, presumably signaling the differentiation of the blob-dominated and interblob-dominated streams in the primary visual cortex. The magnocellular-dominated stream becomes distinct at earlier stages (by E122), as judged by connectional and histochemical criteria.

In area V2, intrinsic connections initially (at E108) involve only deep layer cells and do not have a clustered organization. By E130, superficial layer cells are involved and the V2 intrinsic connections have a patchy distribution; by E145, an adult-like pattern is present. The projection from V2 to V1 passes through an early stage (up to E133) of originating principally from deep layer cells, and thereafter originating from superficial as well as deep layers.

We found evidence for changes in dendritic morphology during development. Most notably, at E118, many neurons in layer 6 which are involved in intrinsic or interareal connections have dendrites that extend well into the superficial layers, even into layer 1, a characteristic not reported in the adult.

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Indexing terms: modularity, lamination, intrinsic connections

The intricate circuitry of the mammalian neocortex is established over an extended period of prenatal and postnatal development. It involves a complex sequence of cell migration, elaboration of dendritic and axonal arbors in specific laminar patterns, and selective pruning of inappropriate connections. These processes have been most intensively studied in visual and somatosensory cortex, particularly in non-primate species (see Katz and Callaway, 1992; O'Leary et al., 1994).

In the macaque monkey and other primates, visual cortex contains a number of unique anatomical features that are of interest from a developmental viewpoint. The lamination of area V1 is more highly elaborated than in other cortical areas, with layer 4 containing at least four distinct sublaminae (4A, 4B, 4Co, 4Cβ) that are related to the pattern of geniculocortical inputs (Hubel and Wiesel, 1977). In the tangential domain (i.e., parallel to the cortical surface), both V1 and V2 have a modular organization that is manifested, in part, by an array of blobs and interblobs in V1 and an array of thick stripes, thin stripes, and interstripes in V2, all of which are visualizable using cytochrome oxidase (CO) histochemistry (Horton, 1984; Livingstone and Hubel, 1984; Tootell et al., 1985). There is a high degree of specificity in the intrinsic connections within each area (Livingstone and Hubel, 1984; Blasdel et al., 1985; Fitzpatrick et al., 1985; Lachica et al., 1992; Yoshioka et al., 1994). In addition, the connections between V1 and V2 involve specific pathways between corresponding types of modules (Livingstone and Hubel, 1987a,b).

Previous studies of the fetal development of visual cortex in the macaque have described several maturational changes in architecture and connectivity. The basic architectonic distinction between areas V1 and V2 in the macaque first becomes discernible around embryonic day 93–100 (E93–E100), out of a 165-day gestation (Rostovic and Rakic, 1984; Rakic, et al., 1991). This is near the end of cortical

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neurogenesis but before neuronal migration is complete (Rakic, 1974, 1975). Tracer injections made in utero have demonstrated that projections from V2 (and other extrastriate areas) to area V1 are present by around E115, but their reported laminar distribution differs from that of the adult (Barone et al., 1995). In V2, staining for acetylcholinesterase (AChE) at E121 reveals an alternating pattern of AChE-rich and AChE-sparse stripes which are likely to be developmental precursors of the adult CO stripes (Barone et al., 1995). In V2, staining for acetylcholinesterase (AChE) at E121 reveals an alternating pattern of AChE-rich and AChE-sparse stripes which are likely to be developmental precursors of the adult CO stripes (Barone et al., 1995). In V2, staining for acetylcholinesterase (AChE) at E121 reveals an alternating pattern of AChE-rich and AChE-sparse stripes which are likely to be developmental precursors of the adult CO stripes (Barone et al., 1995).

The advent of the post-mortem diI method for tracing connections in the CNS (Godement et al., 1987) makes it feasible to study the prenatal development of neural connectivity without the necessity of technically difficult in utero tracer injections. Here, we have used this method to analyze the development of intrinsic connections in V1 and V2 as well as extrinsic connections between V1 and V2 in the fetal macaque. Our general objectives were to analyze the precision of initial connections with respect to layers and modules, to test for major differences in the timing at which each pathway is established and refined, and to examine the incidence and timing of sculpting and regressive events vs. sprouting and expansive events. The diI method is effective at labeling in both anterograde and retrograde directions and is well suited for qualitative assessments of cortical connectivity patterns. As a natural adjunct to the study of neural connectivity, we examined cortical architecture at many developmental stages and identified several interesting changes in laminar organization of V1 that have not previously been reported.

### MATERIALS AND METHODS

Brains of six rhesus monkeys (Macaca mulatta) were obtained from Loma Linda University Department of Prenatal Biology under protocols approved by the Animal Care and Use Committee at Loma Linda University and at the California Institute of Technology. Six brains from M. fascicularis and three brains from M. nemestrina animals came under approved protocols from the University of Washington Department of Biological Structure. The rhesus and nemeistra macaques were timed pregnancies; for the fascicularis macaques, gestational age was estimated in utero by palpation and by postmortem examination of features such as myelination of axon tracts, eyelid opening, degree of closure of sulci, and length and weight of the animals. No inconsistencies were found when these brains were compared to those from timed pregnancies. The pregnant mothers were anesthetized with barbiturate anesthesia, and the fetus was immediately perfused after cesarean section. Most of the animals were perfused intracardially with 4% paraformaldehyde in phosphate buffer, but a few brains were fixed only by immersion in paraformaldehyde. Both methods produced fixation that allowed good diI labeling.

The brains were dissected to isolate pieces of tissue which contained V1 alone or both V1 and V2 from the operculum and from calcarine cortex. Single diI crystals (0.4–0.75 mm across) were introduced into these pieces using fire-polished glass needles. The crystals were large enough to label all layers when they were pushed below the pial surface into the middle layers of cortex. Smaller crystals generally failed to produce robust labeling of long-distance connections. After injection, the brains were left in a 1% fixative solution containing 30% sucrose for 2–3 days. The brains were cut into 50-μm thick slices on a vibratome. Most of the brains were perfused intracardially with 10% formalin, and some were immersed in 30% sucrose for 2–3 days. The brains were cut into 50-μm thick slices on a vibratome at 4°C. The sections were postfixed in 1% osmium tetroxide for 1 h, dehydrated through graded ethanol, and embedded in Epon. The sections were cut on a sliding microtome and stained with toluidine blue for orientation. The sections were mounted on slides in 0.1 M phosphate buffer; the diI was viewed with excitation and barrier filters for rhodamine fluorescence, and the bisbenzimide was viewed with filters for UV fluorescence. One in six sections was stained for Nissl substance with cresyl violet. CO staining was done using the method of Tootell and Hamilton (1989). Reconstructions of labeled sections were made by capturing successive fields of view under the microscope (at 10×) using a frame grabber linked to computer control of a motorized microscope stage (Ludl, Hawthorne, NY) using LabView (National Instruments, Austin, TX) and nustep (nuLogic, Needham, MA) software running on a Macintosh computer. Frames were collected at a regular spacing and were assembled into videomontages using custom software on a UNIX workstation. The range in brightness of labeling in most sections was greater than could be accommodated by a single camera setting. In order to provide high sensitivity for frames containing only faint labeling while minimizing saturation in other frames containing dense

### TABLE 1 Number of Injections by Age and Location

<table>
<thead>
<tr>
<th>Case</th>
<th>Species, age determination</th>
<th>Age</th>
<th>Developmental landmarks</th>
<th>V1 injections</th>
<th>Illustrations</th>
<th>V2 injections</th>
<th>Illustrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M. nemestrina; timed</td>
<td>E106</td>
<td>Last neurons born</td>
<td>1/2</td>
<td>Fig. 4</td>
<td>3/2</td>
<td>Fig. 10</td>
</tr>
<tr>
<td>2</td>
<td>M. fascicularis; est.</td>
<td>E118</td>
<td>V2 AChE stripes</td>
<td>3/3</td>
<td>Fig. 5, 6, 17</td>
<td>1/1</td>
<td>Fig. 5</td>
</tr>
<tr>
<td>3</td>
<td>M. mulatta; timed</td>
<td>E118</td>
<td></td>
<td>2/2</td>
<td></td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M. mulatta; timed</td>
<td>E122</td>
<td></td>
<td>0/1</td>
<td></td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M. mulatta; timed</td>
<td>E126</td>
<td></td>
<td>1/1</td>
<td>Fig. 16A</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M. nemestrina; timed</td>
<td>E180</td>
<td></td>
<td>1/2</td>
<td></td>
<td>2/1</td>
<td></td>
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<tr>
<td>7</td>
<td>M. fascicularis; est.</td>
<td>E180</td>
<td></td>
<td>1/2</td>
<td></td>
<td>1/1</td>
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<tr>
<td>8</td>
<td>M. nemestrina; timed</td>
<td>E133</td>
<td></td>
<td>3/4</td>
<td></td>
<td>3/3</td>
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<td>9</td>
<td>M. fascicularis; est.</td>
<td>E145</td>
<td>CO blobs present</td>
<td>2/2</td>
<td>Fig. 7A</td>
<td>3/3</td>
<td>Fig. 8, 12</td>
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<tr>
<td>10</td>
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<td>E165</td>
<td></td>
<td>2/2</td>
<td>Fig. 7B</td>
<td>3/3</td>
<td>Fig. 13, 14</td>
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<tr>
<td>11</td>
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<td>E159</td>
<td></td>
<td>2/2</td>
<td>Fig. 15B</td>
<td>2/3</td>
<td>Fig. 15</td>
</tr>
<tr>
<td>12</td>
<td>M. mulatta</td>
<td>P0</td>
<td></td>
<td>3/3</td>
<td>Fig. 18</td>
<td>4/6</td>
<td>Fig. 9</td>
</tr>
<tr>
<td>13</td>
<td>M. fascicularis</td>
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<td></td>
<td>1/1</td>
<td></td>
<td>2/2</td>
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<td>14</td>
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<tr>
<td>15</td>
<td>M. fascicularis</td>
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<td></td>
<td>0/1</td>
<td></td>
<td>1/2</td>
<td></td>
</tr>
</tbody>
</table>

1Successful injection attempted.
labeling, the contrast range for separate frames was adjusted individually. Consequently, there are artifactual transitions in image brightness at the borders of many frames, but these can be readily distinguished in the montages by their sharpness and by their consistent orientation.

Reconstructions were made for cases judged to have successful transport of label. Unsuccessful cases usually were attributable to insufficient dye deposition, inclusion of white matter in the injection, unduly short incubation time, or excessive artifactual labeling of neurons (through an intermediary of non-neuronal cells in layer 1) from dye absorption into the pia. Another problem was the presence in some cases of regions where the dye was blurred instead of discretely restricted to neuronal processes, possibly the result of incomplete fixation or of partial drying of the tissue during handling. Cases with any of these problems were evaluated for consistency with other results, but are not counted in the tabulation of cases which support our conclusions (Table 1).

RESULTS

Lamination

Examination of Nissl-stained sections at successive developmental stages revealed several maturational changes that have not been previously reported. At the earliest stage examined (E108), both V1 and V2 have a distinctive cytoarchitecture (Fig. 1). The pattern in V2 is similar to the six-layered pattern characteristic of adult V2 (left side of Fig. 1A), and the border between V1 and V2 is nearly as sharp as in the adult. The pattern in V1 at this stage shows many of the characteristics of the highly differentiated adult pattern, as has been reported by Zielinski and Hendrickson (1992), but it differs from the adult in two significant respects. First, layer 4C appears as a uniformly dense granular layer, without any clear distinction between layers 4Ca and 4Cp. Even more striking is the pattern immediately above cell-sparse layer 4B. Instead of the single cell-dense layer 4A characteristic of the adult, the E108 pattern includes two cell-dense layers and an interposed cell-sparse layer (Fig. 1A,B). Note that the more superficial cell-dense layer does not extend to layer 1. We interpret the lower cell-dense layer to be layer 4A, because its juncture with layer 4 of V2 is similar to that in the adult. The other two layers (question mark in Fig. 1B) have no identified counterpart in the adult.

Several changes take place in the laminar pattern of V1 in the following 10 days. Specifically, by E118 (Fig. 2, top left panel) the extra layers above layer 4A are no longer present; layer 4C is divisible into distinct sublayers (4Ca and 4Cb); and layer 4B contains a distinct sublamination that is manifested by alternating dark and light bands in its upper half (Fig. 2, top left panel). The alignment of these extra bands in relation to layer 4 of V2 (not shown) suggests that they are indeed part of layer 4B rather than 4A. This distinctive sublamination of layer 4B persists through prenatal development (E126–E155, in Fig. 2), but is only marginally discernible right after birth (P7). Evidence for sub-lamination of layer 4B was also seen after diI injections, as labeled fibers in layer 4B were segregated into upper and lower compartments.

Because of these maturational changes in lamination of V1, it is important to have independent evidence regarding the identity of the different sublaminae of layer 4. Such evidence was obtained from one case at E122 (case 4) where CO histochemistry was successfully applied to tissue obtained soon after perfusion. In a section from V1 of this hemisphere (Fig. 3A), the characteristic tripartite configuration of layer 4 is evident, including a thick CO-dense band corresponding to layer 4C, a paler region corresponding to layer 4B, and a thin dark band corresponding to layer 4A. Layer 4C appears banded, with a region of slightly less dense staining in the middle of the layer. This may be an early manifestation of the three sublaminae within layer 4C that have been reported in the adult (Yoshioka et al., 1994; Wong-Riley, 1994). The staining in layer 4A includes fine-grained fluctuations similar to the “honeycomb” pat-
tern characteristic of the adult (Horton, 1984). In the adjacent Nissl-stained section (Fig. 3B, a 40-μm frozen section), layer 4B lacked the distinct sublamination previously illustrated in Figure 2. However, sublamination of layer 4B was evident in thicker vibratome sections taken from a different block of the same hemisphere, suggesting that its absence in Figure 3B can be attributed to suboptimal staining rather than individual variability. In the superficial layers of V1, there were no systematic variations in CO staining suggestive of blobs and interblobs (Fig. 3A), which fits with previous evidence for the late onset of CO blobs (Horton, 1984; Purves and LaMantia, 1993).

V1 intrinsic projections

Successful diI deposits led to extensive retrograde and anterograde labeling over distances of many millimeters (see Materials and Methods). Anterograde labeling included fine terminal ramifications, and retrograde labeling included the fine details of dendrites and axon collaterals.

At E108, intrinsic projections in V1 were present, but were sparse in density and limited in extent. Layer 6 neurons up to 2 mm from the injection site were labeled, along with fewer layer 4B cells and a very few superficial layer cells (case 1, Fig. 4). Layers 4B and 5 have concentrations of labeled fibers slightly above that seen in the other layers. More extensive labeling was evident after V1 injection at E118. Labeled neurons in the superficial layers and in layer 6 were found more than 3 mm to each side of the injection site (case 2, Fig. 5). In laminar organization, the connections were close to their mature form, as seen in a section near the injection site (Fig. 5A) and in a section 1.1 mm away (Fig. 5C). Labeled neurons were predominantly in layers 2, 3, 4B, and 6, and fibers spread farthest in layers 4B and 5 and least in 4C and 4A. A few transient characteristics were also seen. The most prominent is the extensive labeling in layer 1, where fibers spread over many millimeters, even traversing the V1/V2 border. This transient layer 1 label persists into the 1st postnatal week. Also, a few subplate neurons were seen near injection sites; their small number and proximity to the injection site suggest that they are not a major source of intrinsic projections at this stage.
Within each layer, the intrinsic connections in V1 had a relatively uniform distribution, with no obvious patchiness. This is particularly evident in sections cut tangential to the cortical surface near a different injection site in case 2 (Fig. 6). Examination at higher magnification revealed that the labeled fibers coursing through the superficial layers were only primitively branched; the tight foci of terminal ramifications that distinguish the mature modular pattern were not yet established. This confluent, rather than patchy, pattern of connections occurred in each of the 11 successful injections (out of 15 attempts) in V1 from seven brains ranging in age from E118 to E133 (cases 2–8).

Patchy connections in superficial V1 were found in both injections made at E145 (Fig. 7A; case 9). Labeled neurons and fibers in the superficial layers were distinctively clustered, the patches measuring 0.3 mm across and having comparable spacing. There were also patches of labeled neurons in layer 4B in register with those in the superficial layers. The pattern of label in the superficial layers can be better appreciated in tangential sections, such as in Figure 7B (E155, case 10). In some regions the labeled cells and fibers appeared as arms of label wrapping partially or completely around unlabeled zones. At their narrowest point the arms were 0.2–0.3 mm wide, but extended 0.6 mm, leaving unlabeled gaps of variable shape and size, 0.3–0.6 mm in their longest dimension. This pattern of label, with dimensions appropriate for surrounding CO-blob-like regions, was found up to 2.5 mm from an injection site. As has been reported using other tracers in the adult (Rockland and Lund, 1983; Lund et al., 1993), this pattern occurred even though the injection was considerably larger than individual blobs or interblobs.

**Projections from V1 to V2**

In one of the three successful V2 injections at E108, a small number of neurons in deep and superficial layers were labeled in V1. The lack of V1 label in the other two cases may have been due to technical factors related to the incubation time and the distance of the injection from the V1/V2 border. At slightly later ages (E118–E130, cases 2, 4, 6, 7, 8) there was extensive retrograde labeling in the superficial layers and layer 4B of V1 after V2 injections, but with no obvious clustering (not illustrated).

Two different patterns of label were seen after two V2 injections at E133 (case 8). In one case, there was extensive retrograde labeling of neurons in the superficial layers and layer 4B, with no indication of a patchy arrangement of the labeled neurons in the superficial layers (Fig. 8A). The other V2 injection in the E133 brain resulted in a much higher density of labeled cells in layer 4B relative to the number of superficial layer cells labeled. Figure 8B shows a section in the center and Figure 8C a section near the margin of the labeled zone, illustrating the range seen in the density of superficial layer cells labeled. This suggests that the segregation of the projections from layer 4B to the thick stripes has begun at E133.

The earliest V2 injections that led to a clustered pattern of cells in the superficial layers of V1 were at age E145 (two of three injections), the same stage at which V1 intrinsic connections first showed a patchy organization. The clustered pattern in V1 is illustrated for a P0 brain (Fig. 9, case 12). Multiple patches are visible in layers 2, 3, 4B, 5, and 6, all in register with one another.

**V2 intrinsic projections**

Extensive intrinsic connections in V2 were evident in the earliest injections attempted (E108, three injections). The initial pattern differed strikingly from that in the adult in two respects. First, labeled neurons were concentrated in deep layers, especially layer 5 (Fig. 10). Second, this band of layer 5 labeling extended continuously up to 4 mm from the injection site, without evidence of the patchiness characteristic of the adult. A few labeled fibers were found in the superficial layers where they coursed, with little branching, amidst the few immature superficial layer neurons which were labeled. By E118, superficial layer neurons contributed to intrinsic projections about equally with deep layer cells (cases 2 and 3; not illustrated).

Patchiness in the intrinsic connections in V2 arose at earlier stages than was the case for intrinsic connections in V1. Two of the nine V2 injections made in brains between stages E126 and E133 resulted in a distinctly clustered pattern of connections, and another two were suggestive of a clustered pattern. Figure 11 shows one example (case 6, E130) that includes two broad patches of labeled cells in V2 (upper part of montage), as large as 2 mm across, separated by a sparsely labeled region measuring 0.7 mm across. The labeling in V1 from this injection (lower part of montage) produced a narrowly confined, continuous band of labeling in the superficial layers, layer 4B, and layer 6 in V1 (note upside-down orientation relative to V2). Figure 12 shows a different pattern of intrinsic V2 connections, arising from the injection in an E133 brain (case 8) that had produced mainly layer 4B labeling in V1 (see Fig. 8B,C). The V2
Fig. 4. Distribution of intrinsically projecting V1 neurons at E108 (case 1) revealed by application of DiI crystals to fixed tissue. A: Photomicrograph of a section 1.3 mm from the injection site shows labeled neurons principally in layer 6 and 1. Labeled fibers are densest in layers 5, 4B, and 1. Note the paucity of labeling in the still-forming superficial layers. B: Central part of a photomicrograph of a bis-benzimide counterstain of the same section shown in A. Scale bar = 0.5 mm.

Fig. 5. Distribution of intrinsically projecting V1 neurons at E118 (case 2). A: Videomontage of a section cut perpendicular to the cortical layers, showing the halo of fluorescence near the injection site as well as a large number of labeled neurons in the superficial layers and layer 6. The sublamination of the labeling in layer 4B is evident at the center of the halo. B: Bisbenzimide counterstain of section in A. C: In a section 1.1 mm away from the injection site, the lack of label in layer 4C is striking. Labeled neurons are widely distributed in the superficial layers, but there is no evidence of clustering. Scale bar = 1 mm.

Fig. 6. Videomontage of a tangential section through the superficial layers of V1 at E118 (case 2). At the fringe of the area containing labeled cells there are regions with more labeled cells than other regions, but there is no evidence of the regular, modular organization of connections seen at later stages. Artifactual jumps in the brightness of label is seen at the borders of the individual frames in the montage. Scale bar = 1 mm.

The size, spacing and density of the clusters seen in the E130 and 133 cases differ markedly from the adult pattern of intrinsic connections in V2. More mature intrinsic V2 connections were present at E145 (case 9). Results of an E145 injection are shown in Figure 13. The top panels show the pattern of DiI label in two nearby sections. Distinct, isolated patches of label were found at distances of up to 3.5 mm away (asterisks). In addition, several patches of label were found within 1–2 mm from the injection site. In the closely spaced clusters of fibers near the injection site, afferent fibers were rudimentary in their degree of ramification. (Note also the patchy distribution of labeled cells in V1 in Figure 13B, demonstrating the clustered nature of extrinsic projections at the same stage that intrinsic connec-

labeling in this case involved irregular clusters of cells approximately 0.7 mm wide (asterisks) in layers 3 and 5, extending up to 4 mm from the injection site (Fig. 12). These clumps of labeled neurons were not accompanied by dense columns of labeled fibers. Taken together, these different labeling patterns suggest that the nascent stripe types in V2 (Barone et al., 1994a,b) differ in their patterns of intrinsic as well as extrinsic connectivity.
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Fig. 7. Patchy pattern of connections in the superficial layers of V1. A: Fluorescence videomontage of an El45 injection (case 9) cut perpendicular to the layers of V1. Patches of labeled neurons are seen in superficial layers and layer 4B. B: Results of crystal injection into tissue from El55 brain (case 10), cut tangential to the cortical surface. The site of injection is at lower left, and around that site the tissue is saturated with dye, creating a halo of bright fluorescence. Outside the halo, the label has an uneven distribution. Arms of label surround gaps which do not contain labeled neurons or fibers. Scale bar = 1 mm.

Injections in V1 become patchy. Figure 14, from a different V2 injection site in the same El45 case, shows that the retrogradely labeled neurons in the distant V2 clusters were in layer 5 and in lower layer 3, as they are in the mature pattern (Rockland, 1985).

Injection of V2 at El55 resulted in the labeling of an adult-like array of columnar clusters of neurons and fibers up to 4 mm from the injection site (Fig. 15; case 10). The spacing of the columns varied from 0.2–1 mm, while the columns themselves varied in width 0.2–0.6 mm. Labeled neurons were mainly in layer 5 and lower layer 3. At the center of the columns, afferent fibers spanned all cortical layers but were less dense in layers 6, 4, and 2. Away from the center of the column, fibers were dense only in layers 2 and 3. These results are in agreement with results on adult intrinsic V2 connections obtained using other tracing methods (Rockland, 1985; Levitt et al., 1994).

Projections from V2 to V1

Our results indicate that the projection from V2 to V1 passes through a stage in which it derives principally from deep layer cells, but this stage lasts longer for the interareal projection than for the intrinsic V2 projection. Numerous deep layer cells in V2 were labeled from the one successful V1 injection at E108. At somewhat later stages (E118–E133), the V2 labeling was similar in distribution but greater in density, as the projection appears to gain strength in the succeeding weeks. An example from an E126 case shows retrograde labeling in V2 almost exclusively in deep layer cells (Fig 16A; case 5). Three more examples of predominantly deep layer origins were observed between E118 and 133, and no counterexamples were found. By E145 substantial numbers of superficial layer cells were labeled in the feedback projection from V2 to V1. The retrogradely labeled cells and afferent fibers had a patchy organization, adding to the evidence that V2 has a modular organization at this stage. The results of a V1 injection into an El59 brain (case 11) is illustrated in Figure 16B. In addition to the numerous retrogradely labeled superficial layer cells, this figure also shows a patchy pattern of anterogradely labeled fibers in the middle and deep layers that ramify more extensively than at earlier developmental stages.

Additional observations

The spatial extent of retrograde labeling can be used to assess the sharpness of the targeting of interareal projec-
Fig. 8. Distinct patterns of retrograde label in V1 after different injections of V2 in the same E133 brain (case 8). A: Large numbers of cells are labeled in superficial layers as well as layer 4B. B,C: In the second example, the label is much more concentrated in layer 4B, indicating that terminals from layer 4B cells predominate at the V2 the injection site. Scale bar = 0.5 mm.

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sections at different developmental stages. The restricted pattern of retrograde labeling in V1 after V2 injections and in V2 after V1 injections (Figs. 8, 11, 13) indicates that orderly topographic projections between areas are established early in development. To obtain a semi-quantitative assessment of the precision of this mapping, we measured the extent of labeled regions, including the most distant clusters of cells but not individual neurons or fibers. The size of the labeled regions in V1 measured 4–5 mm in six fully reconstructed cases between E118 and E145, whereas in two E155–P0 cases, the labeled regions were 7–8 mm across. Injection sites in these cases varied between 0.4 and 0.6 mm, but their size was not correlated with age. Given that there is a 64% increase in the area of V1 during this
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Fig. 9. Label in V1 and V2 resulting from V2 injection at P0 (case 12). On the V1 side, labeling in superficial layers has a patchy pattern. A column of fibers is seen in V2. Unlike the other illustrated cases, this one was cut roughly parallel to the V2 stripes. Scale bar = 0.3 mm.

Fig. 10. V2 intrinsic connections at E108 (case 1). A: Labeled neurons form a dense, continuous band in layer 5. The apical dendrites of these cells extend beyond the few labeled cells in the superficial layers to layer 1. B: Bisbenzimide stain of an adjacent section. Scale bar = 1 mm.

Fig. 11. Pattern of intrinsic connections in V2 at E130 (case 6). A: Videomontage of label in V2 and V1 resulting from V2 injection at E130. In V2 (upper part of A), two broad bands of label are found which measure 1.5–2 mm across. In V1 (lower part of A), a tight region of retrograde labeling is found in the superficial layers with no patchy pattern evident. Labeled fibers are dense in layers 4B and 5. B: Central part of reconstruction of bisbenzimide stain of same section. Scale bar = 0.5 mm.

period, and thus about a 30% increase in linear dimensions (Purves and LaMantia, 1993), much if not all of the increase in extent of label would be a passive consequence of physical expansion in surface area, rather than any establishment of new connections. Thus, it appears that the fidelity of the V1 to V2 projection does not change dramatically during prenatal development. Similar conclusions were reached in studies of the postnatal development of the projection from area 17 to area 18 of the cat (Price et al., 1994; Kennedy et al., 1994).

Many of the deep layer cells in V2 which project to V1 have apical dendrites that extend into layer 1 (Fig. 16A). Similarly, layer 6 cells which project intrinsically in V1 at E118 have dendrites that extend well past layer 3 (Fig. 17), and some can be seen to extend into layer 1. This was not reported in previous studies of mature layer 6 cells in V1 or V2 (Lund, 1973; Lund and Boothe, 1975; Valverde, 1978; Lund et al., 1981). Determining how late in development such dendrites persist was made difficult by the extensive elaboration of processes, but one layer 6 cell in V1 with layer 1 dendrites was found in a P0 specimen.

A final observation concerns the distinctive architecture of layer 4A. In CO stains, layer 4A is a latticework of stained regions around pale hollows (Horton, 1984). It has been
suggested that these hollows contain "cones" of neurons in 4A, whereas the sides of the web are composed of processes passing through 4A (Peters and Sethares, 1991). Supporting this conclusion is the bundling of processes around the cones in 4A that was revealed in a P0 brain (Fig. 18, case 12). This pattern was seen as early as E130, although it was less pronounced.

**DISCUSSION**

During prenatal development of areas V1 and V2, we found changes in laminar structure, in the basic morphology of projection cells, and in the lamination of projections within and between areas. In addition, both intrinsic and extrinsic projections showed a transition from initially continuous patterns to adult-like, patchy connections. The timing of these changes relative to developmental landmarks reported in other studies is illustrated in Figure 19.

Two key relationships to note in this figure are 1) that axons involved in horizontal intrinsic connections and interareal projections are being extended even as the last cortical cells are still migrating into position (ca. E110), and 2) that intrinsic and interareal projections in V1 and V2 develop their patchy organization around the time that lateral geniculate nucleus (LGN) afferents begin to segregate into ocular dominance stripes. In both V1 and V2, there is evidence that the components of the magnocellular-dominated stream (layer 4B and the thick stripes) are distinct before the blob- and interblob-dominated pathways are distinct from one another. By a week before birth these cortico-cortical projections are close to their mature configuration, although fine modifications presumably occur during postnatal development.

**Development in the radial dimension**

Previous studies have suggested that the border between V1 and V2 becomes discernible somewhere between E93 and E100 (Kostovic and Rakic, 1984; Rakic et al., 1991), consistent with our finding of a very distinct border at E108. The laminar pattern of V1 during embryonic development includes cell-dense and cell-sparse layers that are not discernible in Nissl-stained sections from adult animals. Layer 4 of V2 had a consistent position relative to a cell-sparse layer in V1 throughout the period studied, and we have used this proximity as a basis for identifying that cell-sparse layer as layer 4B, an approach validated by the identification of layer 4B in the CO stain. This suggests that there is a transient sublamination of 4B during later embryonic development, and there is a transient cell-dense layer above layer 4A at E108. A transient sublamination of layer 4B was reported in CO staining at E143 (Horton,
DEVELOPMENT OF V1 AND V2 CONNECTIONS

Fig. 14. Example of a cluster of intrinsically projecting cells in V2 at E145. This example is from a second injection in the same brain as illustrated in Figure 12. A: Labeled neurons are dense in layers 3 and 5. B: Bisbenzimide stain of the same section. Scale bar = 0.25 mm.

Fig. 15. Clustered intrinsic connections in V2 at E155 (case 10). Videomontages of sections 0.9 mm apart. Columns of dense label, made up of ramifying fibers and neurons in layers 3 and 5, are seen in each section. Scale bar = 1 mm.

1984) along with several other laminar features not seen in the mature CO pattern. A band of CAT-301 staining in lower layer 4B and upper 4Ca in the adult (DeYoe et al., 1990) might be a persisting correlate of this early developmental feature. The laminar assignments we have proposed need to be tested with other methods, including histochem-
With regard to the development of connections in different layers, our most striking finding was the delayed maturation of intrinsic and extrinsic projections from the superficial layers in V2 relative those from the deep layers. Initially, connections within V2 and from V2 to V1 arise predominantly from deep layers. By E118, substantial numbers of superficial layer cells are involved in intrinsic V2 connections, but the projection from V2 to V1 derives predominantly from deep layer cells as late as E133. The time difference in superficial cells projecting intrinsically versus projecting from V2 to V1 might reflect a delay in V1 becoming a competent target for innervation by fibers from superficial layers. The dense webs of fibers seen in the white matter between V1 and V2 (Fig. 11) suggests that these fibers may have a waiting period in the cortical subplate as occurs for geniculocortical projections (Rakic, 1977; Shatz and Luskin, 1986).

Evidence for delayed maturation of superficial layer connections relative to deep layer connections has been reported in humans (Burkhalter, 1993) and in rodents (Ivy and Killackey, 1981; Coogan and Burkhalter, 1988). In contrast, two recent studies (Meisserel et al., 1991; Barone et al., 1995) have reported that the projection from V2 to V1 in the macaque initially involves a transient, excessive projection from the superficial layers. Their results were obtained using multiple large injections of fluorescent retrograde tracers into V1. One possible explanation for the discrepancy between their study and others (particularly the present one) is that their injections may have led to more extensive uptake by fibers in the white matter just under V1 where, as suggested above, the projection from superficial layers of V2 might be transiently concentrated. Another possibility is that the projection from superficial layers of V2 involves notably sparse or diffuse projections that can be detected by multiple large tracer injections in vivo but not by our more focal postmortem deposits. Although the diI method has been noted for its sensitivity (Godement et al., 1987), it may be that afferent axons need a minimum number of terminal branches to absorb enough dye to be detected. Analysis using anterograde tracers should distinguish among those and perhaps other alternatives. In any event, it appears that projections from the superficial layers are qualitatively less mature than those from deep layer cells over a significant period of prenatal development.

Fig. 16. Origins of projection of V2 to V1. A: At E126 (case 5) neurons are principally in the deep layers of V2, and the dendrites of these cells reach layer 1. B: At E159 (case 11) retrogradely labeled neurons are prominent in the superficial layers. Deep to them are clusters of afferent fibers dense enough to saturate the video camera (white regions). Scale bar = 0.5 mm in A, 1 mm in B.
Segregation of streams of processing in V1

Extensive intrinsic connections in the superficial layers of V1 have formed by E118, 2 weeks after the birthdate of the last generated neurons in V1. As it has been estimated to take more than a week for these superficial cells to migrate into position (Rakic, 1975), it is likely that some connections are forming even as cells are still migrating.

Connections within the superficial layers of V1, and cells projecting from those layers to V2, have a patchy organization by E145 (Figs. 7, 13). Patchy connections in superficial V1 at E140 have also recently been reported Yoshioka (1994). This corresponds well with the stage at which cytochrome oxidase blobs are first discernible (cf. Purves and LaMantia, 1993; Horton, 1984). Before E140 it appears that connections in superficial V1 have a confluent, rather than a patchy, pattern (Figs. 5, 6). This suggests that the modular pattern is the product of regressive forces, as has been reported for intrinsic connections in the cat striate cortex (Callaway and Katz, 1990). If clusters are produced by differential addition of connected cells, rather than retraction of connections, then the density of labeled cells between patches should be similar before and after patches are evident. A comparison of the density of labeling in Figures 5 and 7 suggests instead that retraction of connections does play a role in producing clustered connections. The dense elaboration of processes later in development makes a quantitative test of this idea difficult in diI material, however.

Employing methods similar to those used here, Burkhalter et al. (1993) investigated the development of connections in human area V1. The earliest age at which they observed major intrinsic horizontal connections in the superficial layers of V1 was in postnatal brains, long after the appearance of the cytochrome oxidase blobs. This contrasts with the much earlier appearance of intrinsic V1 connections reported here for the macaque.

We obtained two lines of evidence that layer 4B, associated with the magnocellular-dominated processing stream (Livingstone and Hubel, 1987a), develops more rapidly than the two compartments (blobs and interblobs) of the
Fig. 18. Dense dil labeling shows the honeycomb structure of layer 4A. A: V1 injection at P0 (case 12) produces a patchy distribution of label in the superficial layers, dense fiber labeling in layers 5 and 4B, and radial fiber label in 4C. B: Higher-power view of A shows that processes passing through layer 4A are bundled together to pass through "cones" devoid of label. Scale bar = 0.6 mm in A, 0.2 mm in B.

Fig. 19. Relation of present results to developmental landmarks. The relative timing of significant events in the development of the cortex which have been previously reported (Rakic, 1974, 1975, 1977; Horton, 1984; Barone et al., 1994a) are shown above the developmental timeline. Below the timeline we catalog the most significant findings of the present study, organized according to the projection involved. Although these developmental processes are presumably continuous, we assign a change in the state of a projection to the earliest point in time at which our data revealed it. The size of the boxes has no significance, except in the case of the two extended boxes which denote that a state of a projection persisted for more than one period studied. The arrows above the timeline indicate that there is some uncertainty as to when precisely those drawn-out processes may be considered concluded.

Superficial layers. First, CO staining shows 4B to be clearly distinct from 4A and 4C by E122 (Fig. 3), which is consistent with evidence that geniculocortical projections discriminate between layers 4C, 4B, and 4A by E124 (Rakic, 1979). Second, the projections from layer 4B to V2 begin segregating within V2 around E133 (Fig. 8), whereas our earliest evidence for segregation of the blob versus inter-blob projections to V2 was not until E145. An earlier development of connections in layer 4B has also been reported for human cortex (Burkhalter et al., 1993). This temporal sequence is consistent with the earlier birthdates of neurons in layer 4B compared to those in the superficial layers (Rakic, 1974).

Development of the modular organization of V2

Recent histochemical evidence for the prenatal development of the modular organization of V2 provides a frame-
work for the interpretation of our results on the development of clustered connections in V2. Barone et al. (1994a) and Sesma and Burkhalter (1994) found AChE-enriched stripes at E121, perpendicular to the V1/V2 border, that were on average, 2.2 mm wide and were separated by 0.5-mm gaps. Barone et al. (1994b) have further reported that tracer injections in V4 at E129 resulted in patches of retrograde label in V2 that coincided with the AChE-enriched bands. This result suggests that the AChE-enriched bands encompass the region that will later split into CO thin stripes and interstripes, both of which project to V4 in the adult (Shipp and Zeki, 1985; DeYoe et al., 1994).

Our results are consistent with these findings, in that the earliest stage of non-uniform intrinsic label in V2 occurred around the same stage at which the AChE stripes develop. One case (case 6, E130) involved wide bands of labeling comparable in size to the AChE-enriched bands. Another case (case 8, E153) resulted in labeling of narrow clusters in V2 (0.5–7 mm), which is the appropriate size for the presumptive thick stripes associated with the AChE-sparse zones; this correlates with the fact that the projection from V1 in this case originated primarily from layer 4B. Altogether, our findings provide circumstantial support for the idea that the thick stripes are distinct before the thin and interstripes have segregated from one another.

**Cell form**

As late as E130, many of the projection cells in layer 6 of V1 and V2 have dendrites that extend into layer 1. Such cells have not been reported in Golgi studies of postnatal macaque V1 and V2 (Lund, 1973; Lund and Boothe, 1975; Valverde, 1978; Lund et al., 1981). These cells themselves may be transient, or the cells may lose their dendritic connections with layer 1. Although Lund et al. (1977) argued on the basis of Golgi material that the dendritic form of layer 6 cells in the macaque is not the result of regressive processes, other reports make it seem likely that this occurs. Shatz and Rakic (1981) found that presumptive efferent cells in layer 6 had apical dendrites which reach the marginal zone (layer 1) at E64, but were unable to follow the fate of these cells. Dendritic retraction has been explicitly reported in studies of the development of other species, for example layer 6 cells in cat cortex (Marin-Padilla, 1971), and deep layer callosal cells in rat cortex (Koester and O'Leary, 1992). The addition of layer 6 cells of areas V1 and V2 of macaques to this list suggests that transient layer 1 dendrites are a common feature of cortical cell development.

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**LITERATURE CITED**


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