Role of Directed Growth and Target Selection in the Formation of Cortical Pathways: Prenatal Development of the Projection of Area V2 to Area V4 in the Monkey

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ABSTRACT
In experiments combining retrograde tracers and histochemistry, we have looked at the prenatal development of the cortical pathway linking areas V2 and V4. Transient expression of acetylcholinesterase in fetal area V2 reveals the separate compartments that project to V4 (temporal directed pathway) and V5 (parietal directed pathway). During early stages of pathway formation, V2 neurons projecting to area V4 are clustered in the appropriate compartments. During the phase of rapid axonal growth, there is a selective increase of connections originating from the appropriate compartments leading to a strongly clustered organization at the peak of connectivity. During this phase, injections involving the white matter also showed clustering, but this was somewhat reduced in comparison to that of gray matter injections. The growth phase is followed by an elimination phase during which there is a tendency for a preferential loss of intercluster connections, which may sharpen the early formed pattern.

These results demonstrate the primary role of axonal guidance and target recognition mechanisms followed by a limited extent of selective elimination during the formation of functional cortical pathways in the primate isocortex. Compared to previous findings, these results suggest that the developmental restriction of callosal connections is not a universal model of cortical development. In the present report, the directed growth and early specification of feed-forward connections contrast with the prolonged remodelling of monkey feedback projections, suggesting two distinct developmental strategies of pathway formation in the monkey.

The role of refinement in the development of association connections has been examined in the projections of striate connections linking cortical layers (Lund et al., 1977; Katz, 1991) the formation of horizontal intrinsic connections (Callaway and Katz, 1990; Lubke and Albus, 1992; Kennedy et al., 1994; Galuske and Singer, 1996), the topography of extrinsic association connections (Kennedy et al., 1994), and the bifurcation of callosal and association projections (Schwartz and Goldman-Rakic, 1982, 1991; Meissirel et al., 1990, 1991).

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cortex to areas 18 and 19 in the cat. These studies suggest that extensive postnatal refinement leads to the adult pattern of connectivity (Bullier et al., 1984a; Price and Blakemore, 1985a,b; Price et al., 1994b).

The visual cortex of the primate constitutes a particularly advantageous model for studying the establishment of functionally defined pathways. It is composed of a number of relatively well-characterized cortical areas that are organized hierarchically (Felleman and Van Essen, 1991; Salin and Bullier, 1995) and in functionally and anatomically segregated streams (Ungerleider and Mishkin, 1982; Van Essen and Maunsell, 1983; DeYoe and Van Essen, 1985; Livingstone and Hubel, 1988; Shipp and Zeki, 1989; Zeki and Shipp, 1989; Lund et al., 1993; Nakamura et al., 1993; Peterhans and Von der Heydt, 1993; DeYoe et al., 1994; Levitt et al., 1994, 1995). Area V2 is the cortical locus from which the two main functional streams diverge. A dorsal parietal stream is directed towards area V5 or MT and the posterior parietal cortex. Neuronal response properties in this stream suggest that it is specialized in motion analysis (Dubner and Zeki, 1971; Zeki, 1974; Maunsell and Van Essen, 1983a,b). The ventral temporal stream is directed towards area V4 and the inferotemporal complex and deals with form and color analysis (Zeki, 1978; Desimone et al., 1985; Desimone and Schein, 1987; Schein and Desimone, 1990). In the adult area V2, these functional pathways occupy adjacent cortical territories, which can be identified with cytochrome oxidase (CO) histochemistry (Tootell et al., 1983; Livingstone and Hubel, 1984). Neurons projecting to V4 and temporal cortices reside in the thick CO bands (DeYoe and Van Essen, 1985; Livingstone and Hubel, 1988; Shipp and Zeki, 1989; Nakamura et al., 1993; DeYoe et al., 1994). In the present study, we have investigated the mechanisms responsible for the segregation of these two pathways.

At early prenatal stages, CO activity is uniform in area V2, so this technique cannot be used to demarcate the V4 and V5-projecting territories. However, throughout the last 2 months of gestation, area V2 exhibits bands of high acetylcholinesterase (AChE) activity running perpendicular to the area V1/V2 border (Barone et al., 1994). Although the AChE bands disappear postnatally, they are still well defined at birth. Studying neonates, we have reconstructed the location and dimensions of the two sets of bands and shown that AChE bands demarcate the territories containing the clusters of V4 projecting neurons. Armed with a marker of V4 projecting territory in area V2, we have investigated the degree of clustering of V4-projecting neurons throughout fetal development. These tracing experiments show that there is clustering of V4 projection neurons from near the onset of pathway formation. The early stages of pathway formation are characterized by rapid axonal growth, leading to a sharp increase in numbers of connections. During this growth phase, the rate of increase of connections from appropriate compartments is considerably greater than that from inappropriate compartments, such that there is strong clustering when peak levels of connectivity are achieved 1 month before birth. Together, these results show that directed growth and target selection play major roles in the formation of the V4-projecting pathway. To distinguish between these two mechanisms, we have compared patterns of connections obtained with injections restricted to the gray matter of area V4 to those patterns obtained with injections involving the underlying white matter. This shows that there is a larger concentration of appropriate fibers in the white matter, indicating directed growth. However, compared to gray matter injections, fetal white matter injections label a slightly larger proportion of inappropriate projections, suggesting that target selection also contributes to the formation of the adult pattern of connectivity. The early growth phase is followed by a phase of elimination of connections. During the elimination phase, there is a tendency for a higher rate of loss of connections between clusters, which might serve to sharpen the early-formed pattern, leading to an adult-like connectivity around birth.

**MATERIALS AND METHODS**

**Surgical procedures and dye injections**

Timed pregnant cynomolgus monkeys were used to provide eight fetuses of known gestational age. Data were collected from six neonates (6–20 postnatal days), three juveniles (2–16 postnatal months), and one adult (Table 1). Pregnant females were premedicated with atropine (1.25 mg i.m.), demethasone (4 mg i.m.), and ixosuprine (2.5 mg i.m.) and prepared for surgery under ketamine hydrochloride (20 mg/kg i.m.) and chlorpromazine (2 mg/kg i.m.). Following intubation, anaesthesia was continued with halothane in an N2O:O2 (70:30) mixture. Artificial respiration was adjusted to maintain the end-tidal CO2 at 4.5–6.0%. Heart rate was monitored, and rectal temperature was maintained at 37°C.

A midline abdominal incision allowed uterotomy to be performed over the posterior part of the brain. Craniotomy was performed using an operating microscope to reveal, under sterile conditions, the lateral part of the pericranial. Aqueous solutions (3%) of fluorescent tracers (fast blue or diamidino yellow) were injected by using Hamilton syringes in a stereotyped fashion. Injections were made on the lateral part of the prelunate gyrus where the central representation of the visual field is located (Gattass et al., 1988). The type of tracer used and the duration of the survival times are provided in Table 2. In all cases, injections were made at a shallow angle to the cortical surface in such a way that the injection spanned about 3 mm. Following injections, bone flaps were closed and the scalp was stitched back in position. Fetuses were replaced in the uterus, and incisions were closed using routine procedures.

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**TABLE 1. Ages at Injection and Perfusion, Histochemistry, and Injections**

<table>
<thead>
<tr>
<th>Cases</th>
<th>Injection-perfusion</th>
<th>CO histochemistry</th>
<th>AchE histochemistry</th>
<th>Injections</th>
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<tr>
<td>B3113</td>
<td>E115</td>
<td>+</td>
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<td>B3115</td>
<td>E106–E116</td>
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<td>+</td>
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<tr>
<td>B3109</td>
<td>E112–E123</td>
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<tr>
<td>B398</td>
<td>E128</td>
<td></td>
<td>+</td>
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<tr>
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<td>E140</td>
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<tr>
<td>B3105</td>
<td>E129–E142</td>
<td>+</td>
<td>+</td>
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<td>PNM16</td>
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<tr>
<td>M45</td>
<td>Adult</td>
<td></td>
<td>+</td>
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</tr>
</tbody>
</table>

1E, embryonic day; PND, postnatal day; PNM, postnatal month.
The pregnant monkey received postoperative medication consisting of a muscular relaxant (ixosuprine chloride) and an analgesic (tiemannium methylsulfate). Fetuses were delivered by caesarian section after a 10–13 day survival period. Animals were deeply anaesthetized before being perfused transcardially with 200 ml of 2.7% saline, 1–3 liters of a 0.5% glutaraldehyde and 8% paraformaldehyde mixture in 0.1 M phosphate buffer, 0.5 liters of 8% sucrose, 0.5 liter of 20% sucrose, and 0.5 liter of 30% sucrose in phosphate buffer. Brains were immediately removed and blocked and parasagittal, horizontal, or tangential 40-μm-thick sections cut on a freezing microtome. Sections were mounted in saline onto gelatinized slides.

Tissue preparation

Two fetuses, three newborns, two juveniles (4 and 16 months old), and one adult monkey were used to study the ontogenesis of AChE expression and the spatial relationships between AChE and CO bands (Table 1). Following perfusion, brains were dissected into appropriate blocks containing area V1 and V2 and physically flattened. Sections were cut tangentially to the surface, and AChE histochemistry was performed by using a modified protocol of Hardy and coworkers (1976) and cytochrome oxidase activity by using the protocol of Silverman and Tootell (1987). Distances between the centers of AChE bands and CO stripes were measured in each animal on several sections. In two of the neonates, one hemisphere was used for reconstructing the AChE and CO bands. CO stripes and AChE bands were drawn with a camera lucida, and reconstruction over several sections were performed by using blood vessels as anatomical landmarks.

Observation procedures

In animals that received tracer injections, sections were observed under ultraviolet (UV) light with oil-immersion objectives by using a Leitz fluorescence microscope equipped with a D-filter set (355–425 nm). Neurons retrogradely labeled by fast blue exhibit a blue coloration of their cytoplasm. Neurons labeled by diamidino yellow exhibit a yellow nucleus. An x-y plotter electronically coupled to the microscope stage was used to trace out sections and to record the positions of labeled neurons. Sections were counterstained with cresyl violet and were projected onto charts of labeled neurons to relate the positions of labeled neurons to histological borders. Total numbers of neurons were counted as nuclei at a magnification of ×100 by using a differential interference contrast (DIC) optics on Nissl-counterstained sections.

Quantitative analysis

A linear density analysis was made of retrogradely labeled neurons in area V2 to determine the degree of clustering. Sections were sampled throughout the full extent of projection zones. In all cases, except for BB96, AChE histochemistry was performed on the adjacent sections. The limits of the AChE bands were drawn using a camera lucida and then were reported onto the charts of labeled neurons obtained from the adjacent section. Counts of labeled cells were performed in each of the AChE bands and interbands. Linear densities of labeled neurons in AChE bands and interbands were obtained by dividing the number of labeled cells by the extent in millimeters of each compartment. In BB96, the limits of the clusters were determined visually, and linear densities were calculated in and between clusters.

Because of changes during development in the size of the cortex and the associated changes in density, linear densities were converted into a labeling index (LI). The volume of cortex containing labeled cells was measured in bands and interbands. This enabled us to calculate the total number of labeled and unlabeled neurons in a cubic millimeter of cortex, which provides the proportion of labeled neurons at different ages: LI = [(labeled neuron density/(labeled neuron density + unlabeled neuron density)] × 100.

The LI was calculated separately in central and peripheral subregions of the projection zone. The central projection zone included all regions with over 70% of the maximum labeling. The peripheral projection zone included the region containing less than 40% of the maximum labeling. A clustering index, ([LI bands - LI interbands]/LI bands) × 100, was used to reflect the percentage differences of labeled cells between AChE compartments.

Statistical analysis

Changes in AChE bands periodicity during development were analyzed by using ANOVA tests. Comparison of linear density values between bands and interbands was performed by using several statistical tests. A nonparametric test (Mann-Whitney U test) first was used to compare the linear density values of bands and interbands obtained throughout the entire projection zone. Heterogeneity of labeled cell density inside area V2 artifactually reduces the significance levels generated by this analytical procedure. To avoid this, a nonparametric paired comparison (Wilcoxon test) was performed on pairs of adjacent AChE bands and interbands throughout the projection zone. Comparison of linear density values obtained following white or gray matter injection was performed by using the nonparametric Mann-Whitney U test. Numbers of bands, interbands, and neurons in each animal as well as the statistical significance levels are reported in Table 2.

RESULTS

Spatial relationship of AChE and CO bands

AChE is strongly and transiently expressed in the supragranular layers of extrastriate cortex of the immature brain (Kostovic and Rakic, 1984; Barone et al., 1994). By cutting the cortex parallel to its surface, it is possible to visualize the tangential organization of AChE expression. At E94, AChE activity appears continuous and shows no hint of band formation (Kostovic and Rakic, 1984; Barone et al., 1994). By birth (E165), AChE expression in area V2 forms bands of high activity running perpendicular to the V1-V2 border. By examining material at different developmental stages between E94 and birth, we have been able to describe the morphogenesis of the transient AChE bands. At E115, the continuous AChE activity in the supragranular layers begins to break up, and small islands of weaker intensity can be detected (Fig. 1). After E115, AChE activity is virtually lost in the paler regions, and, by E123, the supragranular AChE expression forms a honeycomb pattern. By E142, the AChE bands appear to be fully formed. A clue to the mechanism of band formation can be gained from the E123 honeycomb pattern, for which there are indications that the AChE-poor gaps are coalescing to form the future AChE-poor interbands (see arrows for the E123 fetus in Fig. 1). Altogether, these results show that band
<table>
<thead>
<tr>
<th>Ages at injection-perfusion</th>
<th>Tracer</th>
<th>Number of labeled neurons</th>
<th>Linear density (neurons/mm)</th>
<th>Global</th>
<th>Center</th>
<th>Periphery</th>
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<tr>
<td></td>
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<td>Bands</td>
<td>Interbands</td>
<td>P</td>
</tr>
<tr>
<td>E186–E196</td>
<td>FB</td>
<td>509</td>
<td>7.28</td>
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<td>E198–E208</td>
<td>FB</td>
<td>7.444</td>
<td>25.1 (106)</td>
<td>20.7 (97)</td>
<td>*</td>
<td>1.41</td>
</tr>
<tr>
<td>E212–E234</td>
<td>FB</td>
<td>7.029</td>
<td>57.7 (28)</td>
<td>62.1 (24)</td>
<td>***</td>
<td>1.64</td>
</tr>
<tr>
<td>E129–E142</td>
<td>FB</td>
<td>7.405</td>
<td>113.2 (28)</td>
<td>50.3 (26)</td>
<td>***</td>
<td>3.60</td>
</tr>
<tr>
<td>E149–E151</td>
<td>DY</td>
<td>3.243</td>
<td>61.2 (16)</td>
<td>12.1 (11)</td>
<td>**</td>
<td>1.71</td>
</tr>
<tr>
<td>PND6–PND19</td>
<td>DY</td>
<td>5.219</td>
<td>64.3 (24)</td>
<td>18.0 (19)</td>
<td>***</td>
<td>1.51</td>
</tr>
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<td>PND10–PND20</td>
<td>FB</td>
<td>2.958</td>
<td>50.3 (15)</td>
<td>21.4 (12)</td>
<td>**</td>
<td>1.79</td>
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<td>PND61–PND71</td>
<td>FB</td>
<td>528</td>
<td>(9)</td>
<td>(4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1E, embryonic day; PND, postnatal day; FB, fast blue; DY, diamidino yellow. In parentheses, number of bands or interbands.

2Significance level of Mann Whitney U test of comparison of band vs. interbands; ns, nonsignificant; *P < 0.05; **P < 0.01; ***P < 0.001.
formation covers a 27 day period beginning shortly before E115 and terminating around E142 (Fig. 1).

An important aim of the present study was to determine the spatial relationship between AChE and CO bands. Fully formed AChE bands were observed in two fetuses (E140 and E142) and four postnatal infant monkeys (Table 1). They showed a mean width of 2.4 mm at birth and a mean center-to-center spacing of 5.5 mm (Fig. 2A). During pre- and postnatal development, the band periodicity remained constant through the 4-month-old infant ($P > 0.05$) and was accompanied by a progressive narrowing of the AChE bands (Barone et al., 1994). In the postnatal animals, center-to-center spacing of the CO bands was approximately 1.9 mm, which was approximately half that of the AChE spacing.

The periodicity of AChE and CO bands suggests that each AChE band might correspond to one of the two sets of CO bands, thick bands or thin bands (corresponding to the thick stripes and thin stripes of other authors; Livingstone and Hubel, 1988). To determine whether this is the case, it is necessary to visualize both the CO and the AChE bands in the same brain. However, AChE bands are present early in development and become progressively weaker around term, finally to disappear several months after birth (Barone et al., 1994). On the other hand, CO bands emerge late in gestation, are weak at birth (Horton and Hockin, 1986), and become much stronger after 1 postnatal month (Dehay et al., 1986). In this way, the spatial relationship of AChE and CO bands can be examined only around birth, when neither is at its maximum strength.

To determine the spatial relationship of CO and AChE bands, we have collected all the relevant sections through the full thickness of the cortex of two neonates. Adjacent sections were processed for CO and AChE histochemistry. This enabled us to make reconstructions of each set of bands by using serial sections and then to superimpose the two reconstructions. An example of the reconstruction process is illustrated in Figure 2B-H. In Figure 2, the two successive AChE sections are shown at left (Fig. 2B,D) and the intervening CO sections at right (Fig. 2C,E). The reconstructions of each set of bands are shown at the bottom (Fig. 2F,G). Blood vessels oriented perpendicular to the cortex match across all four sections and are crucially important for the reconstruction procedure.

The superimposition of the two reconstructions (Fig. 2H) shows that one of two CO bands is located in the AChE interband and that the intermediate CO band is centered on the AChE bands. These reconstructions show that the AChE and CO bands maintain a fixed spatial relationship. Distinguishing thick and thin CO bands on visual criteria is not only possible in the macaque (Livingstone and Hubel, 1987; DeYoe et al., 1990). The CO band located in the AChE interbands in Figure 2 appears to be marginally thicker than the CO band centered on the AChE bands. This suggests that the CO bands located in the AChE interbands correspond to the thick stripes, which are known to project to area MT and the parietal cortex, whereas the CO bands centered on the AChE bands correspond to the so-called thin stripes, which along with the interstripes are known to project to area V4 and temporal cortex (Van Essen and Gallant, 1994). The physical appearance of the CO bands therefore provides indirect evidence that AChE labels the V4-projecting compartments. Direct confirmation of this was provided by axonal tracing experiments (see below).

### Description of injection sites

Injections were made in a stereotyped fashion in presumptive area V4 (see Materials and Methods). Histological examination after cresyl violet staining enabled us to determine those injections in which the zones of uptake were restricted to the cortical gray matter along their entire extent (Fig. 3, Table 2). A number of injections did involve the white matter. The E112 injection at its deepest went 800 $\mu$m into the white matter. The white matter injection at E123 involved massively the underlying white matter, the center of the zone of uptake reaching 1.0 mm into the white matter over much of the length of the injection site. This injection site could involve fibers going from area V2 to area V5. The white matter injections at E129, E140, and E142 encroached by several hundred micrometers into the white matter.

A postinjection survival time of 10–12 days is required to obtain optimal retrograde labeling (Meissirel et al., 1990, 1991; Barone et al., 1995). The age at injection and not at perfusion is taken as determining the projection pattern (see Discussion).

### Relationship of AChE bands and V4 projection neurons

The reconstruction of CO and AChE bands and their respective periodicities show that the AChE bands are in correspondence with a subset of the CO bands. The size of the CO bands centered on AChE bands suggests that they corresponded to the so-called thin CO stripes of other authors. However, we and others find that CO band width differences alone are not sufficiently clear to permit unambiguous classification (Livingstone and Hubel, 1987; DeYoe et al., 1990). The problem of classification of CO bands on width criteria alone is accentuated in the immature brain, in which enzyme activity levels are low. To determine which set of CO bands superimpose with the AChE bands, we examined the fetal distribution of retrogradely labeled neurons following fluorescent dye injections in presumptive area V4 on the prelunate gyrus.

Injections led to dense labeling of area V2 neurons, which at all ages were principally located in the supragranular layers (Kennedy et al., 1996). Figure 4 shows the tangential distribution of retrogradely labeled cells in V2 following injections at E129 and E140. In the E140 fetus (Fig. 4A), most retrogradely labeled neurons are organized in clusters of high density. Comparison with the adjacent section reacted for AChE histochemistry shows that the clusters of labeled neurons are largely restricted to the regions of the AChE bands. This result and similar findings obtained from the E129 injection (Fig. 4B) demonstrate that AChE-rich bands delineate the cortical territories that project to area V4 and confirm that they correspond to the future thin and pale CO stripes.

### Developmental changes in labeling patterns of V4-projecting neurons

The strong clustering of projections from area V2 to area V4 as early as E140 was unexpected in view of current concepts of exuberance of early-formed connections. However, comparing the degree of clustering in area V2 at E140 with that at E129 (Fig. 4) suggests that labeled neurons in AChE interband regions could be more numerous in the younger fetus. This raises the possibility that adult-like clustering emerges from an earlier stage where there is a less pronounced organization in clusters. In this section, we
Fig. 1. Acetylcholinesterase (AChE) expression in presumptive area V2 in the posterior bank of the lunate sulcus of the E115, E123, and E142 fetus and newborn monkey. The cortex was physically flattened, and serial sections (section 1 superficial and 5 deep) were cut parallel to the cortical surface (anterior is at the top). In the E115 fetus, AChE in superficial layers is strongly expressed, with islands of weaker activity. By E123, the AChE activity forms a honeycomb pattern; arrowheads indicate coalescing AChE-poor bands. Here, tangential sections are necessary to reveal the developmental changes in the AChE pattern, because, in more perpendicular sections of the E123 fetus (the upper part of sections 4 and 5), the bands, but not the honeycomb pattern, are seen. At E142, AChE bands are fully formed. PND, postnatal day. Scale bar = 2 mm.
Figure 1 (Continued.)
shall examine the development of area V4 projections by examining retrograde labeling patterns in AChE bands and interbands at different developmental stages (Table 2). In the youngest fetus (injection E106, perfusion E116) resulted in few labeled neurons in area V2, indicating that this age is near the onset of pathway formation (Fig.
5A). At the time of perfusion (E116), no obvious clustering of labeled neurons was observed, and there were no discontinuities in the AChE activity.

During the following 6 days, there was an increase in numbers of connections, such that injection at E112 (Fig. 5B) led to a larger number of labeled neurons than at E106. At perfusion (E123), AChE activity was discontinuous, and visual inspection of the plots of labeled cells suggests that V4 projection neurons display a clustered distribution matching with regions of high AChE activity. At E129 (Fig. 5C), there is a further increase in numbers of labeled neurons that appear to be selectively located in the AChE band regions. Meanwhile, the numbers of labeled neurons in the interbands appear stationary, so overall clustering is more accentuated. By birth (Fig. 5D), labeled neurons between clusters are rare and the degree of clustering appears adult-like.

The patterns of labeled projection neurons suggest that pathways might exhibit some degree of clustering from very early stages of their formation. The degree of clustering can be examined in histograms of numbers of neurons in bands and interbands (Fig. 6). In the fetus injected at E112 and perfused at E123, labeled neurons appear to be clustered in the regions of the AChE bands. By E129, there is a large increase in the numbers of labeled neurons in the AChE bands, whereas, in the interbands, density appears rather similar to that in the E112 fetus. At E140, numbers of neurons between bands have dropped, which accentuates the clustering.

Quantitative analysis of cluster formation

An important aspect of our results is that clustering in area V2 differs according to its location within the labeled region. Elsewhere we have defined the region of a cortical area that contains retrogradely labeled neurons following an injection in a target structure as the projection zone (Kennedy et al., 1989, 1994; Salin et al., 1992; Barone et al., 1995). Labeled neurons show peak densities at the centers of projection zones, and there is a smooth decrease in density towards the periphery of the projection zone. In the present study, the periphery of the projection zone was defined as those regions corresponding to less than 40% of the peak density and the center of the projection zone as those regions containing more than 70% peak density values.

For each experimental case, we compared the density of labeled neurons within regions of high and low AChE activity (which we refer to as bands and interbands). Labeled neurons were counted in sections made perpendicu-
Figure 4 (Continued.)
lar to the cortical surface. This involved calculating the linear density (i.e., the number of labeled neurons per millimeter of cortical surface) in bands and interbands throughout the projection zone.

Injections in presumptive area V4 were elongated (see Materials and Methods), giving rise to extensive projection zones in area V2. Computing and pooling neurons in all labeled bands and interbands throughout the projection zone generated a global estimation of the degree of clustering for the projection zone of each injection. Quantitative comparisons of labeling in bands and interbands are provided for all animals injected, including injections involving the white matter.

Because the linear density measure pools values from the entire projection zone and because this includes labeling from a large number of bands and interbands, it is a powerful method for detecting small differences in densities of labeled neurons within bands and interbands (Table 2). Several tests were performed to ensure that differences, particularly in the youngest animal, were statistically significant. A nonparametric Mann-Whitney U test first was used to compare the density values of bands and interbands.
Fig. 5. Fetal and neonatal distribution of retrogradely labeled cells in successive sections of area V2. Dots represent labeled neurons, and dark bars under the labeled cells indicate the tangential extent of the AChE bands obtained from the adjacent section processed for AChE histochemistry. In the youngest fetus (E106), at the age of perfusion (E116), AChE activity shows a continuous pattern of expression. Scale bar = 1 mm.
Fig. 6. Developmental changes in the linear density of area V4 projection neurons in AChE bands and interbands. AChE bands are shown by dark bars. Histograms were prepared from two adjacent sections, and neurons were counted in 200 μm bins. Fetuses: injected E112, perfused E123; injected E129, perfused E142; injected E140, perfused E151. Ordinate: numbers of neurons.

This gave significance levels of $P < 0.05$ for E112 and E123 and $P < 0.01$ for all other ages, except for the white matter injection at E123 (Table 2). However, the significance levels in this test were reduced by global density gradients across the projection zones going from peak values at its center to minimal values at the periphery. Given the theoretical importance attached to density difference between bands and interbands in the E112 fetus, we carried out further tests. An analysis was performed on pairs of adjacent AChE bands and interbands throughout the projection zones. A nonparametric paired comparison (Wilcoxon test) of linear density returned significance levels of $P < 0.01$ for E112 (all other ages, except the white matter E123 injection, $P < 0.001$).

So far, our results show that there is a clustering of V4 projection neurons in area V2 at the early stages of pathway formation. Furthermore, there is a steady increase in labeled neuron density during early pathway formation. Labeled cell changes in the density of labeled neurons in bands and interbands (Fig. 6) suggest that the developmental segregation process is the consequence of higher rates of increase in density in the bands compared to interbands. However, the comparison of densities at different ages is complicated by the fact that there is a large expansion of the cortex between E112 and E140, essentially resulting from increasing cell size. Hence, the high densities at early fetal ages could in fact correspond to low proportions of labeled neurons. To compare proportions of labeled neurons at different fetal ages, we have computed a labeling index (LI) for the band and interband compartments. By dividing the number of labeled neurons by the total number of neurons (labeled and unlabeled), we have computed the percentage of labeled cells (i.e., the LI) in bands and interbands (see Materials and Methods). Pooling values throughout the projection zone gives global LI values for bands and interbands (Fig. 7A).

The LI allows an absolute comparison of labeling across ages and provides an improved understanding of the developmental changes of connectivity. In Figure 7A,B, all white and gray matter injection values are included. At E106, the global LI is less than 0.2%, indicating that very few neurons in area V2 have contacted their target in area V4 and confirming that this age is very near the onset of pathway formation (Table 2). Global LI values in the bands double over the following 6 days, to reach 0.4% by E112. Global LI values in bands increase at E123, and peak values of 3.6% are obtained at E129. Global LI values in the interbands also increase between E112 and E129, but the rate of increase is far more modest than in the bands, and peak values are 1.6% at E129. The global LI values drop at E140 to near-adult levels both in bands and in interbands.

By comparing global LI values in bands and interbands, it is possible to calculate a global clustering index for each injection (see Materials and Methods). The clustering index computed throughout the projection zone returns a value of 18% at E112 (Fig. 7B). Clustering increases in a smooth fashion to reach maximal, adult-like values of 80% by E140.

Cluster formation in the central regions of the projection zones was more pronounced than in the periphery at all
Fig. 7. Developmental changes of labeling index (left column) and clustering index (right column) throughout the global (A,B), central (C,D), and peripheral (E,F) regions of the projection zone. For the global projection zone, labeling index (LI) curves for bands (solid line) and interbands (dashed line) are drawn through the interanimal means. Cross symbol at E106 designates LI values obtained in the youngest fetus, when AChE bands and interbands could not be distinguished. White bars, white matter injections; gray bars, gray matter injections. WM, white matter; GM, gray matter; PND, postnatal day. Arrow indicates birth.
ages, as shown by comparing central and peripheral LI and clustering indices (Fig. 7C–F). Note that, when there is both a gray and a white matter injection for a given age, only the gray matter injection is included in Figure 7C–F. In the E106 fetus, the LI in the center of the projection zone was 0.5%. At E112, the LI of the bands and interbands in the center of the projection zone were 0.9% and 0.6%, respectively, which gave a clustering index of 37.6%, nearly twice that of the cluster index computed from the global LI. Hence, although the overall shape of the curves for the central LI did not differ from that of the global LI, the clustering indices were significantly higher in the central regions, particularly at younger ages. For the two oldest animals (PND 11 and PNM 2), we obtained measurements only in the central projection zone. Here clustering was found to be marginally improved (compared to late fetal stages and the second neonate) and reached values of 96%.

Cluster formation in the peripheral regions of the projection zones was much poorer than in central regions (cf. Fig. 7D and F). In the two youngest fetuses (E112 and E123), there was no cluster formation in the periphery of the projection zone, in contrast to the approximately 40% clustering in the central regions. From E129 onwards, the periphery of the projection zones returned clustering indices that were on average about 20% lower than in the center of the projection zones.

**Influence of depth of injection**

During development, inappropriate axons can grow towards a cortical target without actually invading the gray matter (Ivy et al., 1979). This leads to different patterns of retrogradely labeled neurons according to whether the injection site is restricted to the cortical gray matter (Innocenti and Frost, 1979; Innocenti et al., 1988). This has important implications for the mechanisms underlying the specification of connectivity, because the failure of inappropriate connections to penetrate the cortical gray matter implies late target selection.

To investigate this issue, we compared labeling in bands and interbands following gray and white matter injections. Paired injections were successfully carried out at three critical stages: first, during the main phase of increasing connectivity (E123); second, nearing the completion of segregation (E140); and, third, in the postnatal animal when the adult pattern of connectivity has been established.

The total number of labeled neurons in the two fetuses was higher following the white matter injection compared to the gray matter injection ($P < 0.05$; Table 2). This was not the case in the neonate, in which the white matter and the gray matter injections returned similar values ($P > 0.05$). The increased numbers of labeled neurons following the white matter injections in the fetus were allocated to the interbands (Fig. 8A,C) and so led to a decrease in the clustering index (Fig. 8B,D). This indicates that inappropriate axons from interbands accumulate in the white matter under area V4. The reduction in clustering following the white matter injection was more pronounced in the periphery of the projection zone than in the center, both in the E140 fetus and in the neonate (Fig. 8B,D).

The increase in numbers of labeled neurons following white matter injections in the fetus reflects the immature status of the fetal connectivity and suggests that large numbers of fibers have yet to grow into the cortical gray matter. The reduced clustering in the periphery of the projection zone of the neonate following the white matter injection indicates a degree of immaturity that is not apparent in the center values. Altogether, these results show that the accumulation of fibers in the white matter of V4 originates principally from the interband neurons.

**DISCUSSION**

The present study shows that the area V2 territories that project to area V4 are determined early in development. Near the onset of pathway formation, V4 projecting neurons are clustered in the appropriate compartments. Cluster formation increases during the main phase of axon growth, when the bulk of connections are being laid down. Before considering the theoretical implications of these results, we first must make a critically appraisal for possible artefacts.

**Technical considerations**

**Defining projection pattern by the date of injection.** Several difficulties stem from the fact that we are using retrograde tracers in a dynamic system in which numbers of connections are changing. Several factors suggest that the pattern of connectivity corresponds more closely to the day of injection than to the day when the fetus is sacrificed. Uptake of the dye occurs only when fibers are disrupted. Hence, growing axons that arrive at the bolus of dye after injection do not pick up the dye in quantities sufficient to lead to retrograde labeling (Kennedy and Dehay, unpublished observations).

Another concern is that connections could be eliminated between the day of injection and perfusion. It has been well documented that axonal elimination without death of the soma is the major mechanism responsible for the elimination of cortical connections during development (Cowan et al., 1984; O'Leary, 1992). Different authors have shown that the fluorescent dyes can be stored in the soma for periods considerably longer than the survival times employed here (Innocenti, 1981; Price and Blakemore, 1985a; Dehay et al., 1988a; Chalupa and Kallayek, 1989). Therefore, we can expect to visualize the majority of connections present at the moment of injection. However, some axons of the area V2 neurons that project to area V4 and that are present at the moment of injection may be eliminated before the end of the survival period. If these axons were present for less than 5–6 days at the site of injection, then the parent neurons would be expected to be very weakly labeled or not labeled at all. This will slightly limit the temporal resolution of our technique when it comes to detecting decreasing numbers of connections.

**Possible variability of capture of injected dyes.** The rarity of fetal material prohibits us from using several fetal brains at each age; therefore, we must address the question of variability. The LI method enables us to compare patterns of labeling across different developmental ages. Such comparison will be valid only if changes in numbers of neurons reflects changes in numbers of connections and not a variability in the efficiency of dye capture. The dyes used in the present study have a number of distinctive advantages: They are extremely sensitive and after injection remain highly localized, with well-defined zones of uptake (Kyupers et al., 1980; Keizer et al., 1983; Bullier et al., 1984b). The injections were performed according to a stereotyped procedure: A bolus of dye was deposited along the length of the injection sites, which were made tangentially to the surface. These 3-mm-long injection sites are comparatively large in the fetal brain, and the sheer size of
Fig. 8. Influence of depth of injection on labeling in bands and interbands. Values presented for fetuses injected on E123 and E140 and a neonate. 

A: Labeling indices from the center of the projection zone. 

B: Clustering indices from the center of the projection zone. 

C: Labeling indices from the periphery of the projection zone. 

D: Clustering indices from the periphery of the projection zone. WM, white matter; GM, gray matter.

the injection optimizes uptake and therefore reduces variability resulting from local factors. Although we cannot exclude some variation of uptake, the major finding of the study is the early segregation at E112 and the progressive increase of this segregation with age (Fig. 7B). This result rests on the observed differences in labeling in AChE bands and interbands within individual injections and therefore is not expected to be influenced by uptake variability.

Directed growth is largely responsible for early segregation

Directed growth refers to instances where growing axons follow stereotyped trajectories, presumably in response to molecular cues (Goodman and Shatz, 1993; Goodman, 1994). Two aspects of the results argue in favor of directed growth contributing to cluster formation during the early development of the V2-V4 pathway. First, following injection in the white matter underlying area V4 near the onset of pathway formation at E112, labeled neurons in area V2 show a clustering index of 36.7% at the center of the projection zone. Second, at a later stage, the E129 white matter injection reveals peak connectivity and shows clustering of 65%, indicating a major increase in the proportion of appropriate V2 fibers in the white matter underlying area V4. The fact that following white matter injections the clustering is more reduced in the center of the projection zone than in the periphery suggests that directed growth exerts a stricter control on axon trajectory of projections showing small divergence. This suggests that formation of clusters and topography might share common mechanisms (Kennedy et al., 1994).
At E112, clustering is 36.7%. The time period prior to E112 when connectivity could be uniform is actually very short. This can be calculated by considering birth dates and migration times of cortical neurons. Birth-dating experiments using S-phase markers show that the onset of generation of the supragranular parent neurons of the V4 projection is around E50 (Rakic, 1981; Dehay and Kennedy, unpublished observations) and that migration time of these neurons to the cortical plate is on the order of 15–20 days (Rakic, 1981). In this way, axon emission of projections to V4 could occur, at the earliest, at E90 (i.e., 5 days before termination of migration; Shoukimas and Hinds, 1978; Schwartz et al., 1991). Given the rate of axonal growth (Stirling and Dunlop, 1995), the minimal time required for axons to cover the distance from V2 to V4 would be on the order of 10 days, so one can predict that the very earliest time at which growth cones of the V2 projection neurons could arrive in the vicinity of area V4 is at E100. Hence, there is a 12 day period during which there could be an even distribution of labeled neurons across the cortex. The very low number of connections returned by the E106 injection (LI is 6% of its peak value) confirms that this age corresponds to the initial stage of pathway formation. Because of the low number of labeled neurons, it is difficult to say whether they are distributed in clustered fashion. More importantly, the absence of ACHE bands at this age makes it impossible to undertake a quantitative investigation of this issue.

**Target selection also contributes to early segregation**

During the final stages of growth, axons that are in the vicinity of their appropriate target grow into the cortex, presumably in response to a molecular recognition mechanism. The evidence in support of the involvement of target selection comes from the dependence of clustering index on the depth of the injection site. In the fetus, white matter injections labeled more neurons in the interbands, leading to weaker cluster formation than did gray matter injections. No such effect is seen in the neonate. This result suggests that only fibers from appropriate compartments penetrate into the target and fibers from inappropriate compartments, which reside in the white matter, are eventually eliminated. It could be that V2 fibers, including those from the interbands, form transient synaptic connections in the subplate underlying area V4 (Marin-Padilla, 1978; Kostovic and Rakic, 1980; Ghosh et al., 1990; Shatz et al., 1990; De Carlos and O'Leary, 1992; Allendoerfer and Shatz, 1994; McConnell et al., 1994).

The fact that the inappropriate fibers (from the interbands) have a reduced ability to invade the cortical gray matter suggests that membrane-bound molecules might distinguish interband and band axons and that these molecules facilitate ingrowth of axons derived from the bands into the target (Götz et al., 1992; Henke-Fahle et al., 1996).

**Role of late selective elimination**

Selective elimination is a late phenomenon resulting in the pruning of early-formed connections after target innervation (Cowan et al., 1984; Easter et al., 1985; O'Leary, 1992). Could it be that there is a selective elimination of connections in the later stages of pathway formation that could serve to sharpen the earlier formed clusters? In support of this is the 30% increase in cluster formation after E129, when numbers of connections are actually decreasing. Although it is very likely that selective elimination does sharpen the early-formed pattern, variability in clustering in the adult (Zeki and Shipp, 1989) makes it difficult to quantify this regressive phenomenon.

**Interspecies comparison**

It is now firmly established that, with the exception of monkey striate cortex, which is devoid of callosal connections throughout its development (Dehay et al., 1988b; Chalupa et al., 1989), the restricted adult pattern of callosal connections emerges from an initially widespread connectivity (Innocenti et al., 1977; Innocenti, 1980; Chow et al., 1981; Ivy and Killackey, 1981; Cusick and Lund, 1982; Rhoades and Fish, 1983; Miller and Vogt, 1984; Chalupa and Killackey, 1989). Clearly the early segregation of the V4 projection suggests a developmental mechanism different from that of callosal pathways, both in primates and in nonprimates. Could it be that directed growth constitutes the hallmark of the development of association pathways linking cortical areas within the same hemisphere? Prior to the present study, development of an association pathway has been examined only in the visual cortex of the cat, where projections of area 17 to areas 18 and 19 in the adult emerge from a continuous distribution in the kitten (Bulliet et al., 1984a; Price and Blakemore, 1985b; Price et al., 1994a,b). However, there is no extrinsic marker in cat area 17 of the territories projecting to areas 18 and 19 such as the ACHE bands in area V2 of the fetal monkey. This prevents the summing of numbers of neurons in patches and interpatches over several sections as in the present study and thereby excludes quantitative verification of whether the projections are truly continuous in the kitten.

**Comparison of the development of feed-forward and feedback pathways in monkey**

The ontogeny of the primate V2-V4 pathway is characterized by an early specification and sets it aside from the development of callosal connections in general as well as the projection of area 17 to area 18 in the kitten. A clue to the significance of these findings is provided by considering the functional categories of corticocortical connections.

Feed-forward and feedback connections can be distinguished anatomically (Rockland and Pandya, 1979; Van Essen and Maunsell, 1983; Casagrande and Kaas, 1994; Rockland, 1994; Singer, 1995). Feed-forward pathways ascend hierarchical levels, are topographically organized, and are involved in the generation of feature-selective receptive fields. Feedback connections are more divergent, associate large ensembles of neurons, and have been implicated in the modulation of receptive field response (Zeki and Shipp, 1988; Desimone and Ungerleider, 1989; Mignard and Malpeli, 1991; Knierim and Van Essen, 1992; Casagrande and Kaas, 1994; Salin and Bullier, 1995).

Feedback projections from extrastriate to striate cortex show protracted development and are not adult-like before 1 month after birth (Kennedy et al., 1989; Barone et al., 1996; Batardiere et al., 1995). The late development of the primate feedback pathways, relying at least in part on selective elimination, contrasts with the present results demonstrating maturation of a feed-forward pathway that is dominated by directed growth of axons and is largely complete by birth.
The greater importance of selective elimination in the development of the projection of area 17 to area 18 in cat compared to the present results in monkey could reflect differences in the organization of the visual pathways in these two species. The distributed organization of the system of the carnivore (Bullier et al. 1994) means that areas 17 and 18 occupy equivalent hierarchical levels, so the projection from 17 to 18 could include both feed-forward and feedback pathways. Consequently, the selective elimination of the early widespread feedback connections in this pathway might obscure early segregated patterns of the feed-forward connections.

CONCLUSIONS

The segregation of the V2-V4 pathway in the primate develops largely by means of directed growth and target selection and depends possibly to a more limited extent on selective elimination. In the primate at least, feed-forward connections, which have to achieve the high levels of precision required for feature extraction, are more concerned with mechanisms of directed growth and target selection than are feedback projections. Development of the ascending visual pathways in lower vertebrates and mammals depends to a large extent on early pathway and target recognition mechanisms (Goodman and Shatz, 1993). This strongly supports the notion that axonal guidance mechanisms are highly conserved throughout evolution (Goodman, 1994). The present results extend the scope of this hypothesis, because the isocortex constitutes a recent major phylogenetic addition to the vertebrate nervous system.

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EARLY SPECIFICATION OF MONKEY CORTICAL PATHWAYS


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