Development of ocular dominance columns in the absence of retinal input

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The initial establishment of ocular dominance columns in visual cortex is believed to involve the segregation of overlapping geniculocortical axons into eye-specific patches based on patterns of correlated activity. However, we found that total removal of retinal influence early in visual development did not prevent segregation of geniculocortical axons into alternating stripes with periodicity normal for ocular dominance columns. Because the patterning of geniculocortical afferents resists this dramatic change in the level, source and pattern of spontaneous activity, we propose that formation of ocular dominance columns relies on molecular cues present on thalamic axons, cortical cells or both.

The property of ocular dominance reflects the relative strength of eye-specific physiological responses in visual cortex. This results from the segregation of left and right eye-specific lateral geniculate nucleus (LGN) axons into alternating stripes in cortical layer 4. Current hypotheses suggest that activity patterns during development drive the maturation of this organizational feature of visual cortex from an initial state in which axons representing the two eyes overlap considerably8,9. Activity derived from visual experience can reorganize ocular dominance columns during the critical period10,11; however, fully formed ocular dominance columns in primates at birth8,9 suggest that patterned spontaneous activity, and not visual experience, drives the initial establishment of ocular dominance columns. A candidate source for this activity is retinal waves10,11, which are much better correlated within an eye than between the eyes. These retinal waves send periodic bursts of action potentials to the developing LGN13,14 and cortex.

To determine whether the retinal or retinal activity are required for the establishment of ocular dominance columns, we developed methods to visualize the patterns of LGN axons in mature ferrets that had been enucleated very early in life. In the absence of eyes, conventional transneuronal transport techniques for visualizing the pattern of ocular dominance columns are impossible. Thus, these experiments used two independent anatomical methods—anterograde LGN injections and retrograde cortical injections—to determine whether ocular dominance segregation occurred. Surprisingly, geniculocortical axons organized into discrete ocular dominance stripes, despite the absence of retinally derived information throughout the course of ocular dominance column formation. We therefore propose that, unlike ocular dominance plasticity during the critical period, the initial establishment of ocular dominance columns relies primarily on activity-independent cues, rather than on specific patterns of correlated activity.

Results
In the ferret, events possibly relevant to development of ocular dominance columns, including segregation of retinal axons into eye-specific layers in the LGN and the initial ingrowth of geniculocortical axons into cortical layer 4, occur between birth (postnatal day 0; P0) and P15 (refs. 15, 16), ages roughly equivalent to embryonic days 42 and 57 (E42 and E57) in cat17. Waves of spontaneous retinal activity, implicated in the segregation of retinal axons by eye in the LGN, are present throughout this period18,19. To evaluate the contribution of retinal input to the formation of ocular dominance columns, experimental animals were binocularly enucleated between P0 and P18 and allowed to mature until at least P70, after the close of the ferret critical period12.

At this time, the dorsal surface of the LGN on one side of the brain was surgically exposed, and a focal injection of an anterograde tracer, biotinylated dextran amine (BDA), was targeted to one of the A layers of the LGN. These LGN injections allowed direct labeling of geniculocortical afferents in adult animals that had been enucleated early in development.

In control adult animals (> P70), BDA injections were largely confined to single LGN layers (Fig. 1a), and the LGN afferents projecting to large regions of primary visual cortex were anterogradely labeled, revealing ocular dominance columns over several cycles of alternating eye-specific domains (Fig. 1b). Their size, periodicity and appearance, both in coronal sections (Fig. 1b) and in surface-view reconstructions of adjacent sections (see below), were similar to ocular dominance columns visualized with transneuronal transport of tritiated amino acids or wheat germ agglutinin-horseradish peroxidase20-22 in normal animals.

Remarkably, injections of single LGN layers in adult animals binocularly enucleated between P15 and P18 (just after layer 4 receives LGN afferents3, roughly equivalent to an E57–E60 cat) consistently revealed alternating patches of label within cortical layer 4 (Fig. 1c). Although labeling in layer 4 seemed thinner and the peak labeling density was lower than in normal animals (probably a general effect of early enucleation on the geniculocortical projection23-25), the size and spacing of patches were virtually identical to those for control animals. The average center-to-center patch spacing in these animals was 741 ± 47 μm (n = 4 animals), which was statistically indistinguishable from controls (612 ± 42 μm, n = 5, Fig. 2). Both measures were simi-
Fig. 1. Focal biotinylated dextran amine (BDA) injections in LGN reveal segregated geniculocortical afferents in striate cortex of normal and enucleated animals. (a) Horizontal section of the right LGN of a normal adult following a focal BDA injection. Tissue subdivisions are outlined based on the adjacent Nissl-stained section (right). The injection site is almost entirely confined to layer A1. P. perigeniculate; A, LGN A layer (contralateral eye input); A1, LGN A1 layer (ipsilateral eye input); C, LGN C layers. Scale bar, 500 μm. (b) Coronal section of visual cortex in a normal animal showing segregated patches of anterograde BDA label in the territorial region of layer 4. Coronal sections include both dorsal and territorial striate cortex. (c–e) Binocular enucleations were performed on young animals (P0–P15), and all BDA injections were made after P70. (c) In a ferret enucleated at P18, clear BDA-labeled patches are evident in dorsal layer 4. White matter is visible near bottom. (d) Ferret enucleated at P0. BDA-labeled patches separated by unlabeled regions are present in both dorsal and territorial layer 4. (e) Ferret enucleated at P0. BDA label patches are seen in both dorsal and territorial layer 4. Labeled bands in center of section are geniculocortical axons in white matter. Scale bar (b–e), 1 mm.

Fig. 2. Anterogradely labeled patches in enucleates have the same spacing as normal ocular dominance columns. Mean center-to-center spacing (± s.e.; all patches sampled every 300 μm though the coronal series) is shown for patch of cortical label following BDA injections into LGN. Animals were grouped into four categories: animals enucleated at P0 (n = 5), P5 or P6 (n = 2) or P15 to P18 (n = 4) and normal controls (n = 5). No significant differences were detected between the groups by ANOVA.

ocular-dominance patches, whereas area 17, the posteriormost portion of the dorsal and territorial surfaces, contains ocular dominance columns similar to those described in cats. In enucleates, both regions were labeled, and the distinction between large and fine columns evident.

Although all generally accepted criteria (laminar location, spacing and periodicity) these segregated patterns could only reflect ocular dominance columns, these patterns in enucleated animals could conceivably reflect segregation across some other functional domain, or a large-scale disruption of the normal cortical topography. For example, afferents from the ON and OFF layers in the normal ferret LGN form patches in cortex. To verify that the segregated patterns of geniculocortical axons reflected topographically appropriate segregation by eye-specific layer, retrograde tracers (fluorescent latex microspheres) were micro-injected into striate cortex. Individual injections were ~100 μm in diameter and, therefore, were probably confined to single columns. If axons originated from a different source or failed to segregate, or if there were some change in geniculocortical topography or other aberrant patterns of projection, these should be immediately apparent in the distribution and laminar pattern of retrograde label.

As expected, in normal animals with normally segregated geniculocortical afferents, most cortical microinjections retro-
Fig. 3. Segregated geniculocortical axons in enucleated form ocular dominance-like stripe patterns. BDA label in cortex following LGN injections was marked across coronal series, and the members of the coronal series were aligned and rotated to provide a surface view of the layer 4 label pattern. Reconstructed views of dorsal (upper) and ventral (lower) layer 4 label are shown at left, with interruptions at the caudal poles. Representative coronal sections are at right. (Arrows indicate the lines in the surface view generated by these sections.) The patterns in control (a) and early-enucleated animals (b; enucleated at P0) show contiguous strips of labeled and unlabeled regions, indicating normal, eye-specific geniculocortical axon segregation. Scale bars, 1 mm.

gradually labeled a small cluster of neurons in either layer A or A1 (spanning both ON and OFF sublaminas), depending on whether the injection was in a contralateral or ipsilateral eye column in cortex (Fig. 4a and b). Occasionally an injection resulted in roughly equal labeling in both LGN layers; presumably, this reflected an injection site spanning the border between two columns (Fig. 4a and d).

As in normal animals, cortical injections in ferrets enucleated between P8 and P21 strongly labeled discrete clusters of cells in zones identified as either layer A or A1 (see below and Methods), indicating both normal topography and a normal pattern of segregated afferents (Fig. 4c). In animals enucleated before P13, the cell-sparse zones between LGN layers do not develop normally25,26, making cytoarchitectonic discrimination of the A layers difficult. For this reason, we used an unbiased technique to identify peaks in the number of labeled neurons and their distance from the ventricular surface to assign the laminar location of labeled cells and to thereby calculate the degree of laminar bias, the segregation index (see Methods). In normal animals, five of six injections yielded a greater than threefold bias in the number of retrogradely labeled cells in A or A1 (Fig. 4d). In enucleated animals, four of seven injections yielded a similar bias (Fig. 4d).

There was no statistical difference between the two groups in the degree of bias (Wilcoxon signed-rank test). Thus, two anatomical techniques, anterograde labeling from LGN injections and retrograde labeling from cortical injections, independently yielded the same conclusion: early binocular enucleation does not disrupt segregation of geniculocortical terminals arising from layers A and A1 in layer 4 of striate cortex.

Discussion

How might segregated patterns of geniculocortical terminals emerge following early removal of retinally generated activity patterns? In the absence of retinal inputs, patterned spontaneous activity could be present in the LGN or cortex alone, or in the geniculocortical loops connecting them. In brain slice preparations, the LGN generates low levels of patterned spontaneous activity beginning at approximately P21 (ref. 31); however, this activity is largely synchronized between LGN layers6. In recordings from the LGN of awake, behaving ferrets at slightly older ages (P24-P27), within-eye correlations are much stronger than between-eye correlations, as might be expected if the observed bursting patterns were driven by retinal waves14. However, removal of both eyes leads to the rapid emergence of highly synchronized bursts of spontaneous activity in which correlations between eye-specific layers are dramatically enhanced. Removing retinally driven activity clearly degrades (although it may not completely eliminate) correlation-based cues for segregating geniculocortical afferents. Although we do not yet know the activity pattern resulting from enucleation at P0, we suspect that the differences in activity patterns between layers would be reduced, as LGN layers would not yet be distinct. Nevertheless, we cannot exclude the possibility that spatially patterned activity in the LGNs or cortices of developing animals may be a sufficient cue for the organization of ocular dominance columns.

Alternatively, the detailed correlational structure of spontaneous activity may not be critical for the initial establishment of segregated geniculocortical terminals. Although most current models invoke some form of correlation-based, activity-dependent remodelling to explain formation of ocular dominance columns3,36, the strong contralateral biases observed in the correlational structure of pre-visual spontaneous activity in ferret LGN14 and in the emergence of orientation columns in striate cortex36 are inconsistent with straightforward Hebbian models based on competition between functionally equivalent ipsilateral and contralateral inputs. Moreover, studies in the visual cortices of very young, visually inexperienced kittens indicate a high degree of organization34. The development of another feature of striate cortex, cytochrome oxidase-rich blobs in layer 2/3 (representing the termination pattern of a specific class of LGN inputs35,36), also seems unaffected by prenatal binocular eye removal37. As blobs lie at the centers of ocular dominance columns in normal macaques38, thalamocortical axons may segregate normally in these animals as well. We propose that segregation of geniculocortical axons by eye-specific layer despite removal of retinal inputs before they segregate in the LGN is most consistent with the presence of intrinsic molecular cues that distinguish ipsilateral from contralateral inputs in either the LGN, striate cortex or both.
Presumably for technical reasons, there have been virtually no tests of the effects of early manipulations of spontaneous activity on the initial events in formation of ocular dominance columns. Binocular injections in P14 cats of tetrodotoxin, which blocks both spontaneous and evoked activity, do disrupt ocular dominance columns. However, as these experiments were initiated at a stage roughly equivalent to P35 in ferret, these experiments did not assess the effects of activity disruption on the initial establishment of ocular dominance columns. In related work on the development of the retinogeniculate synapse, brain-wide activity blockade disrupted eye-specific segregation, whereas activity blockade limited to the retina and begun later in development had more modest effects. A general problem is that blockade of action potential activity results in increased nonspecific process outgrowth that may not reflect normal developmental events. Thus, even if tetrodotoxin were applied earlier in development, nonselective growth associated with activity blockade may mask precise axonal targeting rather than disrupt it.

Although often referred to as 'eye-specific stripes', ocular dominance columns can also be thought of as the cortical representation of distinct LGN neuron populations that correspond to nasal and temporal retina. Without the benefit of retinal input after P0, cells of the LGN layers can assume distinct identities early in development. Our findings indicate that segregation of the axons arising from these layers in cortex can proceed without retinal influence. As an alternative to correlation-based models for ocular dominance development, we propose that axons arising from each LGN layer are molecularly distinct, and that interaction with corresponding cortical cues determines the termination pattern of each set of axons. Potential axon-guidance cues are present in the developing visual cortex and are implicated in thalamic axon guidance at the areal level. Other neocortical molecular markers persist even without thalamic input. In the olfactory bulb, axons can segregate into over 1000 distinct loci in the absence of correlated neuronal activity. These observations, taken together with the wealth of attractive and repulsive axon guidance molecules, suggest that LGN and cortex have a molecular repertoire capable of segregating axons into stripes without requiring specific spatial patterns of correlated activity.

Temporally patterned spontaneous activity may nevertheless have an important permissive role in the segregation process, allowing cortical or geniculate neurons to produce or respond to putative molecular cues. Rather than providing detailed pre- and postsynaptic correlations, the role of patterned activity may be to control patterns of gene expression through patterned calcium fluxes, as has been suggested in the developing spinal cord and in the immune system.

**METHODS**

Eunectes. Normally pigmented, sable ferrets, Mustela putorius furo (Marshall Farms, North Rose, New York), of both sexes were used for all experiments. All surgical procedures were performed under aseptic conditions in accordance with a Duke University Institutional Animal Care.

**Fig. 4.** Retrograde label of LGN by microinjection of fluorescent microspheres in visual cortex demonstrates normal topography and eye-specific termination patterns of geniculocortical axons. (a) Possible labeling patterns resulting from injections made into visual cortex with normally segregated or unsegregated geniculocortical axons. Injections in a contralateral ocular dominance domain (C) generate LGN label biased toward the A layer of the LGN, and injections in an ipsilateral ocular dominance domain (I) generate LGN label biased toward the A1 layer of the LGN. Injections made in the border between ocular dominance domains (B) or in cortex lacking eye-specific segregation of geniculocortical axons should result in no LGN label bias. (b) Sagittal section through LGN of a normal animal following cortical microinjection of red fluorescent latex microspheres. Retrograde label is confined to a small patch of layer A (white circle), indicating a contralateral dominance of the injection site. (c) Retrograde labeling following injection of fluorescent microspheres in an animal enucleated at P14. As in (b), the cortical injection labeled a small patch of cells confined to layer A (white circle), indicating segregation of geniculocortical axons at the cortical injection site. The tightly clustered label also suggests that enucleation did not degrade the topographic specificity of the projection. Abbreviations as in Fig. 1. Scale bar (b, c), 250 μm. (d) Segregation indices (see Methods) based on retrograde injection patterns indicate a high degree of eye-specific segregation for both normal and enucleated animals. A higher index value indicates a greater LGN laminar bias at the injection site; a segregation index of 0.5 indicates a 50:50 bias in the number of labeled cells between LGN layers. No significant segregation index difference was detected between early-enucleated (□, enucleated at P0 or P9; ○, enucleated at P14 or P15; △, enucleated at P21; n = 7) and control adult (□, n = 6) groups (Wilcoxon signed rank). Both enucleated and control groups contained animals with either very high or very low segregation indices. (Low indices probably resulted from injections near borders between ocular dominance domains.)
Tracer injections. In anesthetized animals (as above), small burr holes were drilled at the appropriate stereotaxic coordinates for a lateral geniculate nucleus and the contralateral visual cortex. Biotinylated dextran amine (BDA 10,000; Molecular Probes, Eugene, Oregon) injections were made by visualizing the LGN by aspiration of a small amount of the overlying cortex and hippocampus. BDA was then iontophoresed (+ 5 μA, pulsed for 5–20 min) into the LGN through a glass micropipet (10–50 μm diameter tip). For cortical injections, glass micropipets were inserted into the brain through a resected dura mater, and pressure injections of fluorescein microspheres (Lumharlo, Naples, Florida) were made.

Histology/immunohistochemistry. After 2–7 days of transport following tracer injections, ferrets were perfused transcardially with 0.9% saline, followed by 2% paraformaldehyde in 0.1 M phosphate buffer and finally, 2% paraformaldehyde and 30% sucrose in 0.1 M phosphate buffer. Injected cortices were unfolded and flattened between two appropriately spaced glass slides for analysis of microscope injection sites. After postfixation (4% paraformaldehyde/30% sucrose in 0.1 M phosphate buffer), the tissue was sectioned on a freezing microtome at 60–120 μm. BDA-labeled tissue was incubated in 10% methanol, 3.5% H2O2 in phosphate buffered saline (PBS) to remove endogenous peroxidase activity. After washing in PBS and treatment with 0.5% bovine serum albumin (Boehringer Mannheim, Indianapolis, Indiana) and 0.25% Triton X-100 (Sigma) in PBS, sections were reacted with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, California), using 3,3’-diaminobenzidine (Sigma) as a peroxidase substrate, with nickel ammonium sulfate and cobalt chloride intensification.

Data analysis/determination of segregation index. Images of histological specimens were acquired using a cooled CCD camera (Princeton Instruments, Trenton, New Jersey) and analyzed using Macintosh G3 and Power Computing computers using IPLab (Scionalytics, Fairfax, Virginia) and NIH Image (public domain, developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/).

Because of the absence of cell-sparse zones between the A layers of the LGN, layers could not be differentiated by cytoarchitectonic alone in early-enucleated animals. Thus measurements of cell locations were used to determine laminar locations of LGN cells retrogradely labeled by cortical injections. Retrogradely labeled LGN cells were sectioned parasagittally, and the distance from the caudal edge of the thalamus to each microscopic-labeled cell was measured. For this analysis, all labeled cells across all LGN sections were combined into the computation of the segregation index for a given cortical injection. The distribution of cell distances from the section edge was fit with an high-order polynomial, allowing discrimination of the LGN lamina from each other by the location of the peaks and troughs in the resulting curve. This technique was validated using cytoarchitectonic determination of laminae in both normal animals and the later-enucleated (P15–P21) animals in this study. Once the number of cells in the individual LGN layers was known, a segregation index (SI) was calculated using the equation,

\[ SI = \frac{A}{A + A1} \]

where A is the number of cells in the A layer of the LGN, and A1 is the number of cells in the A1 layer of the LGN.

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