Involvement of Subplate Neurons in the Formation of Ocular Dominance Columns

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During development of the mammalian visual system, axon terminals of lateral geniculate nucleus (LGN) neurons, initially intermixed within layer 4 of the visual cortex, gradually segregate according to eye preference to form ocular dominance columns. In addition to LGN axons and layer 4 neurons, subplate neurons may also participate in interactions leading to column formation. Deletion of subplate neurons before the formation of ocular dominance columns prevents the segregation of LGN axons within layer 4. Thus, interactions between LGN axons and layer 4 neurons are not sufficient; subplate neurons are also required for formation of ocular dominance columns in the visual cortex.

OCULAR DOMINANCE COLUMNS IN layer 4 of the cat's visual cortex begin to form ~3 weeks after birth and are complete by about 6 weeks (*1*). The anatomical basis for column formation is the eye-specific segregation of axons from the lateral geniculate nucleus (LGN) within layer 4 (*2, 3*). This segregation can be prevented by pharmacological blockade of action potentials within the visual pathways (*4*), suggesting that activity-dependent interactions between LGN axons and their targets, the neurons of cortical layer 4 (*5–8*), are essential for the formation of ocular dominance columns.

Subplate neurons might also influence developmental events within layer 4. These neurons are present only in the developing cerebral cortex, and their cell bodies are located immediately below cortical layer 6 in the white matter (*9*). These cells send axonal collaterals into the cortical plate, primarily into layers 4 and 1 (Fig. 1) (*10, 11*); physiological studies suggest that these collaterals may synaptically activate layer 4 neurons (*12*). At this time, LGN axons have branches within both the subplate and cortical layer 4 (*13*).

To study the dependence of ocular dominance columns on subplate neurons, we deleted subplate neurons during the first postnatal week by injecting the excitotoxin kainic acid (*14*). At this age, most LGN axons and their target neurons are in place, but LGN axons have not yet begun to segregate (*1, 13, 15*). The ablation of subplate neurons was confirmed by MAP2 immunostaining (*16*) (Fig. 2, A and B). It is likely that the injected kainic acid diffuses over several hundred micrometers within the white matter because subplate neurons underlying roughly half of primary visual cortex were typically deleted by this procedure. The histological organization of the cortical plate appeared almost entirely unaffected after injections of kainic acid into the white matter (Fig. 2, C and D). Cortical plate neurons themselves are evidently not susceptible to the toxic effects of kainic acid during the first postnatal week, because injection directly into layer 4 had no effect on MAP2 immunostaining (Fig. 3A) or on the histological appearance of the cortex (Fig. 3B). Similar injections into one adult animal caused massive cell death of cortical neurons (*17*), which is consistent with previous descriptions of kainate neuronal susceptibility acquired during development (*18*). These results suggest that kainic acid injected into the white matter selectively eliminates subplate neurons while the neurons of the cortical plate appear unaffected.

Subplate neurons were ablated during the first postnatal week, and the pattern of the geniculocortical projection was examined (*19*) at times after columns normally would have formed (7 weeks of age or later) (*1*). Normally, geniculocortical axons representing the two eyes have segregated from each other by postnatal day 44 (P44), so that periodic patches of autoradiographic label that represent the injected eye are evident in layer 4 (Fig. 4A). Ocular dominance patches in layer 4 are known to be more distinct in the cortex ipsilateral to the injected eye (*3, 20*). Therefore, the eye injections were routinely made ipsilateral to the hemisphere in which subplate neurons had been ablated in order to create the most stringent conditions for detecting alterations in ocular dominance column formation.

Deleting subplate neurons in posterior visual cortex at P2 affected the pattern of transneuronal labeling at P70 (Fig. 4B). Normal ocular dominance patches were visible in layer 4 in anterior regions. By contrast, in posterior regions overlying the kainic acid injection sites (verified by the presence of two deposits of fluorescent microspheres coinjected with the kainic acid), a continuous, labeled band was present in layer 4 (Fig. 4B). Transneuronal labeling within layer 4 overlying the injection sites was uniform in eight of the nine animals examined. In one animal there was a subtle

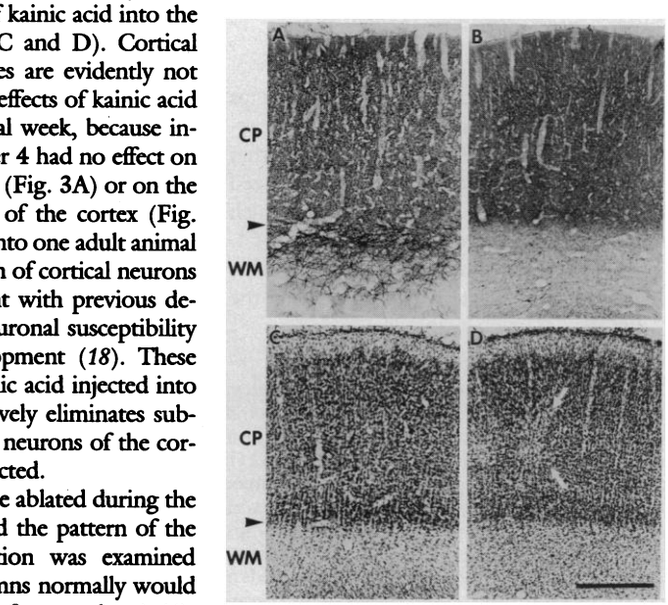

Fig. 2. Effects of neonatal kainic acid injections into the white matter (WM) on the cellular organization of the visual cortex. (**A**) MAP2 immunostaining of a section through the visual cortex of a normal P19 cat. All of the immunostaining in the white matter is associated with subplate neurons, which form a dense immunoreactive band immediately below the cortical plate (CP; arrowhead indicates border between CP and WM). (**B**) A section through the visual cortex of a P19 animal that was injected with kainic acid at P7. MAP2 immunoreactive subplate neurons were eliminated without affecting neurons in the cortical plate. (**C** and **D**) Sections adjacent to those shown in (A) and (B), respectively, stained with cresyl violet, indicate that the histology of the cortical plate appears essentially normal after kainic acid injections at P7. About 2 weeks after injections, small areas of layer 4 often appear less densely MAP2-immunostained than the surrounding tissue (D, white arrows). Sections are horizontal, and the medial bank of area 17 (primary visual cortex) is to the top. Injection sites were verified by the presence of coinjected fluorescent microspheres. Scale bar, 500 μm.

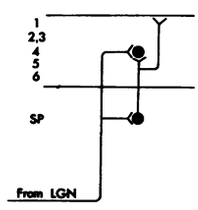

Fig. 1. Hypothetical relations between LGN axons, layer 4 neurons, and subplate neurons during ocular segregation in the cat visual cortex. LGN axons have branches within both the subplate (SP; future white matter) and layer 4 at birth (*13*). During fetal and neonatal development, subplate neurons have extensive axonal projections into the cortical plate, primarily to layers 4 and 1 (*10*).

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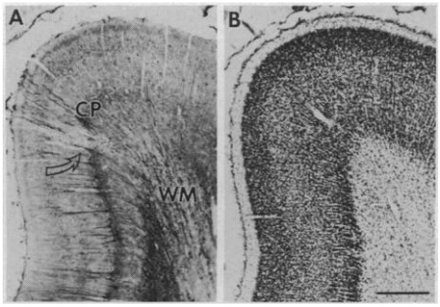

Fig. 3. Cortical plate neurons are insensitive to kainic acid–induced excitotoxicity during the first postnatal week. (**A**) A section through the visual cortex of a P14 animal that received an injection of kainic acid into the cortical plate at P7 (the deposit of latex microspheres at the injection site is indicated with a curved arrow). MAP2 immunoreactivity of neurons in the cortical plate and subplate neurons in the white matter appear normal. (**B**) Cresyl violet staining of the section adjacent to the one shown in (A) reveals normal histological organization of the cortical plate even in the immediate vicinity of the kainic acid injection site. Scale bar, 750 μm.

periodicity to the labeling, which perhaps indicated partial segregation of geniculocortical afferents. Each cortical region that lacked segregation was consistently localized over the region of white matter into which kainic acid had been injected (Fig. 4C). Thus, wherever subplate neurons were missing, geniculocortical axons representing the two eyes remained unsegregated within layer 4 (*21*).

The dense and uniform accumulation of transneuronal labeling in layer 4 after kainic acid injections suggests that LGN axons do not simply degenerate when they fail to segregate (Fig. 4, D and E). LGN neurons projecting to cortex in the vicinity of the kainic acid injections retrogradely transport the fluorescent microspheres and can be detected 6 to 8 weeks later, indicating that the neurons survived (*17*). Although geniculocortical axons overlying subplate-ablated regions fail to segregate into ocular dominance columns, their restriction to layer 4 appears to be normal, indicating that the laminar specificity of the geniculocortical projection is not obviously affected by neonatal kainic acid injections.

Our observations suggest that subplate neurons are required for LGN axons to form ocular dominance columns within cortical layer 4. This interpretation requires that the effects of kainic acid be specific to subplate neurons, as is supported by several lines of evidence. Layer 4 neurons are unlikely to be directly affected by kainic acid because an injection into layer 4 itself during the first postnatal week does not destroy cortical neurons (Fig. 3) (*22*). Nor is it likely that kainic acid directly alters geniculocortical axon terminals, as normal ocular dominance columns form even when kainic acid is injected directly into layer 4 (*21*).

Other cell types, such as cholinergic and noradrenergic afferents to visual cortex and young astrocytes (*23*), have been implicated in the process of ocular dominance plasticity during and after the critical period in visual cortex. These cell types continue to be present after kainic acid injections, as indicated by immunostaining with antibodies against choline-acetyltransferase and glial fibrillary acidic protein (*17*). Thus, evidence favors the interpretation that a selective loss of subplate neurons interferes with the segregation of geniculocortical axons representing the two eyes in layer 4.

Given that the formation of ocular dominance columns requires ongoing patterned neural activity (*5, 24*), subplate neurons might participate in the process of axonal segregation by modulating activity-dependent interactions between LGN axons and layer 4 cortical neurons. Synaptic modifications in visual cortex, as in hippocampus, may require a minimum amount of postsynaptic membrane depolarization, possibly through activation of *N*-methyl-D-asparate (NMDA) receptors (*7, 8, 25*). Because subplate neurons have extensive axonal branches within layer 4 during perinatal development (Fig. 1) (*10*), and because analysis of current-source density recordings suggests that these axons can monosynaptically activate layer 4 neurons (*12*), subplate neurons

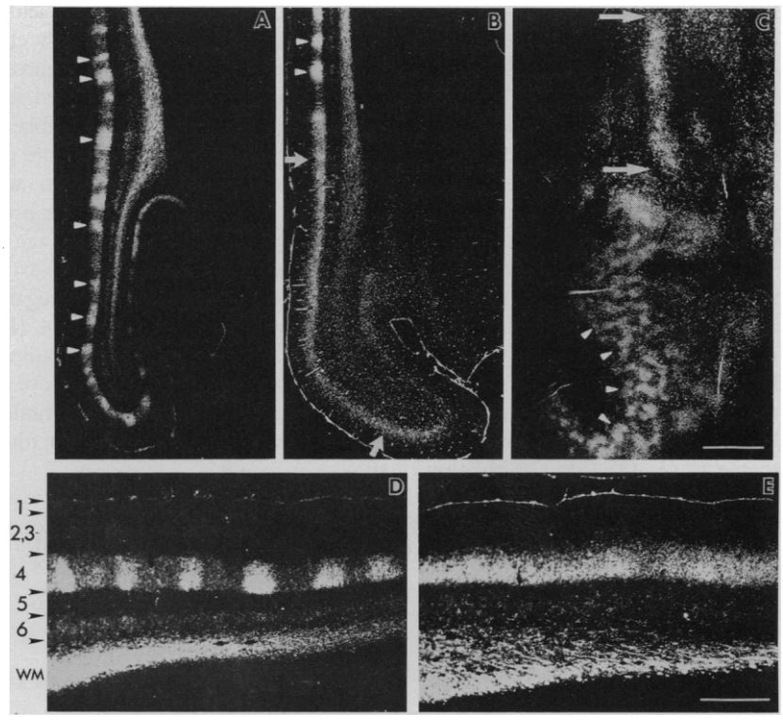

Fig. 4. Effect of subplate ablations on the segregation of geniculocortical axons into ocular dominance columns. Each of the panels shows a single section through primary visual cortex; in (A) through (C), anterior is to the top; in (D) and (E), anterior is to the left. (**A**) The normal pattern of ocular dominance columns in layer 4 at P44, seen in a horizontal section revealed by transneuronal transport of [^{3}H]proline injected into the ipsilateral eye. The periodic pattern of autoradiographic labeling representing the terminals of LGN axons (arrowheads) extends to the posterior pole as a narrow band within layer 4 of the cortical plate. (**B**) The pattern of transneuronal labeling at P70 in an animal that received injections into the posterior half of the visual cortex at P2. Ocular dominance patches formed normally in anterior visual cortex (arrowheads), but are absent from the region of cortex overlying the kainate-injected area (region between white arrows), where the label is continuous. (**C**) A section through the visual cortex of a P100 animal that received kainic acid injections into the white matter underlying anterior visual cortex at P5. This tangential section was obtained from a flat-mount preparation of the visual cortex after the transneuronal transport of WGA-HRP injected into the ipsilateral eye (*19, 20*). The flat-mount technique allows large areas of layer 4 to be included in a single section. Normal ocular dominance columns (labeled patches) are present in the posterior visual cortex (arrowheads) but not in the anterior regions where subplate neurons were deleted [area between the white arrows; this region corresponds to layer 4 along the medial bank of primary visual cortex and in normal animals contains ocular dominance patches (A) (*20*)]. The remaining large regions devoid of label correspond to areas where the plane of section did not pass through layer 4 and therefore did not contain labeled LGN axon terminals; these regions were labeled in adjacent sections. (**D** and **E**) Comparison of the geniculocortical projection to the visual cortex of normal (P44) and subplate-ablated (examined at P55 after P6 ablation) animals. Ocular dominance columns did not form in the subplate-ablated animal; however, several other features of the geniculocortical projection, such as the laminar restriction of afferents to layer 4, appear unaffected by the procedure. Scale bars for (A) and (B) [in (C)], 2 mm; for (C), 3 mm; and for (D) and (E) [in (E)], 1 mm.

could alter the excitability of layer 4 neurons either by direct synaptic inputs or by affecting NMDA receptors in layer 4. Examining the effect of subplate neurons on synaptic responses in the visual cortex should lend insight into how these neurons influence geniculocortical interactions during the critical period.

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14. The consequences of white matter kainic acid injections were examined in 16 animals: the extent and specificity of ablations were evaluated in seven animals 1 to 2 weeks after injection; the effect of the ablations on the segregation of geniculocortical afferents was examined in the remaining nine animals. Neonates (between P2 and P7) were initially anesthetized by inhalation of halothane (3 to 4% in O_2) and maintained under halothane anesthesia (1 to 2% in O_2) during surgery. Two injections (0.5 μl) of kainic acid and microspheres [10 mg of kainic acid per milliliter in 0.9% saline with 10% v/v rhodamine-labeled microspheres (Lumafluor, Inc., New City, NY)] separated by 1 to 2 mm in the anteroposterior dimension were made into the white matter underlying visual cortex with a 30-gauge needle (coordinates: 5 mm anterior to the lambdoid suture, 2 mm lateral to the sagittal suture, 2 mm deep).
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16. Brains from seven animals injected with kainic acid between P2 and P7 were processed for histology and immunocytochemistry 1 to 2 weeks after injection. For methodological details see J. J. M. Chun and C. J. Shatz, *J. Cell Biol.* **106**, 857 (1988).
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19. The projection patterns of LGN axons in animals injected with kainic acid into the white matter were examined at P47 (two animals), P48 (one animal), P55 (three animals), P60 (one animal), P70 (one animal), and P100 (one animal). The transneuronal pattern of labeling in visual cortex after an ipsilateral eye injection (2 mCi of [^{3}H]proline in 45 μl of 0.9% saline) was revealed by autoradiography (*1*). In one animal (injected with kainic acid on P5) the pattern of LGN axon terminals in primary visual cortex was revealed by transneuronal transport of wheat germ agglutinin–horseradish peroxidase (WGA-HRP) (*20*).
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21. The lack of segregation of geniculocortical afferents within layer 4 in the subplate-lesioned animals is probably not a direct effect of the kainic acid on LGN axons because injections of kainic acid directly into layer 4 (two animals) or layer 1 (one animal) did not prevent segregation. Nor is the failure of column formation due to a reorganization of the retinogeniculate projection, as retinal ganglion cell axons are restricted to the appropriate eye-specific layers throughout the LGN in kainic acid–injected animals.
22. Kainic acid injections into the white matter in neonates produce long-term histological changes in layer 4. By several months after injection, many of the small stellate cells are missing from layer 4. Because injection of kainic acid into the cortical plate during the first postnatal week has no effect on cortical organization, the subsequent changes in cortical histology are likely to be secondary to changes (such as altered geniculocortical interactions) caused by the absence of subplate neurons.
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Technical Comments

Age-Associated Inclusions in Normal and Transgenic Mouse Brain

Two research groups have used transgenic techniques in attempts to induce cerebral amyloidogenesis in mice (*1, 2*). In (*1*), the transgene construct included the human amyloid precursor protein (APP) promoter and sequence encoding the amyloid β protein (βAP). After 6 months of age, these transgenic mice developed clusters of βAP-positive granules primarily in the hippocampus. Ultrastructurally, these lesions consisted of fibrillar material, perhaps within dendrites (*1*). In recent immunohistochemical studies of a laminin-binding protein (LBP) in brain (*3*), we noted that inbred C57BL/6J mice exhibited abnormal clusters of LBP-like immunoreactive granules that were similar in appearance and distribution to the βAP-positive granules in βAP-transgenic mice (*1*). Seen with a light microscope, they resemble periodic acid–Schiff (PAS)–positive particles that have been described in hippocampus of aged C57BL/6 mice and aged nude mice (*4*).

In normal adult C57BL/6 mice, the clusters predominated in the hippocampus (Fig. 1A) and were located mainly in the stratum lacunosum moleculare of region CA1 and in the molecular layer of the dentate gyrus, although there was some variability in distribution among individuals. The clusters were also present in piriform cortex and cerebellum and were occasionally observed in the diencephalon, striatum, and amygdala. In adult mice, clusters usually ranged from 30 to 60 μm in diameter and consisted of 40 to 100 granules (Fig. 1A). Each granule was typically between 1 to 3 μm in diameter, although larger granules appeared with increasing frequency in older mice.

In addition to having LBP-like immunoreactivity, the granules appeared to be immunoreactive with polyclonal antisera directed against βAP, somatostatin, and neurotensin (*5*). However, when appropriate synthetic peptides were used to preabsorb the antibodies, staining of granules was not blocked even though staining of normally immunoreactive elements (such as somatostatin- and neurotensin-containing neurons) was eliminated, as was staining of βAP in concurrently processed brain tissue from individuals with Alzheimer's disease (Fig. 2, A, B, and C). Affinity purification of the antibody to LBP reduced immunoreactivity of the granules, while it enhanced the typical pattern of LBP-immunoreactivity in brain, which suggests that LBP epitopes also are not expressed in these clusters. Moreover, occasional batches of normal rabbit sera and control ascites fluid stained the granules. The granules also stained with monoclonal antibodies to synaptophysin and to laminin B2 chain, and a small percentage stained for phosphorylated neurofilaments (*5*), but we did not perform preabsorption controls with these antibodies. The granules did not stain with monoclonal antibodies that recognize βAP, APP, microtubule-associated protein (MAP2), and calbindin D28, nor