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Immunoreactivity for a calmodulin-dependent protein kinase is selectively increased in macaque striate cortex after monocular deprivation

(visual system/phosphorylation/central nervous system plasticity)

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ABSTRACT **Immunocytochemical methods were used to localize type II Ca^{2+}/calmodulin-dependent protein kinase in the macaque primary visual cortex. Neurons that stain for the kinase include both pyramidal and nonpyramidal cells and they appear to form a subset of cortical neurons. They are densely packed in layers II and IVB, somewhat more sparse in layers III, IVCβ, and VI, and nearly absent in layer V. In normal animals the distribution of kinase-positive cells within each layer is relatively uniform. However, in animals in which one eye is removed 7–14 days before sacrifice or sutured shut for 9 or 11 weeks, the cells in layer IVCβ are divided into alternating lightly and darkly stained bands. Comparison of immunocytochemically stained sections with adjacent sections stained for the mitochondrial enzyme, cytochrome oxidase, reveals that the kinase staining increases in ocular dominance columns originally driven by the removed or closed eye. These findings suggest that either the concentration of type II Ca^{2+}/calmodulin-dependent protein kinase or its accessibility to the antibody probe increases dramatically and selectively in neurons of macaque primary visual cortex that have been deprived of their normal visual input. This may indicate that changing levels of activity in cortical neurons can alter their regulatory machinery.**

Biochemical studies of regulatory processes in brain have provided strong evidence that protein phosphorylation systems controlled by several different intracellular messengers regulate many aspects of neuronal function, including synaptic efficacy and membrane excitability (1–3). Type II Ca^{2+}/calmodulin-dependent protein kinase may regulate certain specialized functions of neurons in the telencephalon (4–6). This is suggested by studies in the rat, which have shown that the kinase is highly expressed in brain and particularly highly concentrated in neurons in the cortex and hippocampus where it comprises 1–2% of the total protein (7). To determine whether the kinase is differentially expressed in particular populations of cortical neurons, we used immunocytochemistry to examine the location of the kinase in macaque primary visual cortex (area 17). This area of cortex is favorable for such studies because of its highly organized architecture in which neurons of various sizes, morphologies, and packing densities form several layers. As in other areas of cortex, the layers in area 17 represent collections of neurons with similar connectional and functional properties (8, 9). For example, visual information is carried from the retina to the dorsal lateral geniculate nucleus of the thalamus and thence via afferent fibers to neurons primarily in layer IV. We find that kinase-positive neurons form a distinct laminar pattern in area 17, indicating that the kinase is differentially expressed in certain populations of cortical neurons.

Further, we find that the pattern of neurons staining for the kinase can be altered by visual deprivation. In addition to the horizontal laminae described above, area 17 is organized into a series of vertical ocular dominance columns in which neurons driven preferentially by visual input from one eye alternate with those driven by the other eye (8, 9). This columnar arrangement arises presumably because thalamic afferents carrying information from each of the eyes alternate as they end in layer IV (10, 11). In monocularly deprived monkeys, kinase staining within layer IV increases in ocular dominance columns previously dominated by the removed or closed eye. These results suggest that the absolute level of type II Ca^{2+}/calmodulin-dependent kinase, or its availability for binding to the antibody, is dramatically increased in neurons deprived of their normal visual input. Thus, short-term visual deprivation can affect biochemical processes in addition to those involved directly with metabolism (12–15) in the deprived neurons.

MATERIALS AND METHODS

Eight cynomolgus monkeys (*Macaca fascicularis*) of ages 14 months to 2.5 years were used in this study. One eye was surgically removed from each of four animals 7, 10, or 14 days before they were sacrificed. In two animals, the lids were sutured closed over one eye 9 or 11 weeks before sacrifice. All animals were anesthetized with sodium pentobarbital and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed, cut into blocks, postfixed in the same paraformaldehyde solution for 4 hr, and placed in 30% sucrose in phosphate buffer overnight. Blocks that included area 17 were sectioned on a freezing microtome at 30 or 40 μm. Sections were pretreated with 5% normal swine serum and 0.3% Triton X-100 in phosphate buffer for 2 hr at 4°C. They were transferred to 6G9 anti-kinase ascites fluid at a dilution of 1:250 in 0.1 M phosphate buffer and incubated for 18–24 hr at 4°C. The specificity of 6G9 has been documented previously (7). They were washed in several changes of phosphate buffer for 1 hr and incubated in a horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako, Santa Barbara, CA) for 2 hr. After they were washed in phosphate buffer for 1 hr, the sections were processed for peroxidase histochemistry by the method of LaVail and LaVail (16) or Adams (17). The sections were mounted, dried, cleared in xylene, and covered with a coverslip. Some immunocytochemically stained sections and adjacent unprocessed sections were stained with thionin. Series of sections from one occipital lobe of two lid-sutured and three enucleated animals, both lobes of a fourth (14-day postenucleation survival), and both lobes of the two normal

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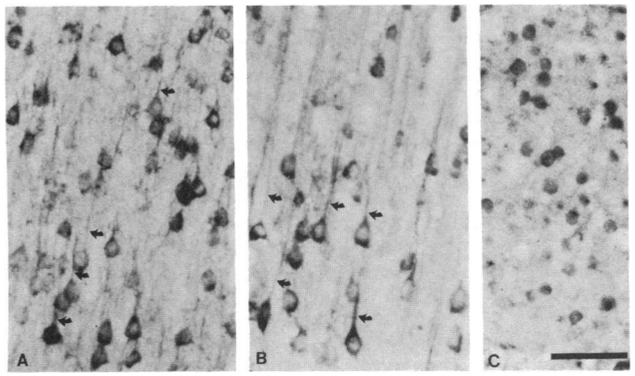

FIG. 1. Photomicrographs of neurons in macaque area 17 that display kinase immunoreactivity. Many stained neurons in layers II (*A*) and III (*B*) have pyramidal-shaped cell bodies and prominent ascending processes (arrows). By contrast, kinase-immunoreactive neurons in layer IVCβ (*C*) have small, round cell bodies and give rise to few stained processes. (Bar = 50 μm.)

animals were sectioned parallel to the opercular surface. Alternate sections were processed immunocytochemically for the kinase and histochemically for cytochrome oxidase. Control sections from each animal were processed as above, except that the 6G9 ascites fluid was replaced with a control ascites fluid prepared from the nonsecreting parent tumor, NS1-SP2.

RESULTS

In area 17, kinase immunoreactivity is seen within the cell bodies of neurons, in the surrounding neuropil, and, to a lesser extent, within proximal neuronal processes. Many immunoreactive neurons, mainly in layers II, III, and VI, have pyramidal-shaped cell bodies and heavily stained ascending processes (Fig. 1 *A* and *B*), while other neurons, particularly in layer IV, have rounded cell bodies and display little staining of processes (Fig. 1*C*). In several of the layers, the number of immunoreactive cells is much less than the total number seen in Nissl-stained sections. Comparison of immunocytochemically processed sections (Fig. 2*B*) with adjacent thionin-stained (Fig. 2*C*) or cytochrome oxidase-stained sections (Fig. 2*A*) reveals a distinct laminar distribution of kinase-positive neurons. These neurons and the surrounding neuropil are most intensely stained in two bands, corresponding to layer II and IVB [lamination according to Lund (18)]. In the intervening layers, III and IVA, immunoreactive neurons are less densely distributed and less intensely stained. The distribution of kinase-positive neurons divides layer IVC into equal halves, with the upper half containing relatively few, lightly stained neurons and the lower half containing a greater number of moderately stained neurons. These correspond to layers IVCα and IVCβ, respectively. Layer VI resembles layer IVCβ in the numbers and staining intensity of immunoreactive neurons, whereas in layer V very few cell bodies are stained. The immunoreactivity of the neuropil in layer V divides it into a superficial unstained layer, VA, and a deeper heavily stained layer, VB. In layer I, heavy staining is found in the neuropil and very rarely in cell bodies. In the subcortical white matter, a few lightly stained neurons are found, mainly within 500 μm of layer VI, while the axons of the white matter are, themselves, unstained.

In sections cut radially through area 17 of normal monkeys, no obvious inhomogeneities in kinase immunoreactivity or in cytochrome oxidase staining are found in any of the layers (Fig. 3 *A* and *B*). However, in animals enucleated unilaterally at least 7 days prior to sacrifice, alternating light and dark kinase-positive bands are evident in layer IVCβ (Fig. 3*D*). The dark bands are 350 $\pm$ 100 μm in diameter, while the light bands tend to be larger and often exceed 500 μm in diameter. Alternating light and dark histochemically stained bands are also obvious in layer IVC and in layer IVA in adjacent sections processed for cytochrome oxidase (Fig. 3*C*). The dark cytochrome oxidase bands correspond to ocular dominance columns driven mainly by the intact eye and the light bands to columns previously dominated by the removed eye (19–21). Comparison of kinase- and cytochrome oxidase-stained sections shows that the dark kinase-stained bands lie in precise register with the light cytochrome oxidase bands (Figs. 3 and 4). Thus, kinase immunoreactivity in columns once driven primarily by the removed eye is greater than in

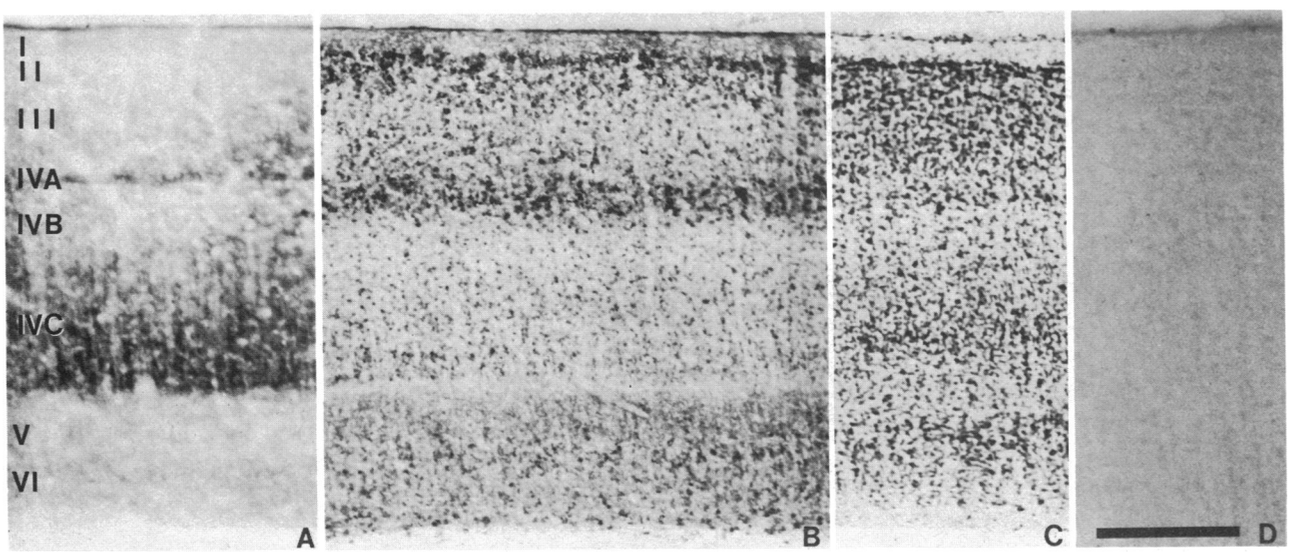

FIG. 2. Photomicrographs illustrating the laminar distribution of kinase-immunoreactive neurons in area 17 of a normal macaque. Comparison of sections processed histochemically for cytochrome oxidase (*A*) or stained with thionin (*C*) with an immunocytochemically stained section (*B*) reveals concentrations of kinase-positive neurons in layers II and IVB. Immunoreactive neurons are also evident in layers III, IVCβ, and VI. In normal macaques the kinase staining within a layer is uniform. Staining of area 17 with a control ascites fluid (*D*) produces a very light, evenly distributed reaction product. (Bar = 500 μm.)

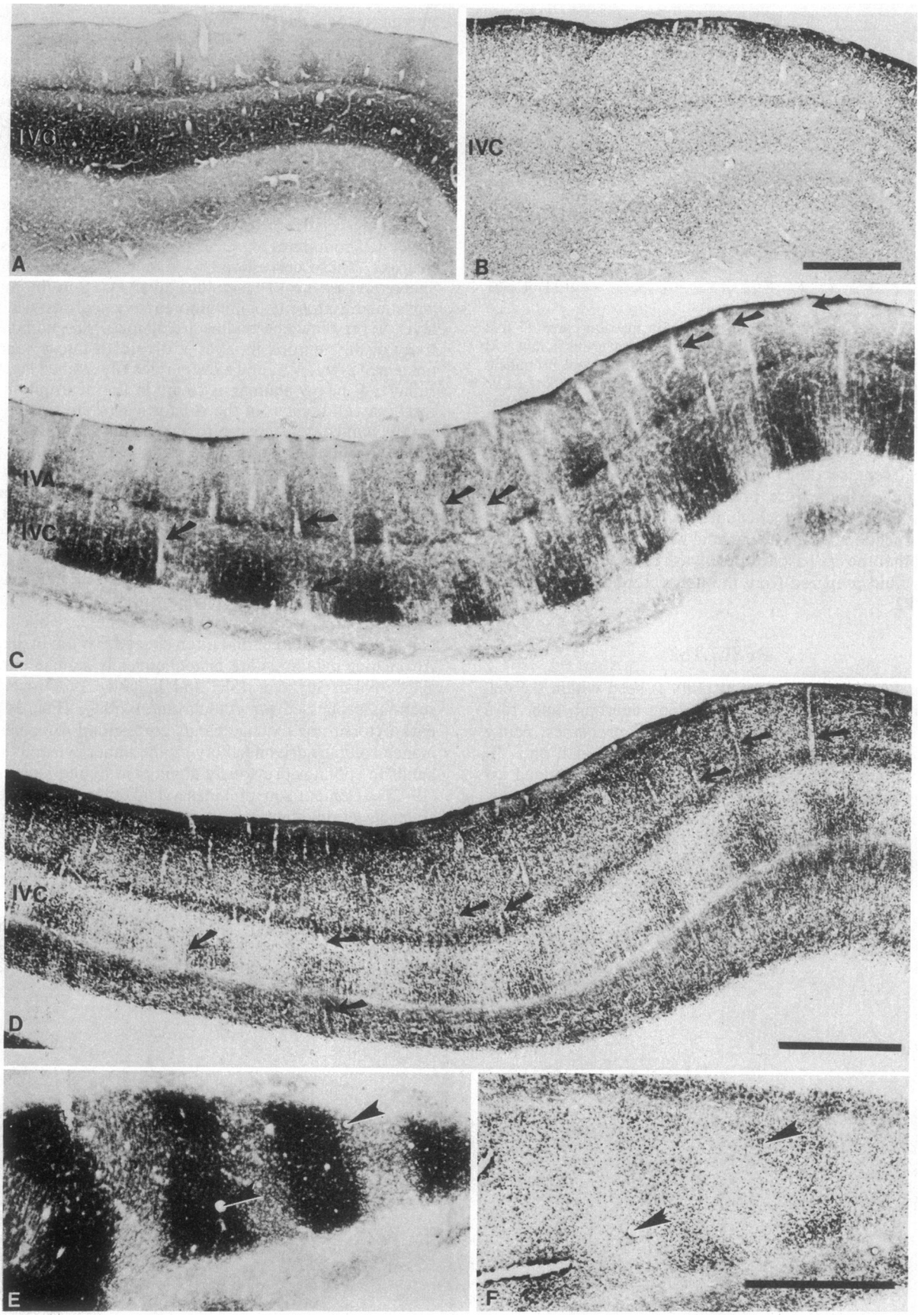

FIG. 3. Photomicrographs of kinase (*B*, *D*, and *E*) and cytochrome oxidase (*A*, *C*, and *F*) staining in area 17 of a normal (*A* and *B*) and a unilaterally enucleated macaque (16 months old) (*C–F*). In sections from a normal monkey, the kinase staining throughout any layer is relatively uniform. In the corresponding cytochrome oxidase-stained section, patchy staining is obvious only in layers II and III, with the remaining layers, including the heavily stained layer IVC, appearing homogeneous. In sections from a monkey enucleated 10 days before sacrifice, the cytochrome oxidase staining in layer IVC is broken up into alternating light and dark bands. The kinase staining in layers II and IVCβ is also divided into light and dark bands. Comparison of the positions of the same blood vessels in the two sections (arrows) reveals that the dark kinase bands

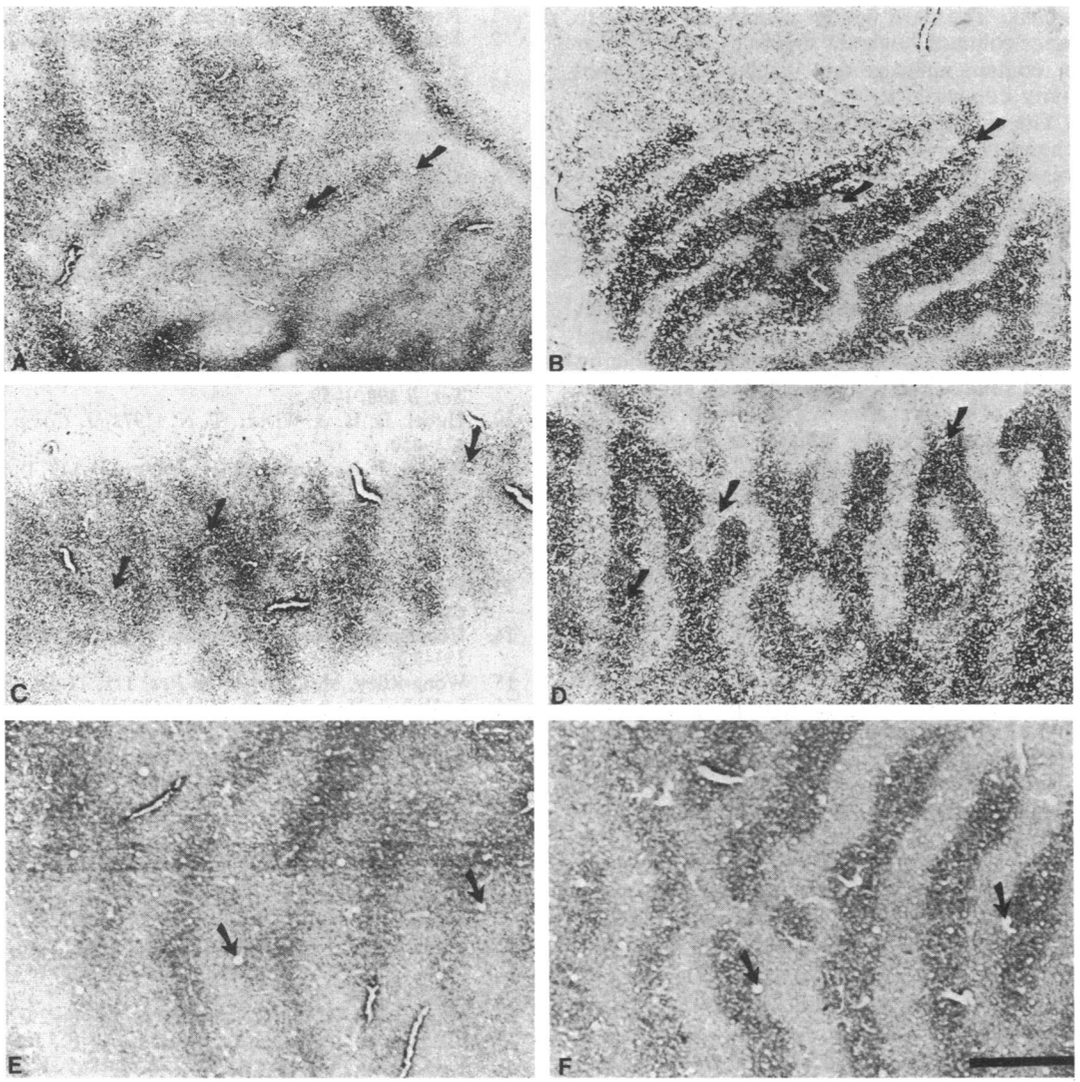

FIG. 4. Photomicrographs of kinase (*A*, *C*, and *E*) and cytochrome oxidase (*B*, *D*, and *F*) staining in tangential sections through area 17 of monocularly deprived macaques. The sections were cut from animals sacrificed 7 (*A* and *B*) or 14 days (*C* and *D*) after eye removal; or 11 weeks after monocular lid suture (*E* and *F*). In layer IVCβ both the kinase staining and the cytochrome oxidase staining are divided into alternating light and dark bands. When the same radially oriented blood vessels (arrows) in the adjacent pairs of sections are lined up it is clear that the dark kinase bands coincide with the light cytochrome oxidase bands. (Bar = 1 mm in *A–D* and 0.8 mm in *E* and *F*.)

columns driven primarily by the intact eye. In addition, comparison of sections from intact and enucleated animals processed simultaneously reveals that kinase staining in deprived-eye columns is greater than in layer IVC of normal animals. The difference after enucleation is due to an increase in intensity of staining within individual neurons of the deprived-eye columns rather than to an increase in the number of kinase-positive neurons (Fig. 3 *E* and *F*). The change does not simply reflect a generalized increase in synthesis of regulatory proteins in the deprived neurons since staining for synapsin I, a neuronal protein that is a substrate for the type II Ca^{2+}/calmodulin-dependent kinase, is unaffected by removal of an eye 14 days prior to sacrifice (data not shown).

The relationship of the kinase immunoreactive bands to the cytochrome oxidase bands is shown clearly in sections cut tangentially through layer IVC (Fig. 4). In four enucleated monkeys, the dark kinase bands interdigitate with the dark cytochrome oxidase bands. The kinase banding pattern is present 7 and 14 days after enucleation (Fig. 4 *A–D*). The same pattern is evident in sections from monkeys sacrificed 9–11 weeks after monocular lid suture (Fig. 4 *E* and *F*).

DISCUSSION

We have used immunocytochemical methods to localize type II Ca^{2+}/calmodulin-dependent protein kinase in cortical area 17 of both normal and monocularly deprived macaques. Kinase immunoreactive neurons have a distinctive laminar distribution and form a subpopulation of neurons in area 17.

Our most intriguing finding is that kinase immunoreactivity is dramatically altered in visually deprived animals. Stained neurons are distributed evenly within individual layers in normal monkeys but occur in alternating lightly and darkly stained bands in layer IVCβ of monkeys that had one eye removed 7–14 days before sacrifice or one eye sutured closed

coincide with the light cytochrome oxidase bands in layer IVCβ. In tangential sections through layer IVCβ (*E* and *F*) the intensity of staining of individual neurons is greater in removed-eye columns than in intact-eye columns. Arrowheads indicate the same blood vessels in *E* and *F*. (Bars = 1 mm.)

for 9 or 11 weeks. The dark bands, which correspond to ocular dominance columns originally driven by a deprived or removed eye, contain neurons that display a heightened immunoreactivity compared to neurons in adjacent intact-eye columns. The simplest explanation for this increase in immunocytochemical staining is that visual deprivation produces an increase in the concentration of the kinase in the deprived neurons. However, it is also possible that the deprivation causes a change in subcellular distribution or molecular structure of the kinase that makes it more accessible to the antibody probe.

The increase in kinase immunoreactivity appears to result from a change in neuronal activity rather than from structural alterations or degeneration, since it can be produced either by enucleation or lid suture. Previous studies have shown that during long periods of survival after eye removal or lid suture (up to 1 year), a reduction in the number of neurons (22), synaptic contacts (22), and dendritic spines (23, 24) occurs in area 17, but these changes have not been observed after postenucleation survival as short as 1 or 2 weeks. Further, our observation that immunocytochemical staining for synapsin I, a substrate protein of the kinase, is not altered 14 days after enucleation suggests that no extensive degeneration has occurred. This result also indicates that there has not been a general increase in synthesis of proteins in the deprived neurons. Recent results by Black *et al.* (25) suggest that adult neurons show activity-dependent plasticity in the expression of certain neurotransmitters. Our results suggest that this plasticity can extend to intracellular regulatory machinery as well.

The strict laminar pattern displayed by kinase-positive cells in area 17 does not correspond to the lamination observed in staining patterns for any known neurotransmitter including γ-aminobutyric acid (26), the peptides somatostatin and neuropeptide Y (27), the monoamines serotonin and norepinephrine (28, 29), and the glutamate-generating enzyme glutaminase (30). Thus, kinase expression appears to occur independently of any one transmitter system. Similarly, although layer IVB in which the neurons are intensely kinase-positive is characterized by a rich projection to and input from the medial temporal visual area of cortex (31), the overall laminar distribution of the kinase does not coincide with any single pattern of connections or physiological types, indicating that the kinase-positive cells display a variety of connectional and functional features. Several characteristics of the type II Ca^{2+}/calmodulin-dependent kinase in rat brain, including its substrate proteins (4, 32, 33), its concentration in particular brain areas (7), its presence in presynaptic terminals (6), its possible involvement in regulation of transmitter release (34), its association with postsynaptic densities (5, 35), and its unusual "switch-like" regulation by autophosphorylation (36, 37) all suggest that the kinase may play an important role in synaptic function, perhaps in modulation of synaptic efficacy. Thus, type II Ca^{2+}/calmodulin-dependent kinase immunoreactivity may be a property of cells with specialized regulatory features that are modulated in an ongoing way by synaptic activity.

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