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# Molecular and Morphological Changes in the Cat Lateral Geniculate Nucleus and Visual Cortex Induced by Visual Deprivation Are Revealed by Monoclonal Antibodies Cat-304 and Cat-301

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**Monoclonal antibody Cat-301 recognizes a surface-associated proteoglycan on subsets of neurons in the mammalian CNS (Hockfield and McKay, 1983). The expression of Cat-301 immunoreactivity on Y cells in the cat LGN is sharply reduced by early visual deprivation (Sur et al., 1988). We employed an immunosuppression strategy (Hockfield, 1987) to further study alterations in the expression of experience-dependent molecules. Newborn BALB/c mice were injected with LGN from dark-reared cats to induce a suppression of the immune response to antigens expressed in visually deprived animals. These mice were then immunized with LGN from normal cats to elicit an immune response to antigens with an expression dependent on normal early visual experience. This strategy permitted the generation of monoclonal antibody Cat-304, which recognizes a surface-associated antigen on neuronal cell bodies and proximal dendrites, and which appears histologically identical to Cat-301. Further analyses show that Cat-304 and Cat-301 recognize different epitopes on the same 680-kDa chondroitin sulfate proteoglycan.**

**We examined the effects of early visual deprivation on Cat-304 immunoreactivity in the LGN and visual cortex of cats. In LGN from normal cats, Cat-304 labels neurons in layers A, A1, and C, in interlaminar zones, and in the medial interlaminar nucleus. In LGN from dark-reared cats, the number of antibody-positive neurons is markedly reduced, and the cross-sectional area of the remaining positive neurons is smaller than normal. In cortical area 17 of normally reared cats, Cat 304-positive neurons are densely distributed in 2 bands, in layers IV and V/VI. Labeled neurons are also present in layers II and III. In area 17 of dark-reared cats, the number of antibody-positive neurons is reduced. The reduction in the number of labeled neurons is most pronounced in layers II/III and V/VI. Antibody-positive neurons are smaller in all cortical layers of dark-reared cats. The changes in the expression of Cat-301 immunoreactivity in dark-reared visual cortex and LGN are identical to those of Cat-304. The laminar differences in the effect of dark rearing on Cat- 301 and Cat-304 expression in the visual cortex provides support for the suggestion that layer IV of cortical area 17 may be less susceptible to prolongation of plasticity by dark rearing than layers II/III and V/VI. Further, the biochemical and histological studies reported provide evidence that early visual experience regulates protein expression in the cat LGN and visual cortex.**

Central visual pathways of the cat mature during the first 3 postnatal months. During this period, the structure and function of visually responsive neurons are determined, in part, by an animal's visual experience. Abnormal visual experience during this period interferes with the acquisition of normal physiological and anatomical properties of neurons in the LGN and visual cortex (Hubel and Wiesel, 1970; reviewed in Wiesel, 1982). Our studies have been designed to test whether these alterations in neuronal structure and function also might be reflected in alterations in the molecular composition of neurons.

Animals raised in the dark from birth provide one deprivation paradigm that has been used to study the experience-dependent development of neurons in the visual system (reviewed in Sherman and Spear, 1982). In the LGN of dark-reared cats, there is a profound reduction in the proportion of electrophysiologically identifiable Y-cells, though the total number of LGN neurons is not reduced (Kratz et al., 1979; Mower et al., 1981a, b; Kratz, 1982). This has been interpreted as a failure of presumptive Y-cells to attain their mature physiological characteristics.

Our previous studies showed a correlation between the loss of this physiologically identified population of neurons and a reduction in immunoreactivity for monoclonal antibody Cat-301. Cat-301 recognizes a surface-associated proteoglycan on subsets of neurons in many parts of the mammalian CNS (Hockfield and McKay, 1983; Hockfield et al., 1983; Hendry et al., 1984, 1988; Zaremba et al., 1989). In the LGN of cats reared in a normal visual environment, Cat-301 selectively recognizes Y-cells (Hockfield et al., 1983; Hendry et al., 1984; Sur et al., 1988; Hockfield and Sur, 1990). Cat-301 immunoreactivity is dramatically reduced in the LGN of animals deprived of normal visual experience from birth by monocular lid suture or dark rearing (Sur et al., 1988). In contrast, adult cats subjected to visual deprivation show no reduction in Cat-301 expression (Sur et al., 1988) and no reduction in the number of functional Y-cells (Sherman and Spear, 1982). Together, these results showed that the development of Cat-301 immunoreactivity in the LGN parallels that of the experience-dependent physiological maturation of Y-cells. However, whether this regulation of Cat-301 im-

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munoreactivity reflected a reduction in the expression of the Cat-301 proteoglycan itself, or simply a loss or masking of the Cat-301 epitope, could not be determined.

The aim of the present study was to generate additional monoclonal antibodies to study further experience-dependent alternations in the expression of molecular species in mammalian visual pathways. Here, we describe Cat-304, a monoclonal antibody generated using an immunosuppression technique (Hockfield, 1987) that recognizes a different epitope on the same chondroitin sulfate proteoglycan identified by Cat-301. Using both Cat-304 and Cat-301, we show molecular and anatomical changes in the LGN and visual cortex of dark-reared cats. These changes include a reduction in the number of neurons that express the Cat-301/Cat-304 proteoglycan, as well as a reduction in the size of antibody-positive neurons. The results presented here provide strong evidence that neuronal activity during the critical period regulates the expression of the Cat-301/Cat-304 proteoglycan itself and not simply the expression of one epitope. They also provide evidence that, as previously proposed (Friedlander et al., 1982; Mower and Christen, 1985), different subpopulations of visually responsive neurons are differentially sensitive to visual deprivation during development.

## Materials and Methods

*Subjects.* Eight 90-d-old kittens were used in this study. For each condition, animals were obtained from at least 2 separate litters. Three kittens were from litters reared in complete darkness, and 5 were from litters reared in a normal light-dark cycle. Animals were anesthetized deeply with sodium pentobarbital and perfused transcardially with sodium PBS (pH, 7.4), followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB) (pH, 7.4). Fixed brains were stored in fixative at 4°C.

*Immunization and fusion.* Material for immunization was obtained by microdissecting the LGN from normal and dark-reared cats. LGN pieces were equilibrated and homogenized in PB. An immunosuppression procedure was used to generate monoclonal antibodies (Hockfield, 1987). Six newborn BALB/c mice were injected every other day, from the day of birth through day 14, with LGN from dark-reared cats (2 mg/50 μl, i.p.). The mice were then immunized on days 28, 32, 37, and 42 with homogenized LGN from normal cats, emulsified 1:1 with Freund's complete adjuvant, by injection into the hind footpads (0.07 mg/50 μl). On day 43, the mice were killed by cervical dislocation, and lymphocytes from popliteal and inguinal lymph nodes were harvested. The nodal lymphocytes from 2 groups of 3 animals were each pooled, fused with NS-1 myeloma cells, suspended in selection medium, and plated at low density onto macrophage feeder layers into a total of 12 96-well plates. Hybridoma supernatants were screened immunohistochemically on free-floating, 25-μm-thick sections of LGN from normal animals. Positive lines were subsequently tested for reactivity on sections of LGN from dark-reared animals. Hybridoma lines of interest were stabilized by 3 rounds of limiting dilution cloning.

*Immunohistochemistry.* Blocks of visual cortex and dissected LGN were equilibrated in 30% sucrose in PB, frozen, and 25- or 50-μm-thick serial sections were cut in a coronal plane. Sets of sections through the LGN and visual cortex were processed for immunohistochemistry (McKay and Hockfield, 1982). Free-floating sections were incubated overnight in primary antibody with a final concentration of 2% Triton X-100, rinsed in PB, and then incubated in HRP-conjugated anti-mouse antibody (diluted in Dulbecco's modified Eagles's medium with 10% fetal calf serum) with 2% Triton X-100 for 2 hr. Following several rinses in PB, sections were reacted with 3,3'-diaminobenzidine (DAB) (0.03% in PB) in the presence of $H_2O_2$ (0.003%). Sections were rinsed, mounted onto slides, and coverslipped. All incubations were performed at room temperature with continuous rotation.

HRP-conjugated goat anti-mouse IgG, IgA, and IgM (Cappel) were used for screening hybridoma supernatants. HRP-conjugated goat anti-mouse IgG (γ-chain specific) and HRP-conjugated goat anti-mouse IgM (μ-chain specific; Cappel) were used in order to determine antibody subclasses. In some experiments, the DAB reaction product was intensified with 0.2% nickel sulfate. The following controls were always performed: monoclonal antibody Cat-301 was used as a positive control and unconditioned tissue-culture media or an irrelevant monoclonal antibody was used as a negative control.

For double-labeling experiments, sections were incubated overnight with monoclonal antibody Cat-301, followed by fluorescein isothiocyanate (FITC)-goat anti-mouse IgG (γ-chain specific; 1:100; Cappel) for 2 hr. Sections were then incubated overnight with monoclonal antibody Cat-304, followed by Texas Red-goat anti-mouse IgM (μ-chain specific; 1:100; Fisher) for 2 hr. Sections were rinsed, mounted onto gelatin-subbed slides, and viewed under epifluorescence with filters appropriate for FITC or Texas-Red fluorescence. In control experiments, no cross reactivity was observed between the subclass-specific secondary antibodies and the inappropriate primary antibody. The order of primary antibody incubation did not affect the observed staining patterns.

*Cell counting and measurements.* Coronal sections through the visual cortex and LGN from normal and dark-reared cats were stained with Cat-304 or Cat-301, counterstained with thionin, and neurons with well-defined nucleoli were counted or measured (see below). Cell counts were performed using a 75× oil-immersion objective. Sections from at least 2 different animals were analyzed for each experimental condition.

In the LGN, 7 successive fields of view, in the vertical plane from dorsal layer A through layers A1 and C, were analyzed from the LGN of 2 normal and 2 dark-reared cats. In cortical area 17, 5 fields of view were scored in each layer from 3 normal and 3 dark-reared cats. The number of antibody-positive neurons was expressed as a percentage of the total neuronal population (based on Nissl staining) in each field of view.

The cross-sectional area of antibody-positive neurons was measured using video-enhanced differential interference contrast optics (Inoué, 1986) in combination with a video-overlay system (Curcio and Sloan, 1986; Williams and Rakic, 1988). A camera (Hamamatsu C2400) was mounted on the microscope, and the field of cells was viewed on a video monitor (Sony PVM-1271Q). The outline of each antibody-positive neuron was traced at a final magnification of 2500× on the screen of the monitor by using a digitizing tablet connected to a computer. The computer then calculated the cross-sectional area of each neuron. Fifty labeled neurons were measured in each layer of area 17 from 3 normal and 3 dark-reared cats, and in the LGN, from 2 normal and 2 dark-reared cats.

*Biochemical characterization.* Partial biochemical purification of the Cat-301 antigen, chondroitinase digestion, SDS-PAGE, and Western blot analysis were performed according to methods described previously (Zaremba et al., 1989). Antigens binding to Cat-304 (an IgM) were detected by blotting the nitrocellulose with undiluted Cat-304 hybridoma supernatant followed by alkaline phosphatase-conjugated goat anti-mouse IgM (μ-chain specific; Cappel).

The Cat-301 antigen was further purified on an immunoaffinity column of Cat-301 antibody (hybridoma supernatant) covalently coupled to Protein G Sepharose 4 Fast Flow (Pharmacia) according to the method of Schneider et al. (1982). Antigen was loaded onto the column in 10 mM PB (pH, 7.4) containing 0.14 M NaCl, 0.01 M EDTA, 0.5% NP-40, and 0.1% SDS (affinity column buffer). After extensive washing, the column was eluted with 0.1 M glycine buffer (pH, 2.2), and fractions were immediately neutralized with 1 M PB (pH, 8; 1:0.13 v/v).

Partially purified antigen was also immunopurified with Cat-304. Aliquots of antigen in affinity column buffer were mixed with beads of anti-IgM Actigel (Sterogene, Inc., Arcadia, CA) that had been equilibrated with Cat-304 hybridoma supernatant. After mixing overnight, the beads were collected and washed, then boiled in electrophoresis buffer for Western blot analysis.

## Results

### *Generation of monoclonal antibody Cat-304 by immunosuppression*

A major goal of this study was to generate additional monoclonal antibodies to experience-dependent molecules present in the cat LGN using a new immunosuppression technique (Hockfield, 1987). In order to suppress the immune response to antigens present in the LGN from visually deprived cats, newborn BALB/c mice were injected with LGN from dark-reared cats. Then, to generate an immune response to LGN antigens with an expression specifically dependent on visually evoked neuronal activity,

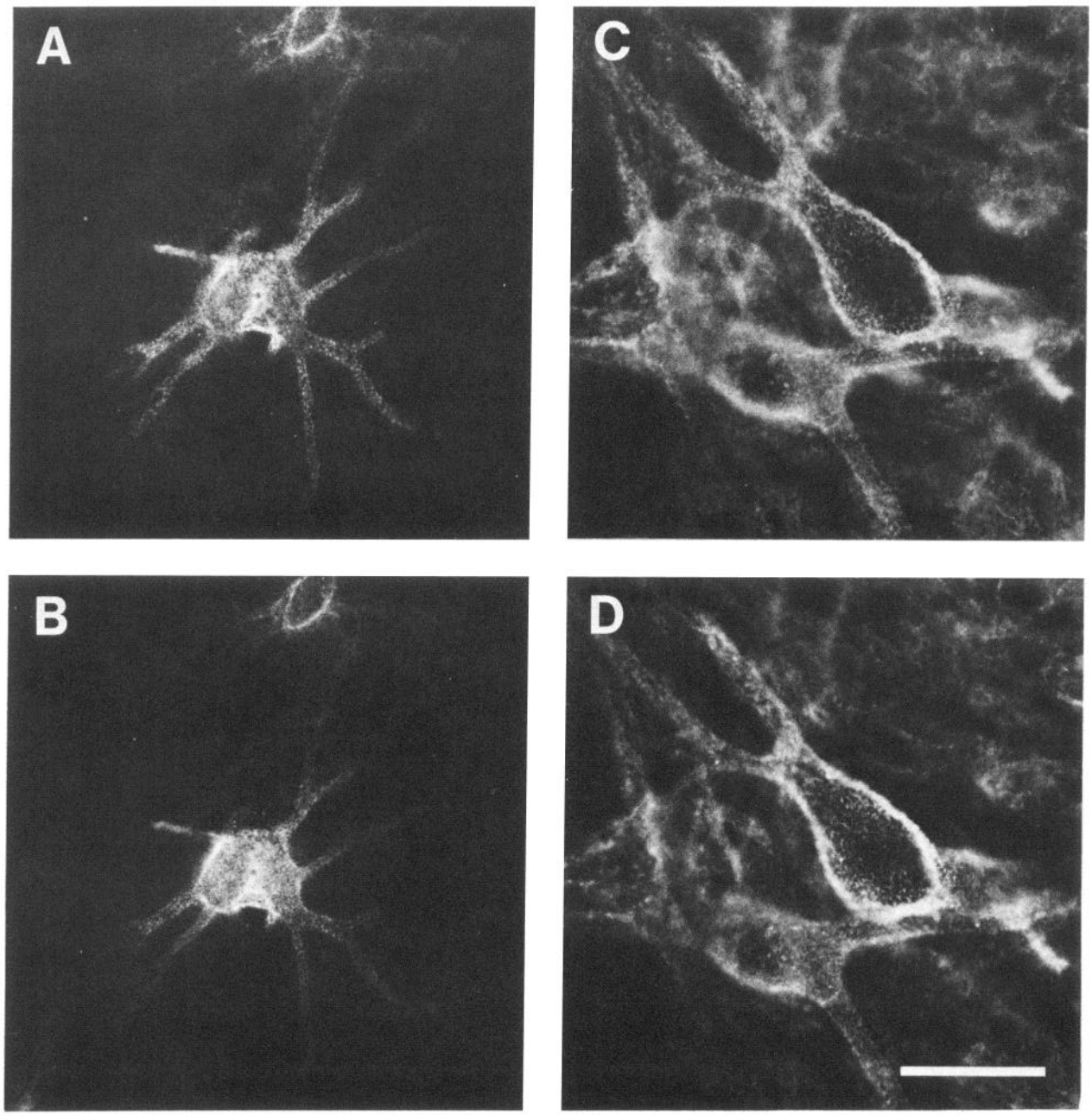


*Figure 1.* Cat-304 and Cat-301 stain the same subset of neurons in CNS: double-labeled neurons in cat cortical area 17 (*A, B*) and spinal cord (*C, D*). Neurons were double-labeled with Cat-304 (*A, C*) and Cat 301 (*B, C*) using subclass-specific secondary antibodies (Texas Red-anti-IgM for Cat-304 and FITC-anti-IgG for Cat-301). Both antibodies label the same population of neurons throughout the CNS, with the same discontinuous pattern of staining over the surface of cell bodies and proximal dendrites. Scale bar = 50 $\mu$m.

the mice were immunized with LGN from normal cats. Hybridomas of interest were selected by differential screening on sections of LGN from normal and dark-reared cats. This strategy resulted in the generation of monoclonal antibodies that recognized antigens in LGN from normal animals that are less abundant in LGN from dark-reared animals. One of these, like monoclonal antibody Cat-301, recognized a surface-associated antigen on a subset of neurons in the cat CNS and has been named Cat-304. Monoclonal antibody Cat-304 stained neurons with a discontinuous pattern over the surface of cell bodies and proximal dendrites (Fig.1), the same pattern described for Cat-301 (Hockfield and McKay, 1983).

Cat-304 is an immunoglobulin of the IgM subclass, which allowed us to perform double-labeling experiments with Cat-301 (an IgG) and subclass-specific secondary antibodies. In these experiments, Cat-304 and Cat-301 double-labeled the same population of neurons throughout the cat CNS. Examples of double-labeled neurons from cortical area 17 and from the spinal cord are shown in Figure 1. In every area of the cat CNS examined thus far (including cerebral cortex, thalamus, brainstem, cerebellum, and spinal cord), all neurons positive for one antibody were also positive for the other. These results suggested that both antibodies either bind to the same antigen or recognize different antigens that have identical surface localizations and

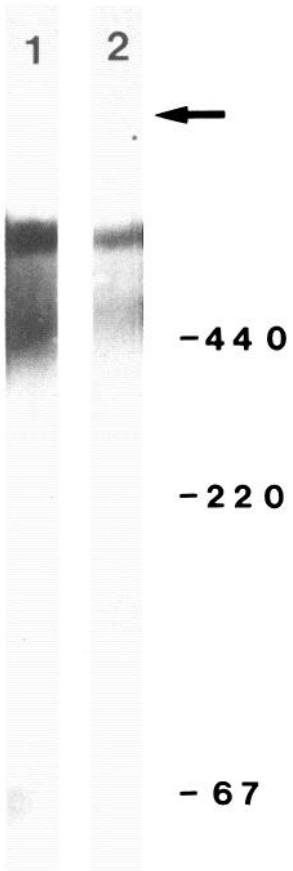


*Figure 2.* Immunoprecipitation shows Cat-304 and Cat-301 recognize the same molecule. Extracts of cat cortical membrane preparations were purified over an affinity column of Cat-301 covalently coupled to Protein G-Sepharose beads. The affinity-purified fraction was electrophoresed on 3–8% gradient SDS-polyacrylamide gels, transferred to nitrocellulose, and stained with Cat-301 (*lane 1*) or Cat-304 (*lane 2*). On the Western blots, both antibodies recognize the same affinity-purified antigen with an apparent molecular weight of 680 kDa. The *arrow* indicates the interphase between the resolving and stacking gels. The biochemical procedures are described in detail in Zaremba et al. (1989).

are co-expressed on the same set of neurons. Several lines of evidence suggested that the antibodies recognize the same antigen but at distinct epitopes.

### *Cat-304 and Cat-301 recognize different epitopes on the same molecule*

On Western blots of cat cortical membrane preparations, Cat-301 and Cat-304 each recognized a broad band of approximately 680 kDa apparent molecular weight (Zaremba et al., 1989). Figure 2 shows that the 680-kDa antigen purified on a Cat-301-antibody affinity column reacted with both Cat-301 (lane 1) and Cat-304 (lane 2). Affinity purification with Cat-304 followed by Western blotting with Cat-301 and Cat-304 gave the same results. Both antibodies, therefore, recognized the same affinity-purified antigen.

Enzymatic deglycosylations provided further evidence that Cat-301 and Cat-304 recognize the same antigen. Previous work has shown that the Cat-301 antigen is a chondroitin sulfate proteoglycan (Zaremba et al., 1989). After treatment of partially purified antigen with chondroitinase ABC, the 680-kDa antigen

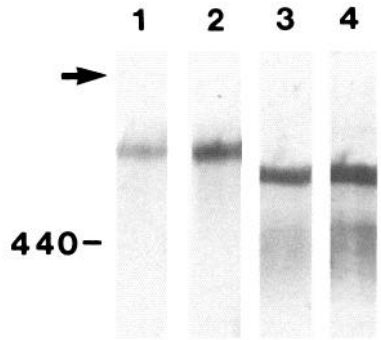


*Figure 3.* The Cat-304 and Cat-301 antigens digested with chondroitinase. Western blots of partially purified antigen (Zaremba et al., 1989) from cat cortical membrane preparations reacted with Cat-301 (*lane 1*) and Cat-304 (*lane 2*). *Lanes 3* (Cat-301) and *4* (Cat 304) show samples of the antigen incubated overnight at 37°C with 50 mU chondroitinase ABC in the presence of protease inhibitors (50 μg/ml pepstatin A and 1 mM PMSF), as described previously (Zaremba et al., 1989). In controls (*lanes 1, 2*) samples of the antigen were incubated in the presence of inactivated enzyme. Digestion with chondroitinase produces a shift in the migration of the antigen but does not destroy the immunoreactivity. Both antibodies recognize the same deglycosylated peptide with an apparent molecular weight of 580 kDa.

was no longer seen on Western blots with Cat-301 or Cat-304 (Fig. 3). Instead, a new band appeared at an apparent molecular weight of 580 kDa that stained with both Cat-301 and Cat-304. The persistence of immunoreactivity following digestion indicated that neither Cat-301 nor Cat-304 recognized the intact glycosaminoglycan component of the proteoglycan.

Several lines of immunochemical and immunohistochemical evidence indicate that, though Cat-304 and Cat-301 recognize the same 680-kDa chondroitin sulfate proteoglycan, they do so at distinct epitopes. First, on Western blots of cat cortical membranes, Cat-304 recognized a 50-kDa band that was neither recognized by Cat-301 (Zaremba et al., 1989) nor immunoprecipitated by Cat-301 from radioiodinated preparations (S. Zaremba and S. Hockfield, unpublished observations). Second, though Cat-301 recognized a closely related, surface-associated proteoglycan in the rodent (Kalb and Hockfield, 1988, 1990), monkey (Hockfield et al., 1983; DeYoe et al., 1987, 1990) and human (Hockfield et al., 1990), Cat-304 showed no cross-reactivity in other species. Third, binding of the antibodies in the double-label immunohistochemical studies was not competitive. Sections incubated in excess of either Cat-301 or Cat-304 did not subsequently show diminished staining with the other antibody. Together, these data indicate that Cat-301 and Cat-304 recognize different epitopes on the same molecule.

### *Dark rearing reduces the number and size of antibody-positive neurons in cat LGN*

In the LGN of normal cats (Fig. 4*A*), Cat-304 recognized neurons in layers A, A1, and dorsal C, in the interlaminar zones and in the medial interlaminar nucleus. The same distribution of antibody-positive cells was observed with Cat-301 (Fig. 4*C*), as previously reported (Hockfield et al., 1983; Hendry et al., 1984; Sur et al., 1988). Detailed analyses have shown that the number of Cat-301-positive neurons in layer A1 is somewhat higher than in layer A (Sur et al., 1988; Hockfield and Sur, 1990), and this was also observed with Cat-304 (Fig. 4,*A, C*). Cats dark reared from birth showed a dramatic reduction in the number

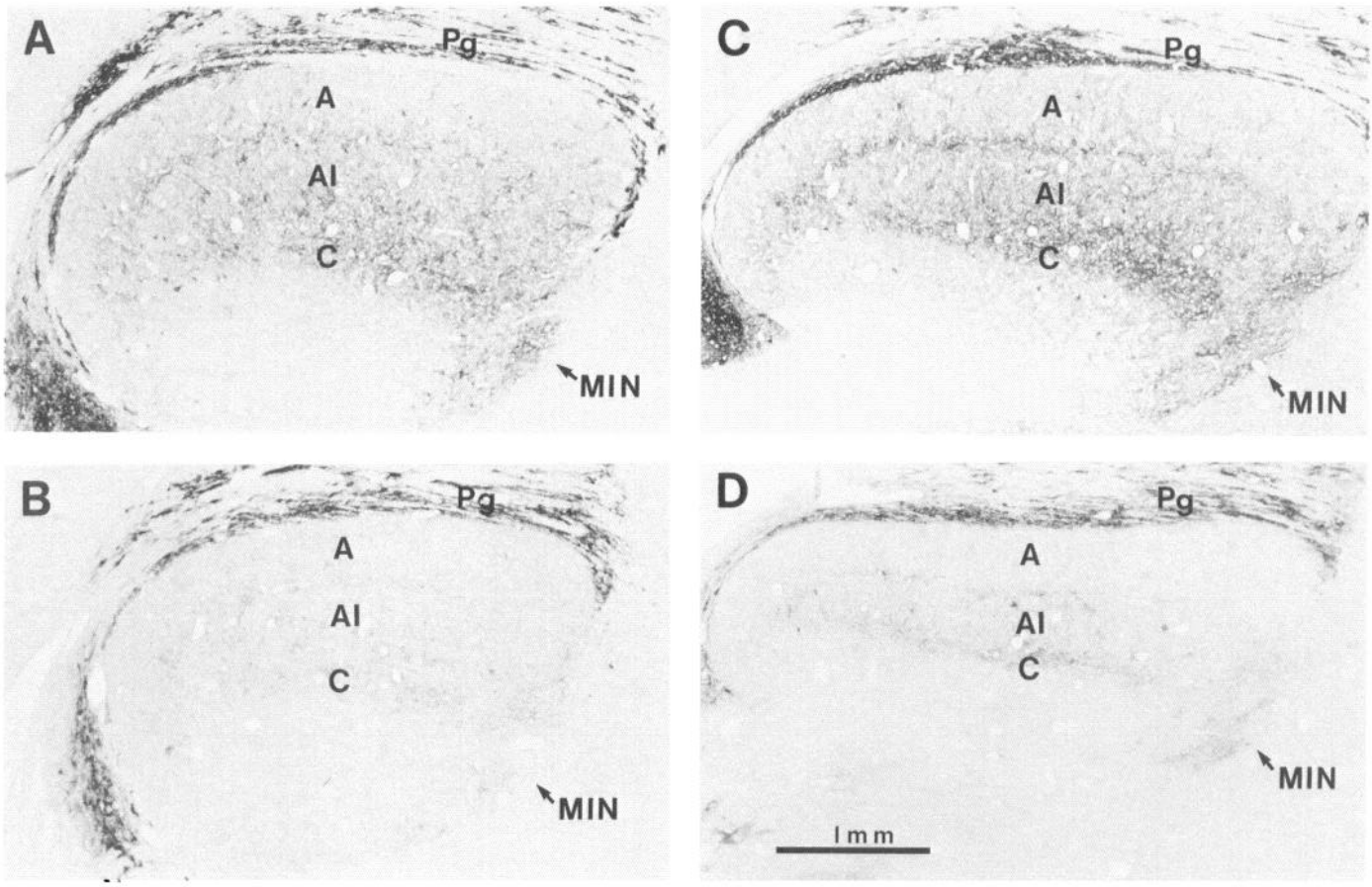


*Figure 4.* Dark rearing reduces the number of antibody-positive neurons in cat LGN. Coronal sections through the LGN of normal (*A, C*) and dark-reared (*DR*) cats (*B, D*) stained with Cat-304 (*A, B*) or Cat-301 (*C, D*). In the normal LGN, cells that stain with Cat-304 (*A*) and Cat-301 (*B*) are found in layers *A, A1,* and *C,* in the interlaminar zones, in the medial interlaminar nucleus (*MIN*), and in the perigeniculate nucleus (*Pg*) that lies dorsal to the LGN. Coronal sections of the dark-reared cat LGN stained with Cat-304 (*B*) and Cat-301 (*D*) show a marked reduction of antibody-positive neurons in all layers of the LGN and in the MIN. Staining of neurons in the perigeniculate nucleus is not affected by visual deprivation. Scale bar, 1 mm.

of Cat-304- (Fig. 4*B*) and Cat-301- (Fig. 4*D*) positive neurons throughout the LGN. The medial interlaminar nucleus, which also contains a large number of antibody-positive neurons in normal cats, showed a substantial reduction in immunoreactivity in dark-reared animals (Fig. 4). In LGN from normally reared cats, Cat-304- and Cat-301-positive neurons represented 11–14% of the total neuronal population, while in LGN from dark-reared cats, antibody-positive neurons represented 2.5–3.5% of the total neuronal population. Dark rearing, therefore, reduced the number of antibody-positive neurons in the LGN by approximately 75% (Fig. 5). The perigeniculate nucleus served as an internal control for the reduction in levels of immunoreactivity. Perigeniculate neurons were stained intensely with both antibodies in normally reared animals (Fig. 4*A,C*), and this immunoreactivity was not reduced following dark rearing (Fig. 4*B,D*).

The cross-sectional area of Cat-304- and Cat-301-positive neurons in LGN from normal and dark-reared cats was measured, as was the area of a sample of cells identified by staining for Nissl substance (Fig. 6). Figure 6*A* is a cell-size frequency histogram of Cat-304-positive neurons from the LGN of normal and dark-reared cats. The same distribution of cell sizes was observed for Cat-301 (Fig. 6*B*). Neurons labeled with either Cat-304 or Cat-301 were larger in sections from normal LGN, ranging from 228.58 to 1167.96 $\mu m^2$ in cross-sectional area, with a mean of 596.02 ± 19.12 $\mu m^2$ for Cat-304 and 600.69 ± 18.15 $\mu m^2$ for Cat 301 (Figs. 6, 7). The labeled neurons from the LGN of dark-reared animals were smaller, ranging from 104.34 to 827.11 $\mu m^2$, with a mean of 339.42 ± 13.88 and 389.12 ± 15.44 $\mu m^2$ for Cat-304 and Cat-301, respectively (Figs. 6, 7). The average size of antibody-positive neurons in dark-reared animals was 40–43% less than in normally reared animals. Measurements of Nissl-stained neurons showed that the mean size of all neurons in normally reared cats was 253.07 ± 14.37 $\mu m^2$ and in dark-reared cats was 200.27 ± 6.26 $\mu m^2$ (Fig. 7). This represented a reduction of 20% in overall cell size in the LGN from dark-reared animals, substantially less than that observed for antibody-positive cells. These results suggested that either (1) dark rearing produced a reduction in the size of the remaining antibody-positive neurons, or (2) the smallest population of antibody-positive neurons preferentially retained immunoreactivity following dark rearing.

### *Dark rearing reduces the number and size of antibody-positive neurons in cat visual cortex*

In cortical area 17 of normally reared cats, Cat-304-positive neurons were densely distributed in 2 bands: a superficial band in layer IV and a deep band in layers V and VI. Labeled neurons

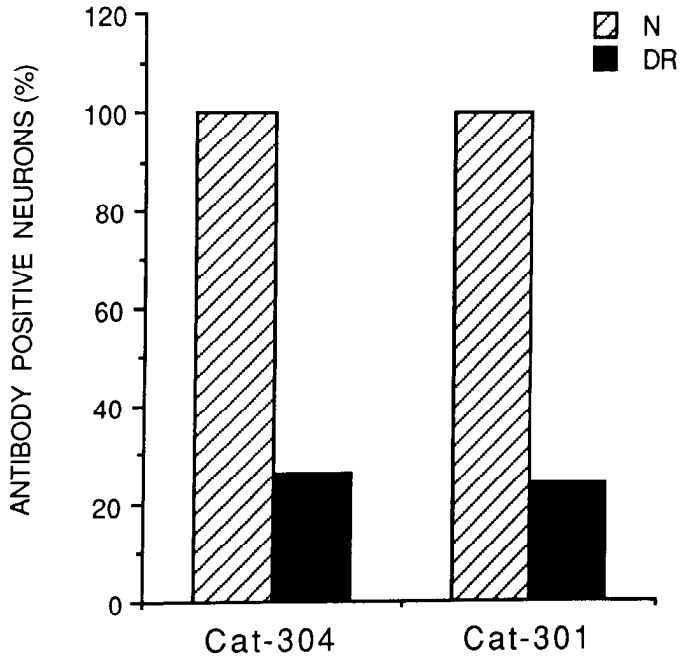


*Figure 5.* Density of antibody-positive neurons reduced in LGN following dark rearing. Coronal sections of LGN stained with Cat-304 and Cat-301 were counterstained with thionin, and neurons with well-defined nucleoli were counted. Density of positive neurons in dark-reared cats (*solid bars*) is compared to normally reared cats (*cross-hatched bars*) and expressed as % of the value from normally reared cats. In normal LGN, Cat-304- and Cat-301-positive cells represent 11–14% of the total neuronal population. Dark-rearing reduces the number of antibody-positive neurons in LGN by 75%. Two dark-reared and 2 normally reared animals were analyzed.

were also present in layers II and III, but at a lower density (Figs. 8*A*, 9*A*). Cortical area 17 of dark-reared cats contained fewer Cat-304-positive neurons than normal, with a marked reduction in layers II and III and in layers V and VI (Figs. 8*B*, 9*B*). Parallel distribution patterns were observed with Cat-301 (Fig. 9*C,D*).

In normal animals, Cat-304- and Cat-301-positive neurons represented 5–10% of the total neuronal population of layers II/III and V/VI and 10–20% of the total neuronal population of layer IV. Area 17 from dark-reared animals showed a reduction in the number of labeled neurons in these layers (Fig. 9*B,D*). The loss of Cat-304- and Cat-301-positive neurons following visual deprivation was most pronounced in layers II/III (74–77% reduction) and V/VI (68–70.5% reduction) compared to that observed in layer IV (24.5% reduction; Fig. 10).

The cross-sectional area of Cat-304- and Cat-301-positive neurons in each layer of cortical area 17 of normal and dark-reared cats was measured. In normally reared cats, the cell-size frequency distribution of Cat-304- (Fig. 11*A*) and Cat-301- (Fig. 11*B*) positive neurons was similar throughout area 17 (open bars), with the mean size ranging from 235.17 to 298.54 $\mu m^2$. The Cat-304- and Cat-301-labeled neurons in area 17 from dark-reared animals were smaller, as indicated by the shift in the cell-size distribution (filled bars), with the mean size ranging from 146.53 to 190.44 $\mu m^2$. This indicated that, in dark-reared animals, the average size of antibody-positive neurons was reduced by 37%. Unlike the differential reduction in the number of labeled neurons between cortical layers, the reduction in cell size following dark rearing was equivalent in all layers.

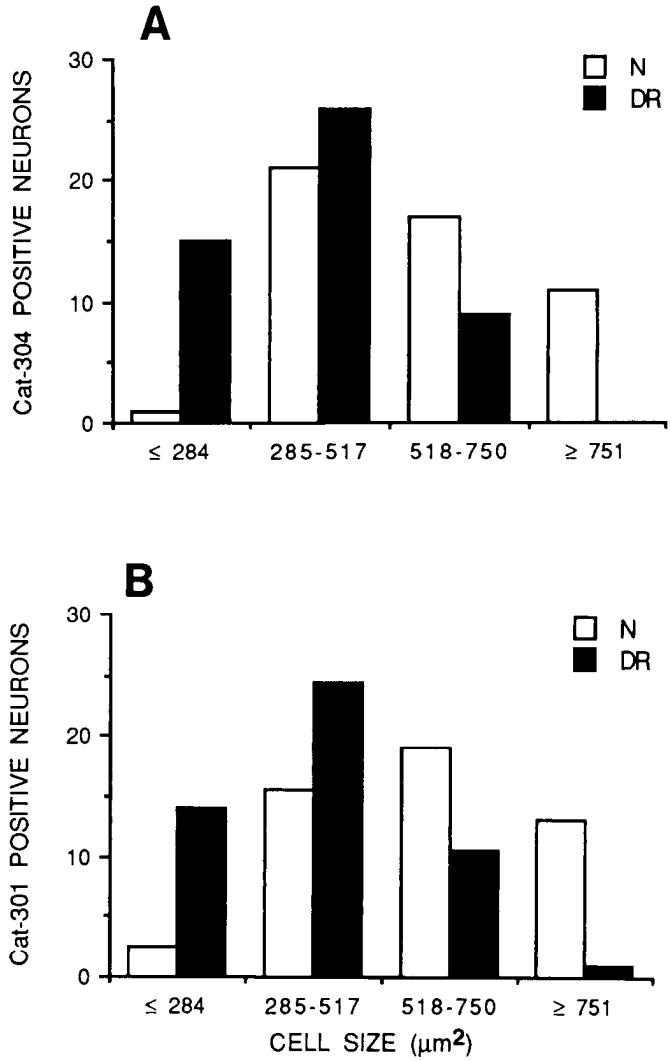


*Figure 6.* Cell-size distribution of antibody-positive neurons in normal and dark-reared LGN. Histograms illustrating the cross-sectional area of Cat-304-positive (*A*) and Cat-301-positive (*B*) neurons in normal (*open bars*) and dark-reared (*solid bars*) LGN. The cross-sectional area of 50 antibody-positive cells was measured through layers A, A1, and C in the binocular segment of LGN of normal and dark-reared cats. Two animals were used for each experimental condition. The distribution of antibody-positive neurons in dark-reared animals is shifted to the left, demonstrating the reduction in cell size in deprived cat LGN. A minimum of 50 cells were measured in each of 2 animals for each condition.

As an internal control for the effects of visual deprivation, antibody-stained pyramidal cells in the hippocampus were also measured. In the same sections in which cells in area 17 were measured and counted, the cross-sectional area of 50 antibody-positive pyramidal cells in the hippocampus was measured and was found not to be different, with a mean value of 539.12 ± 19.65 $\mu m^2$ in normal and 536.14 ± 19.15 $\mu m^2$ in dark-reared cats.

## Discussion

A major focus of this laboratory has been the identification and characterization of experience-dependent molecules in the mammalian CNS. Our previous work showed that Cat-301 immunoreactivity is dramatically reduced in the LGN from dark-reared cats compared to normally reared animals (Sur et al.,

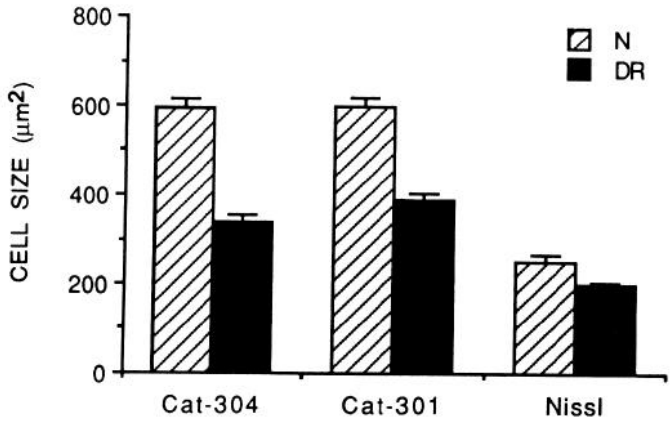


*Figure 7.* Cat-304- and Cat-301-positive neuron size in dark-reared versus normal LGN. This histogram illustrates the cross-sectional area of Cat-304- and Cat-301-positive neurons in normal (*cross-hatched bars*) and dark-reared (*solid bars*) LGN. The size of Cat-304- and Cat-301-positive neurons in dark-reared LGN is smaller than in normally reared LGN. Each *bar* represents the mean value from data shown in Figure 6. In adjacent sections stained for Nissl substance, neurons were measured from both experimental conditions, and a small difference in the cell size was observed, indicating that dark rearing most significantly affects the subset of neurons recognized by Cat-304 and Cat-301. Vertical lines indicate SEM.

1988). We have employed an immune suppression strategy (Hockfield, 1987) to generate a new monoclonal antibody, Cat-304, which also exhibits differential staining of neurons in the LGN and visual cortex. The histological and biochemical studies reported here demonstrate that Cat-304 recognizes a distinct epitope on the previously described neuronal surface-associated proteoglycan identified by monoclonal antibody Cat-301.

We have previously reported that levels of Cat-301 immunoreactivity in the CNS are dependent on an appropriate pattern of neuronal activity during a circumscribed period in early postnatal development. Disruption of normal patterns of activity leads to a reduction in Cat-301 immunoreactivity in the cat LGN (Sur et al., 1988) and in the hamster spinal cord (Kalb and Hockfield, 1988, 1989, 1990). However, it could not be determined whether the observed reduction in immunoreactivity reflected a reduction in levels of the Cat-301 proteoglycan itself or a reduction or masking of the Cat-301 epitope. The generation of another monoclonal antibody to a distinct epitope on the Cat-301 proteoglycan has permitted us to address this issue.

Double-label immunofluorescence shows that, in all areas of the cat CNS examined, Cat-304 and Cat-301 recognize the same population of neurons. The reduction in staining following visual deprivation is the same with both antibodies, demonstrating that the epitopes recognized by both antibodies are regulated by early visual experience. Our biochemical studies presented here and in a previous paper (Zaremba et al., 1989) indicate that Cat-301 and Cat-304 recognize independent epitopes on the polypeptide core of a chondroitin sulfate proteoglycan. In another study, early visual deprivation was shown to regulate the state of phosphorylation of a microtubule-associated protein (Aoki and Siekevitz, 1985), but not the expression of that protein. Our results strongly suggest that the reduction in immunoreactivity we observe consequent to visual deprivation reflects a reduction in the expression of the Cat-301/Cat-304 proteoglycan itself and not simply a loss or masking of the

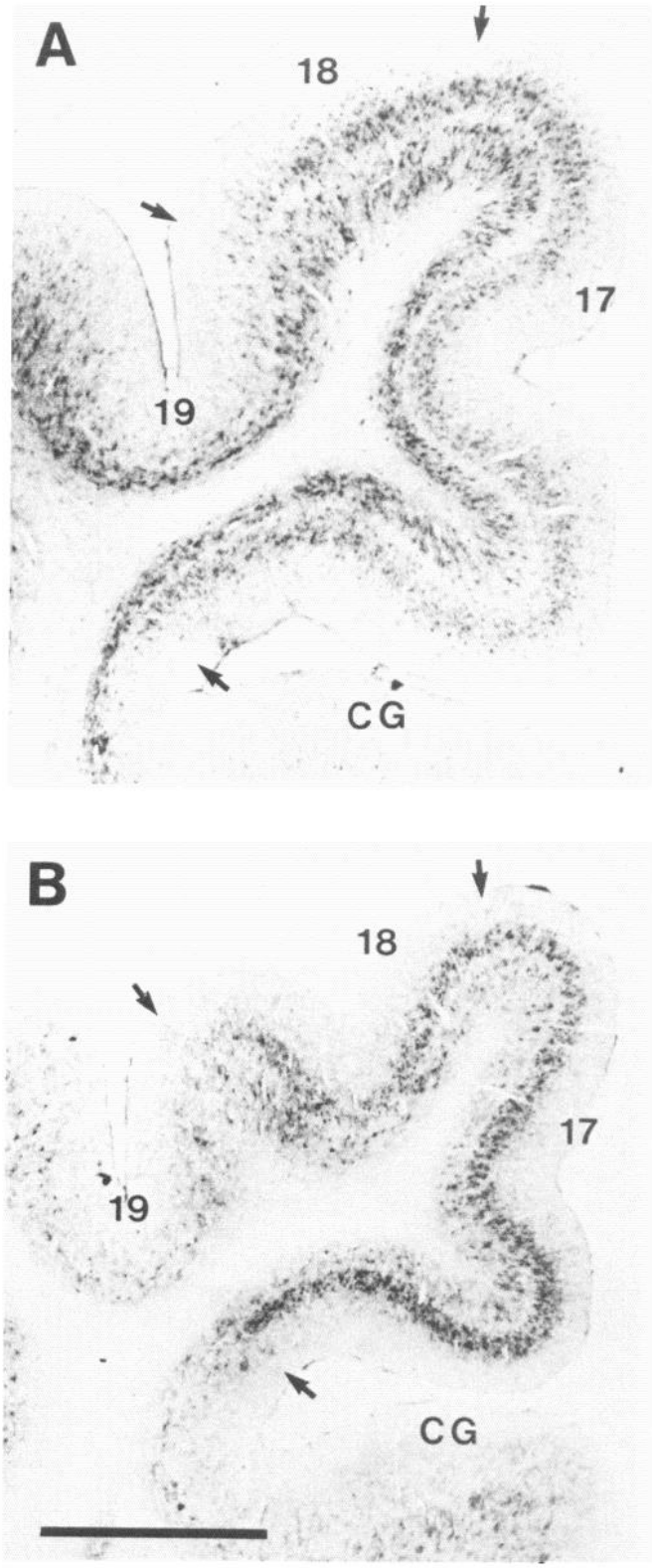


*Figure 8.* Dark rearing reduces the number of antibody-positive neurons in visual cortex. Coronal sections through cortical area 17 of normal (*A*) and dark-reared (*B*) cats stained with Cat-304. In normally reared cats (*A*), Cat-304-positive neurons are densely distributed in 2 bands: a superficial band in layer IV and a deep band in layers V and VI. Labeled neurons are also present in layers II and III. Cortical area 17 of dark-reared cats (*B*) contains fewer antibody-positive neurons than normal, with a noticeable reduction in layers II/III and V/VI. *Arrows* indicate the position of the borders between adjacent cortical areas. Scale bar, 2.5 mm.

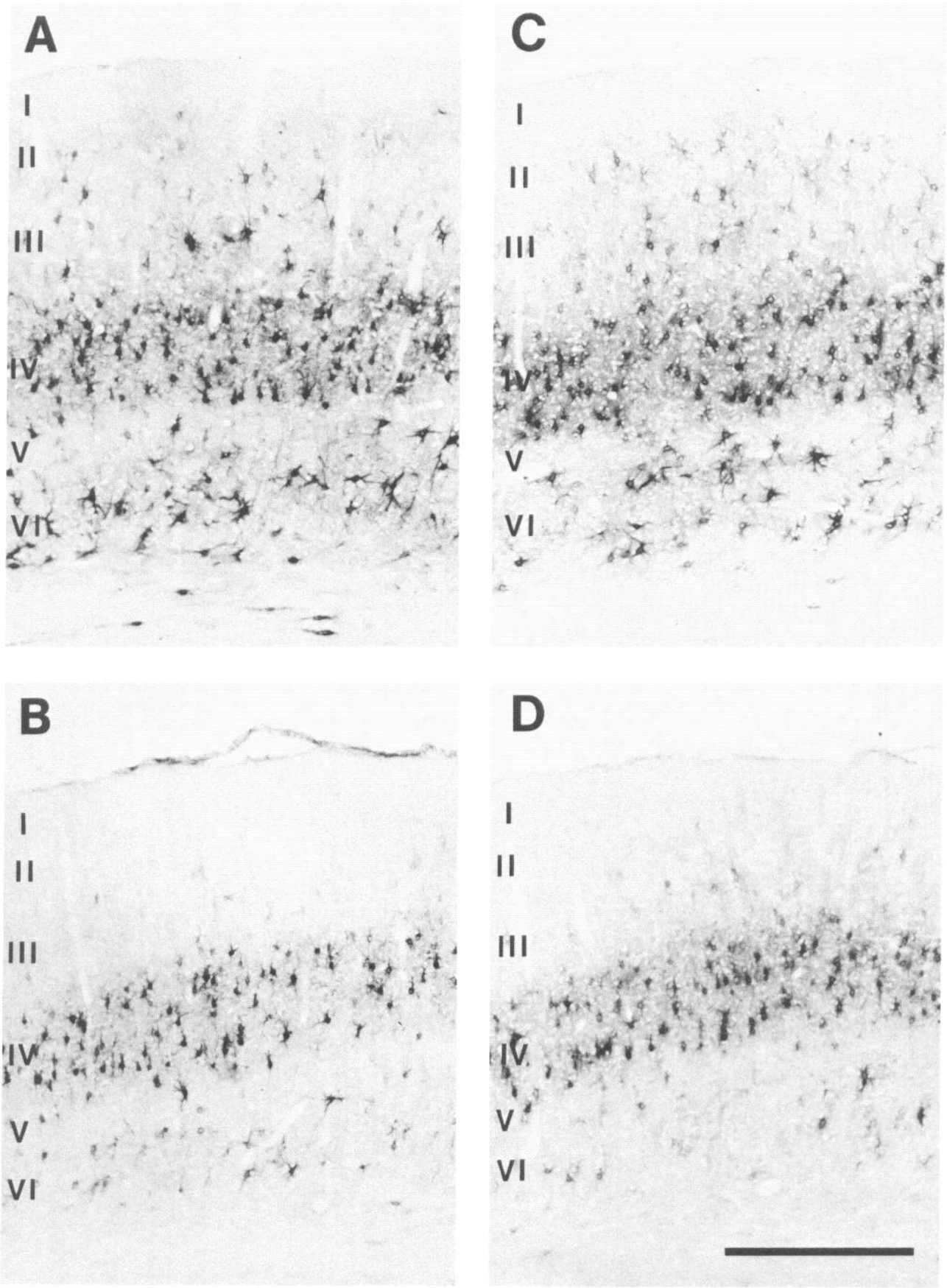


*Figure 9.* Higher magnification of area 17 stained with monoclonal antibodies Cat-304 and Cat-301. Micrographs of Cat-304-positive (*A*) and Cat-301-positive (*C*) neurons in cortical area 17 from a normally reared cat show a dense population of neurons in layers IV and V/VI. In normal cats, area 17 also contains a significant population of labeled neurons in layers II/III. Area 17 from dark-reared animals (*B, D*) shows a pronounced reduction in the number of Cat-304-positive (*B*) and Cat-301-positive (*D*) neurons in layers II/III and V/VI. Scale bar, 0.5 mm.

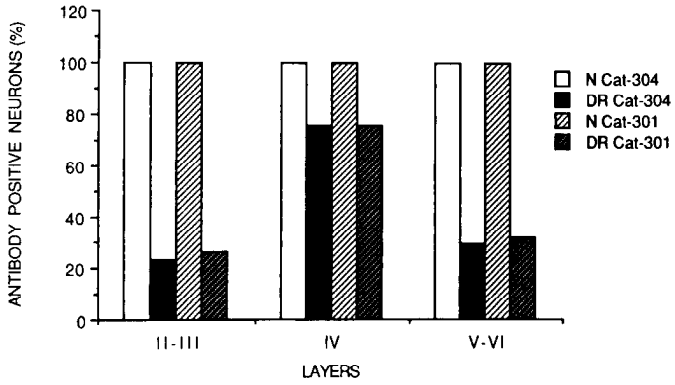


*Figure 10.* Density of antibody-positive cells in area 17 reduced following dark rearing. Density of antibody-positive neurons in area 17 from dark-reared (*DR*) cats was compared to area 17 from normally reared (*N*) cats and expressed as % of the normal value. In normal animals, Cat-304- and Cat-301-positive neurons represent 5–10% of the total neuronal population of layers II/III and V/VI and 10–20% of the population of layer IV. The loss of Cat-304- and Cat-301-positive neurons is most pronounced in layers II/III and V/VI. Three dark-reared and 3 normally reared animals were analyzed.

epitope. The regulation of protein expression has important implications for the cellular mechanisms that might mediate activity-dependent changes in neuronal phenotype.

### *Dark-rearing-induced changes in LGN*

Monoclonal antibodies Cat-304 and Cat-301 recognize 11–15% of the neurons in the LGN from normal cats. Multiple criteria indicate that Cat-301-positive LGN neurons are class I, or Y-cells (Hockfield et al., 1983; Hendry et al., 1984; Sur et al., 1988; Hockfield and Sur, 1990). In the LGN of dark-reared cats, the numbers of Cat-304- and Cat-301-positive neurons are reduced in parallel, by approximately 75%. This reduction in the number of antibody-positive neurons corresponds to the previously reported reduction in the number of physiologically identified Y-cells in the LGN of dark-reared cats (Kratz et al., 1979; Mower et al., 1981a, b; Kratz, 1982). The small number of antibody-positive cells remaining in the LGN of dark-reared animals may represent the few electrophysiologically identified Y-cells with normal spatial and temporal properties present in visually deprived animals (Kratz et al., 1979; Mower et al., 1981a, b; Friedlander et al., 1982; Kratz, 1982).

In addition to the reduction in the number of antibody-positive neurons, we also show here that the size of the remaining

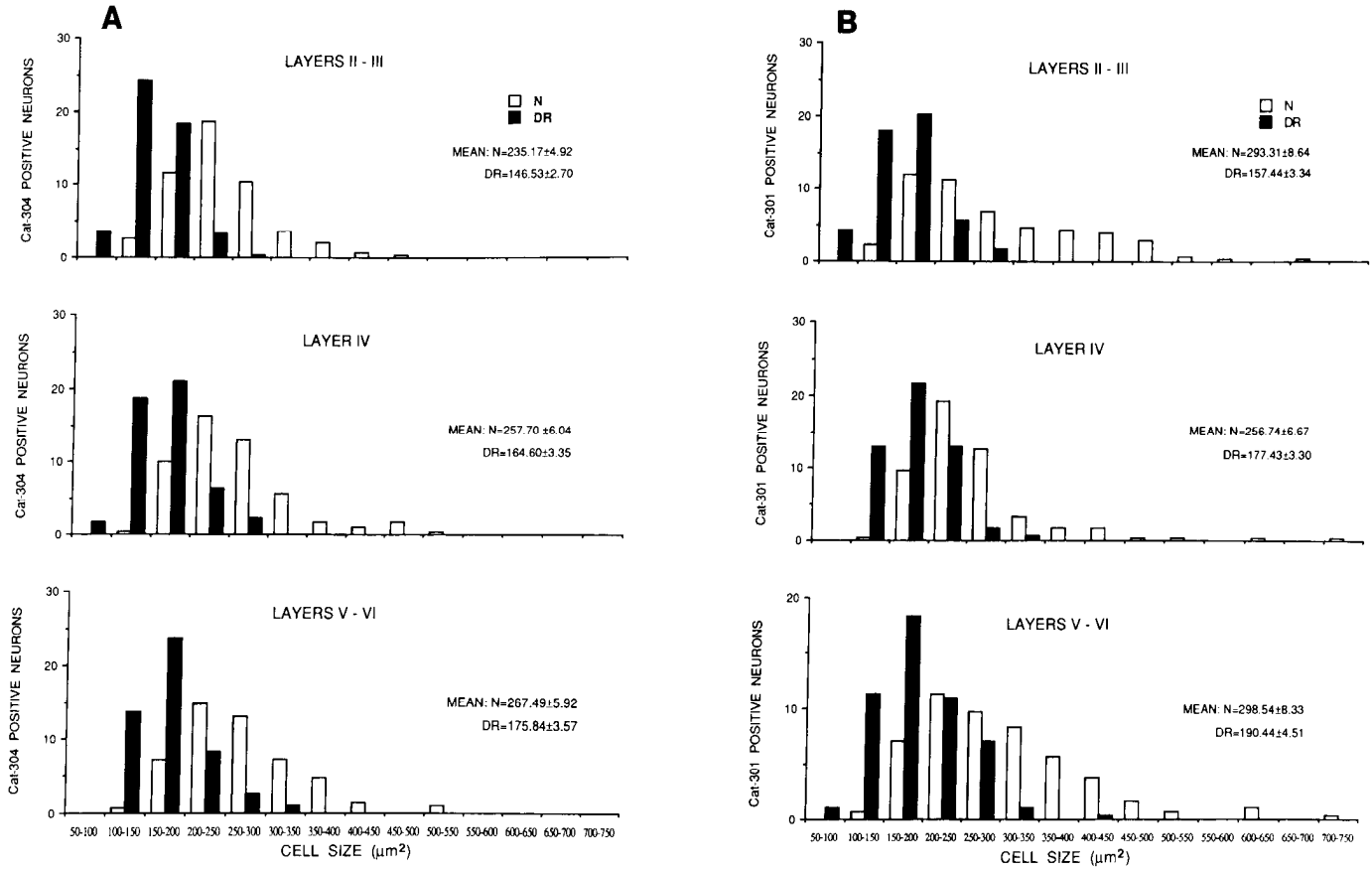


*Figure 11.* Cat-304- and Cat-301-positive neuron size in visual cortex from dark-reared and normal cats. These histograms illustrate the cross-sectional area of Cat-304-positive (*A*) and Cat-301-positive (*B*) neurons in area 17 from normal (*open bars*) and dark-reared (*solid bars*) cats. The cross-sectional area of 50 Cat-304- and 50 Cat-301-positive neurons in each layer of cortical area 17 of normal and dark-reared cats was measured. The distribution of cell sizes in normal animals is similar throughout area 17. In dark-reared animals, the cells are smaller, as indicated by the shift to the left in the cell size distribution. A minimum of 50 cells in each layer of area 17 were measured in 3 animals for each rearing condition.

Cat-304- and Cat-301-positive neurons in the LGN of dark-reared cats is smaller than in the LGN from normally reared cats. A selective reduction in size of LGN Y-cells after dark rearing has not been previously demonstrated. However, Friedlander et al. (1982) have shown that, following monocular deprivation, the few physiologically normal Y-cells identified in deprived layers have cell bodies significantly smaller than normal. Other studies comparing cell sizes in normal and dark-reared cat LGN assayed the total population of neurons by staining for Nissl substance. Kratz et al. (1979) reported that the loss of electrophysiologically identified Y-cells in cats raised from birth to 16–18 weeks in complete darkness occurs without a change in cell size. In another set of experiments, Kalil (1978) reported that dark rearing transiently affects the growth of LGN neurons. The reduction in cell size is maximal at 12 weeks (approximately 25% less than normal), but when dark rearing was extended beyond 3 months, the mean cell size recovered almost completely to normal. In the present study, we also found, based on Nissl staining, a reduction in overall cell size of 20% in 90-d-old, dark-reared cats. However, by using antibodies to assess the effects of visual deprivation on a molecularly distinct subset of neurons, a more profound effect was observed. Immunostaining with Cat-304 and Cat-301 shows a 40–43% reduction in cell size of a subpopulation of neurons, presumably the remaining Y-cells. This is consistent with the conclusions of Friedlander et al. (1982), who suggest that the reduction in cell size observed after monocular deprivation is due to a selective effect on Y-cells.

*Dark-rearing-induced changes in visual cortex*

In cortical area 17 of normal cats, Cat-304- and Cat-301-positive neurons are densely distributed in 2 bands: a superficial band in layer IV and a deep band in layers V and VI. Labeled neurons are also present, but at a lower density, in layers II and III. Dark rearing results in a reduction in the number of antibody-positive neurons in area 17, with a more pronounced reduction in layers II/III and V/VI than in layer IV. The size of antibody-positive neurons is also decreased following dark rearing, by approximately 65%, and this decrement is observed in all layers.

The differential reduction in the number of Cat-301-/Cat-304-positive neurons among layers is intriguing when considered in the context of reports of differential plasticity between cortical layers. Several studies have shown that dark rearing prolongs the physiological susceptibility to monocular deprivation beyond the normally defined critical period (Cynader and Mitchell, 1980; Mower et al., 1981a, b, 1983; Cynader, 1983; Mower and Christen, 1985). If an animal is subjected to monocular deprivation after it is reared in a normal visual environment for 3 months, little ocular dominance shift to the open eye is observed. However, if an animal is subjected to monocular deprivation after it has been dark reared for 3 months (or longer), a clear ocular dominance shift toward the open eye is observed. Significantly, this shift is most pronounced *outside* of layer IV; a large proportion of cells recorded in layer IV (>70%) remain responsive to the closed eye, while in layers II, III, V, and VI, most cells are driven predominantly by the open eye (Mower and Christen, 1985). Dark rearing also has no effect on benzodiazepine receptors in layer IV (Shaw et al., 1987). This suggests that neurons in layer IV (in contrast to those in other layers) largely do *not* remain in a plastic state beyond the normal critical period as a result of dark rearing.

The expression of the Cat-301/Cat-304 proteoglycan is experience dependent in the cat LGN; visual deprivation during the first 3 months of life produces an irreversible loss of immunoreactivity and an irreversible change in electrophysiological properties of LGN Y-cells (Sur et al., 1988). Our present observations, that dark rearing leads to a reduction in the number of antibody-positive cells in visual cortical layers II/III and V/VI, may similarly reflect an irreversible change in molecular and physiological properties of cortical neurons. An alternative explanation consistent with the studies described above is that dark rearing delays development of neurons in cortical layers II/III and V/VI (but not layer IV). Neurons in layers outside of layer IV may remain in an immature, plastic state, which is reflected in a delay in Cat-301/Cat-304 expression. One might then predict that visual experience after dark rearing would induce normal levels of proteoglycan expression in layers II/III and V/VI. To resolve this question, we are currently examining Cat-301/Cat-304 expression in the visual cortex of animals dark reared from birth, then brought into the light for an extended period of time.

The observed reduction in cell size in the LGN and throughout the layers of the visual cortex is, at present, difficult to interpret. Previous studies have not shown that the Y-cells that remain in the LGN after dark rearing are different from those found in normally reared animals (see Sherman and Spear, 1982). However, because electrophysiological properties are reported as a range for each cell type and because analysis depends on adequate sample representation, subtle differences within a subpopulation might escape detection. Such changes might be revealed by more detailed analyses of these preparations.

The results presented here, together with our previous studies, indicate that early sensory experience regulates the expression of specific surface-associated molecules. The regulation of these molecular species correlates with the acquisition of mature anatomical and physiological properties of central visual neurons. We have previously suggested that the expression of the Cat-301/Cat-304 proteoglycan may be involved in stabilizing the mature synaptic structure of neurons (Hockfield et al., 1989; Zaremba et al., 1989). Proteoglycans in other tissues have been suggested to play a role in stabilizing tissue structure as adhesive barrier molecules (see Hockfield, 1990). The characterization of molecular changes that correlate with significant periods in development may further our understanding of the molecular basis of activity-dependent alterations in neuronal phenotype.

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