Effects of Monocular Enucleation on Parvalbumin in Rat Visual System during Postnatal Development

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PURPOSE. To re-evaluate the hypothesis that the expression of the calcium-binding protein parvalbumin (PV) in a subpopulation of γ-aminobutyric acid (GABA)ergic neurons is an appropriate molecular marker for the effect on ocular dominance plasticity of monocular deprivation during the postnatal sensitive period.

METHODS. Long-Evans rats underwent monocular enucleation immediately before eye opening (postnatal day [P] 14). Immunohistochemical analysis using anti-PV antibody was performed on the superior colliculus (SC) and lateral geniculate nucleus (LGN) at P45. In the visual cortex (VC) developmental changes in immunoreactivity were also examined at the ages of P17, P20, P27, and P45. Northern blot analysis for PV mRNA was also performed at P45. Changes in PV expression in the visual system of these rats were evaluated by use of a computer-based quantitative technique.

RESULTS. PV-immunoreactive neurons were present in the SC and VC, whereas only a few were found in the LGN. The monocular enucleation at the onset of the sensitive period markedly reduced PV immunoreactivity in the neuropil of the SC, contralateral to the enucleated eye when examined one month later. No consistent and significant change in PV immunoreactivity was found in either the LGN or the VC. The number of PV-immunoreactive neurons in the VC rapidly decreased to the adult level during the middle of the sensitive period. The expression of PV mRNA in these central visual structures was not affected by early monocular enucleation.

CONCLUSIONS. Expression of PV is developmentally regulated, and marked changes in its protein expression in the SC can be induced by monocular enucleation. Contrary to the original hypothesis, monocular enucleation did not consistently affect the expression of PV in the rat VC. The expression of PV is probably regulated by multiple factors, not merely by binocular competition. (Invest Ophthalmol Vis Sci. 1999;40:2535–2545)

It is well known that visual experience early in life affects the elaborate functional architecture of the central visual structures. Closure of one eye during the early postnatal period (i.e., the sensitive period) results in a reduction in the number of binocular cells in the visual cortex (VC; ocular dominance plasticity).1 Animals deprived of vision in one eye have therefore been regarded as a good model of pattern-deprivation amblyopia. Many efforts have been made to establish effective treatment of amblyopia by use of this animal model.2–6 For this effort, it is important to elucidate the molecular mechanisms for maintaining a high level of cortical plasticity during the sensitive period.

Previous in vitro studies have suggested that intracellular Ca2+ could play important roles as a second messenger in the regulation of synaptic plasticity in the VC.7,8 As a substance that either modulates or mediates the actions of Ca2+, many neurons contain a variety of cytosolic calcium-binding proteins such as parvalbumin (PV), calbindin D-28k, and calretinin.9 It is therefore possible that these calcium-binding proteins also play an important role in the regulation of synaptic plasticity. Among these, PV is known to possess functions of Ca2+ buffering and to be widely distributed in the central nervous system.9,10 It has been reported that PV is associated with fast-firing neurons11 and is localized in a subpopulation of γ-aminobutyric acid (GABA)ergic neurons that represent 50.8% of the GABAergic neurons in the rat VC.12 Although various experimental manipulations, including the injection of kainic acid13 and occlusion of blood flow14 were performed to examine the changes in expression of PV, it is still unclear how the expression of PV is regulated in the central visual structures.

Interestingly, Cellerino et al.15 showed a reduction of PV immunoreactivity only in the binocular region (Oc1B) of the rat VC after monocular deprivation during the sensitive period. They proposed an intriguing hypothesis that PV in the VC is involved in the regulation of ocular dominance plasticity. Because PV immunoreactivity significantly and selectively decreased in the binocular region of the VC, they concluded that the reduction in PV immunoreactivity was not simply caused by decreasedafferent activity but resulted from binocular competition. Binocular competition, a process of axons from two eyes competing with each other for the limited amount of trophic factors and/or synaptic sites in the VC, remains a
leading idea for explaining experience-dependent modification of ocular dominance. Cellerino et al. therefore concluded that PV could be a suitable molecular marker for the modification in the VC.

However, in another study, investigators have shown no alteration in glutamic acid decarboxylase (GAD) immunoreactivity in the kitten VC after monocular deprivation or enucleation in the sensitive period. The study suggested that monocular deprivation during the sensitive period did not affect the synthesis of GABA in neurons, a large percentage of which should possess PV in the cytosol. To determine whether GAD and PV are regulated independently in the VC, we quantitatively examined the expression of PV and effects of monocular enucleation during the sensitive period in the central visual structures of the rat, including the primary VC. Some results of the present study have been presented in an abstract form.

METHODS

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental protocols were approved by the Animal Care and Use Committee of the Osaka Bioscience Institute.

Immunohistochemistry

Twelve Long–Evans rats underwent monocular enucleation (n = 9) or lid suturing (n = 3) under gas anesthesia (a mixture of 66% nitrous oxide, 32% oxygen, and 2% halothane) at postnatal day (P) 14, immediately before natural eye opening. The same number of litters were used as the control. At P17, P20, P27, or P45, the animals were deeply anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg) and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). In three additional animals, we used 4% paraformaldehyde in 0.2 M cacodylate-HCl containing 50 mM CaCl2 to examine the effects of Ca2+ in the fixative on immunoreactivity. The brains were removed and postfixed for 24 hours with the same fixative. Then the sections obtained at 250-50-μm thickness on a freezing microtome. The free-floating sections were reacted with ABC reagent for 45 minutes (Vectastain; Vector). After three washings (15 minutes each) in PBS, sections were transferred to 0.05 M Tris buffer and incubated with 0.05% dianisobenzidine tetrahydrochloride and 0.005% H2O2 to visualize the peroxidase reaction product. The reaction was stopped by transfer of the sections into Tris buffer. After several washings with PBS, the sections were mounted on glass slides, cleared, and coverslipped. The sections from the two hemispheres were always immunostained together to eliminate errors in the comparison caused by differences in processing. The sections nearest to the immunostained sections were always stained with thionine to examine the cortical laminar structures and the border of the monocular region (Oc1M) and binocular region (Oc1B).

Measurement of the Number of Neurons Immunoreactive for PV in the Primary VC

Oc1M and Oc1B in the rat primary VC were identified on Nissl-stained sections by the criteria of Reid and Juraska. Using a computer-based imaging system (Provis AX-HDTV; Olympus, Tokyo, Japan), we obtained immunostained images taken at a magnification of ×40, and printed them out as photographs (Pictography 3000; Fuji Film, Tokyo, Japan). We also input the data for locations of immunopositive cells into computer files by using a computer-based neuroanatomical analysis system (NeuroLucida; MicroBrightField, VT) and measured the number of immunopositive neurons by using software that accompanied the system (Neuroexplorer). We processed only strongly labeled neurons in our analysis. We set five regions of interest (ROIs; approximately 500-μm width from layer II to layer VI) slightly overlapping each other in the Oc1M and/or Oc1B and normalized the cell counts by an area of 0.5 mm². A comparison was made between control and deprived animals for the number of immunopositive neurons in monocular and binocular regions in both hemispheres of the VC. The difference between hemispheres in the number of immunopositive neurons of each region was evaluated statistically by use of the paired t-test. For ontogenic analysis and a comparison against naive control specimens, statistical comparisons were made using one-way analysis of variance. The Bonferroni–Dunn post hoc test was used for comparison when significance was indicated by analysis of variance.

Injection of Kainic Acid into the VC

Under gas anesthesia, animals (7 weeks of age; n = 4) were fixed in a stereotaxic apparatus, and a small hole was drilled over the VC (6.5 mm posterior to the bregma, 1.5 mm lateral to the midline). After the durotomy, a small amount (2 μl) of kainic acid solution (0.33 mg/ml of saline) was pressure-injected at a 1-mm depth from the brain surface by use of a glass micropipette. The animals were revived after the hole had been covered with a dental acrylic and the wound had been sutured closed. An antibiotic (ampicillin sodium, 20 mg/kg body weight) was injected subcutaneously daily during the survival period. Three days after the surgery, the animals were perfused as described previously for the immunohistochemical examination.

Northern Blot Analysis

Six Long–Evans rats that underwent monocular enucleation at P14 were killed by decapitation after 1 month. The brain was
rapidly removed from the skull, and the VC (posterior to the corpus callosum, 5 mm in width), superior colliculus (SC), and lateral geniculate nucleus (LGN) were dissected out. Total RNA was extracted from each brain tissue by the acid guanidine isothiocyanate-phenol chloroform method. Total mRNA (15 μg) was subjected to electrophoresis through 1% agarose-formaldehyde (0.44 M) gel and was transferred to a nylon filter (Hybond N, Amersham, Oakville, Canada) by electroblotting. The filter was prehybridized for 2 days at room temperature in prehybridization buffer containing 50% formamide, 5 × SSC, 50 mg/ml sheared and denatured salmon sperm DNA, and 5 × Denhardt’s solution. PV probe (gift from Paul Epstein) was radiolabeled with [32P]-α-deoxycytidine triphosphate (Amer- sham) by the random priming method (Pharmacia LKB Biotechnology, Piscataway, NJ). Hybridization was performed at 42°C in prehybridization buffer supplemented with a radiolabeled probe. Each filter was washed at 62°C in 0.2 × SSC and 0.1% sodium dodecyl sulfate solution for 15 minutes and exposed to x-ray films (duPont, Wilmington, DE) at −80°C for 2 to 4 days.

Evaluation of PV mRNA in the Rat Visual System

The autoradiographic signals corresponding to specific hybridization of PV mRNA were analyzed by use of a computer-based image analysis system (NIH Image, ver. 1.57; National Institutes of Health, Bethesda, MD). To normalize the densitometric values of signals, we calculated the ratio of the density of PV to that of the glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene, which was constantly expressed under the conditions of the current experiments, for each sample. The ratios were evaluated statistically by the paired t-test between the hemispheres ipsilateral and contralateral to the enucleated eye.

RESULTS

Effect after 1 Month of Monocular Enucleation on the Expression of PV in the SC

Previous physiological study has shown that the sensitive period of monocular deprivation in rat is from P14 to P42. In the present study, animals were thus subjected to monocular vision for 1 month during the entire sensitive period, and its effect was examined at P45. PV-immunoreactive neurons of various form and size were found throughout the layers of the SC (Figs. 1B, 1C). In control animals without manipulation of visual inputs, the staining of the neuropil was found in the superficial gray layers of the SC (Figs. 1A, 1B, 1C). A relatively smaller number of immunoreactive neurons were found in the most superficial gray layer of the normal SC (Figs. 1B, 1C). In the monocularly deprived, the size of the contralateral SC was reduced markedly by degeneration of input fibers (cf. Figs. 1A versus 1D, right). We also observed that the staining of the neuropil in the superficial gray layers of the SC was reduced markedly by degeneration of input fibers (cf. Figs. 1A versus 1D, right). In the monocularly deprived, the size of the contralateral SC was reduced markedly by degeneration of input fibers (cf. Figs. 1A versus 1D, right). We also observed that the staining of the neuropil in the superficial gray layers, which was highly reduced, but staining in the ipsilateral SC could not be readily discriminated from that in the control specimens (Figs. 1A, 1D, left). A faint band of staining was observed in the middle portion of the shrunken SC (Fig. 1D). In contrast, the number of immunoreactive neurons was increased in the superficial gray layer of the contralateral SC, although that in the intermediate and deeper nonvisual layers was not affected (Figs. 1E, 1F). In short, immunoreactivity in the SC was markedly downregulated after denervation of afferents for 1 month.

Effect of Monocular Enucleation on the Lateral Geniculate Nucleus

Only a few PV-immunoreactive neurons were found in the LGN of normal animals (Figs. 2A, 2B). Dense staining with terminal-
like dots was observed in the LGN, whereas bundles of retinal fibers that ran into the LGN showed no immunoreactivity (Fig. 2). In monocularly deprived animals, the LGN contralateral to the enucleated eye shrank markedly; however, no change in the pattern of PV immunoreactivity was found (Figs. 2C, 2D). The terminal-like dots and only a few immunopositive neurons were observed to a similar extent on both sides of the LGN (Figs. 2C, 2D).

**Effect of Monocular Enucleation on the Primary VC**

No change in the pattern of PV immunoreactivity was found in the primary VC of monocularly deprived animals. PV-immunonegative neurons were scattered evenly throughout the layers, except in layer I (Figs. 3A, 3B). The shape of PV-immunonegative neurons varied, taking bipolar, bitufted, and other forms (Fig. 3D). Stained immunoreactive fibers with appearance similar to terminal dots were clearly observed in layer I (Fig. 3C). The neuropil in layer II was most densely stained, and a faint band of staining in layer V was recognizable in the immunonegative neuropils throughout layers II-VI (Figs. 3A, 3B). All these staining patterns were indistinguishable from those in the control animals. To examine quantitatively the difference between the side contralateral to and the side ipsilateral to the monocularly enucleated eye, we counted the numbers of PV-immunonegative cells in the VC on photomicrographs. A quantitative comparison with the control animal was also performed.

In the rats that underwent monocular enucleation, on day P45 the number of PV-immunonegative neurons in the VC ipsilateral to the enucleated eye was 36.15 ± 1.25 (mean ± SD) cells/0.5 mm² in the Oc1M and 34.32 ± 3.19 cells/0.5 mm² in the Oc1B. The numbers of PV-immunonegative cells in the contralateral VC were 34.99 ± 1.08 cells/0.5 mm² in the Oc1M and 34.03 ± 2.00 cells/0.5 mm² in the Oc1B. Thus, we found no significant difference in the number of PV-immunonegative neurons between the right and left VC. These numbers of neurons immunonegative for PV were not different from those in the corresponding regions in the control VC (Table 1).

As a control, we stained a series of neighboring sections with an antibody against the protein of an immediate early gene, zif268 (Zif268). In naive animals Zif268-immunonegative neurons were found in layers II to VI of the VC. The density was highest in layer IV. We found in monocularly deprived animals that Zif268-immunonegativity was reduced markedly only in the VC contralateral to the enucleated eye (Figs. 4A, 4B). The reduction was markedly strong in layers II/III (Fig. 4B).

**Northern Blot Analysis**

To examine further the effects of monocular enucleation on the PV expression, we performed northern blot analysis by use of total RNA isolated from each hemisphere of the SC, LGN, and VC of rats subjected to monocular enucleation during the early postnatal period. PV mRNA was expressed constitutively in the SC, LGN, and VC of both deprived and undeprived control rats. Based on ribosomal 18s and 28s RNAs as markers, the size of PV mRNA was estimated to be approximately 0.7 kb, which corresponds to the previously reported mRNA size for this gene in rats.22 Next, we determined whether the expression of PV mRNA in the primary VC is dependent on neuronal activity driven by visual inputs. In this series of experiments, monocular enucleation was performed on the right eye. Therefore, in these rats, visual input to the left VC should be weaker than that to the right VC. However, as shown in Figures 5A and 5E, the expression of PV mRNA was not significantly different between the two hemispheres of the VC, even 1 month after monocular enucleation. Furthermore, no difference was found when these data were compared with those for the control animals without monocular enucleation. The same pattern of results was obtained for the SC and LGN (Figs. 5B, 5C). It is well known that the level of zif268 mRNA expression is regulated by visual inputs in the primary VC of the rat, cat, and monkey.23 We confirmed the effect of monocular enucleation on zif268 expression using the same blotted filters used for analyzing the PV mRNA. The expression of zif268 mRNA was significantly decreased in the left hemisphere of the VC compared with that in the right hemisphere of the VC (paired t-test, P < 0.05; Figs. 5A, 5D).

**Sporadic Reduction of PV Immunoreactivity**

Intriguingly, regions without PV immunoreactivity were found in various sizes (approximately 100-1500 μm width in the
coronal sections) and sporadically throughout the cerebral cortex. The sporadic reduction was found in 24 observations from 43 sections examined. The absence of PV-immunoreactive neurons was observed especially in layers II/III to IV (Fig. 6A). The stained fibers in layer I resembling terminal dots were also depleted in these regions, but the immunoreactive neuropil in layers II to VI was indistinguishable from that in the normal areas. Using antibodies against Zif268 protein or calbindin D-28k, a different calcium-binding protein, we stained sections neighboring those that contained the regions without PV-immunoreactive neurons. No reduction was found in the number of Zif268- or calbindin D-28k–immunoreactive neurons in the corresponding regions (Fig. 6B).

Additional Control Experiments for PV Immunoreactivity

It is reported that the concentration of Ca$^{2+}$ in the solution of preparations is critical for the detection of PV in samples. Therefore, we examined the effect of Ca$^{2+}$ change in the perfusion solution on the frequency of the sporadic reduction in PV immunoreactivity. We added 50 mM CaCl$_2$ to the perfusate and performed the usual immunostaining for PV. However, this degree of Ca$^{2+}$ change did not affect the occurrence of the sporadic reduction, in that we found the usual immunostaining.

Magloczky and Freund reported the selective loss of PV-immunopositive cells in the contralateral hippocampus after unilateral kainate injections into the CA3 subfield. So we examined this possibility in the rat VC. An amount of 2 ml of 0.33 mg/ml kainate solution was injected unilaterally into the VC of rats during the sensitive period. PV immunohistochemistry, however, showed a reduction in staining only in the region surrounding the toxin injection site (Fig. 7A). No consistent change in PV immunoreactivity was found in the opposite hemisphere of the VC (Fig. 7B).

Development of PV Immunoreactivity in the Monocularly Enucleated Rats

The presence of PV-immunopositive neurons was detectable in the VC of P17 rats (Figs. 8A, 8B). The PV-immunopositive neurons were observed in the layers from II/III to VI, and a band of dense staining of neuropil was clearly identified only in layer V (Figs. 8A, 8B). In P20 animals, the number of PV-immunopositive neurons was increased markedly, and the staining of the neuropil in layer II-VI was also increased, though the dense band in layer V was still visible (Figs. 8C, 8D; Table 2). The number of PV-immunopositive neurons at P17 and P20 were significantly larger than that in P27 and P45 (Table 2). By P27, the PV immunoreactivity reached the adult pattern (Figs. 8E, F). Little difference was found between P27 and P45 (Figs. 8G, H).

Table 1. Results of Quantitative Analysis of PV-Immunopositive Neurons in the VC on P45 after Monocular Enucleation at P14

<table>
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<tr>
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<th>Oc1M</th>
<th>Oc1B</th>
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<tr>
<td>Control</td>
<td>34.41 ± 2.97</td>
<td>34.80 ± 1.42</td>
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<tr>
<td>Ipsilateral enucleation</td>
<td>36.15 ± 1.25</td>
<td>34.32 ± 3.19</td>
</tr>
<tr>
<td>Contralateral</td>
<td>34.99 ± 1.08</td>
<td>34.03 ± 2.00</td>
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The mean ± SD in the ROI is shown in cells/0.5 square millimeters.
The PV-immunoreactive neuropil expanded throughout all layers (Figs. 8G, 8H). Both the cell size and number of PV-immunoreactive neurons were reduced when compared with those in rats of younger age (Figs. 8E, 8F versus 8A, 8B and 8C, 8D). Neither hemispheric difference nor difference between monocularly deprived and control animals in PV immunoreactivity was found at any stage examined during development.

**DISCUSSION**

In this study we evaluated whether the expression of a calcium-binding protein PV and its mRNA was modified in the rat VC by monocular enucleation during the sensitive period. A marked change in PV protein expression was found in the SC contralateral to the enucleated eye. Contrary to the original hypothesis, no significant change in its protein or mRNA was found in either the Oc1M or Oc1B of the primary VC. The characteristic cortical band of PV-immunostaining in cortical layer V was expanded to cover cortical layers II through VI with development. We showed that this developmental process was also not influenced by monocular enucleation.

**Changes in the Subcortical Structures**

In the present study, we confirmed previous findings\(^{25-27}\) that PV immunoreactivity was decreased and the cell density of PV-immunoreactive neurons was increased in the superficial layers of the SC, only on the side contralateral to the enucleated eye. Its expression in the ipsilateral SC was not affected by monocular enucleation. Additionally, we found here that in animals subjected to monocular enucleation, the expression of PV mRNA was not different between contralateral and ipsilateral sides of the SC. Because the number of immunopositive neurons was increased, some neurons had to enhance their expression of PV mRNA. Counteracting this increase, neurons that lost their dendritic arbors by deafferentation may have undergone a reduction in their PV mRNA expression. Another possibility is that removal of PV immunoreactivity in the neuropil of afferent fibers and terminals unmasked the outline of immunopositive neurons and thus seemingly increased the number of immunopositive neurons in the contralateral SC. Further analysis by use of in situ hybridization of PV mRNA is necessary to solve this problem.

It is interesting, in either case, that fetal or neonatal enucleation was shown not to change the relative proportion of PV cells in the contralateral superficial gray layer.\(^{28}\) Previously, we reported that monocular deprivation during the sensitive period reduced the binding activity of 3-adrenergic receptors in the contralateral SC.\(^{29}\) However, this reduction in receptor-binding activity was not found when monocular enucleation was performed during the first few days of postnatal development. We considered this to be caused by the stabilization of ipsilateral projections from the retina, which projections are usually eliminated by programmed cell death that takes place thereafter. The marked reduction in afferent activity may be compensated by this reorganization of afferents. Thus, it is possible that activity-dependent modification of expression takes place only during a limited period (i.e., the sensitive period) for a variety of molecules, including PV, in the rat SC.

In the LGN, both PV immunoreactivity and its mRNA expression were found to be stable, although the volume of contralateral LGN was decreased slightly by the monocular enucleation. Similar to a previous report\(^{25}\) our results indicated that the PV in the LGN was free from the effect of monocular enucleation.

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**Figure 4.** Reduction in the number of Zif268-immunopositive cells in the VC observed at P45 after monocular enucleation at P14. Coronal sections ipsilateral (A) and contralateral (B) to the enucleated eye were immunostained with an anti-Zif268 antibody. The reduction is prominent in layers II/III and layer V. Scale bar, 100 μm.
Changes in the Primary VC

Previous immunohistochemical studies showed that a subpopulation of GABAergic neurons in the primary VC of rodents, cats, and monkeys expressed PV. In the primary VC of the adult monkey, PV immunoreactivity in the neuropil was reported to be reduced in denervated ocular dominance columns after monocular enucleation, although no change was found in the number of immunoreactive neurons. Another group reported that PV immunoreactivity was reduced in the ocular dominance column that corresponded to the eye injected with tetrodotoxin. This reduction in PV immunoreactivity was observed even when monocular deprivation was performed at the adult stage. Therefore, the observed reduction was probably simply caused by the reduced activity in the functional columns, bearing no correlation with ocular dominance plasticity. In the rat, however, it has been reported that monocular lid suturing significantly decreases the numbers of PV-immunoreactive neurons and neuropil only in the Oc1B of the VC. In this case, it was considered that the reduction was not caused by decreased neuronal activity itself but resulted from binocular competition. These investigators also mentioned that PV immunoreactivity was a useful immunohistochemical marker for binocular competitive modification in the VC, because the visual cortices of dark-reared and binocularly deprived rats showed normal PV immunoreactivity. Our results, however, indicate that significant reduction of the number of PV-immunoreactive neurons was detected in neither the binocular nor monocular portions of the VC by monocular enucleation during the sensitive period. We further compared changes in PV-immunoreactive neurons induced by monocular lid suturing with those by enucleation and found no that difference was caused by the variation in the manipulation of visual afferents (data not shown). In neighboring sections, we constantly showed significant and restricted reduction in the expression of Zif268 after monocular enucleation. Therefore, we concluded that the expression of PV is independent from the neuronal activity or binocular competition, at least in the rat VC.

The results of the present study differ fundamentally from those reported for primates, in which PV immunoreactivity was significantly affected by the manipulation of visual inputs. This discrepancy is probably caused by species differences, which include an ordinarily smaller number of cortical projections through the LGN, the absence of functional columns, and differences in development of inhibitory systems in the rat VC.

Development

It has been reported that the first neurons immunopositive for PV appear in layer V of the VC at approximately P11. During
postnatal development, the pattern of PV immunoreactivity in the VC changed dramatically. In the early postnatal period until P17, PV-immunoreactive neuropils were found selectively in layer V. This band of immunoreactivity started to expand at approximately P20 and reached the adult pattern by P27. The pattern of development of PV immunoreactivity is different from that of GAD immunoreactivity, although PV-immunoreactive neurons probably represent a large percent of GAD-immunoreactive cells. It is therefore possible that the characteristic staining of fibers in layer I of the mature VC is derived from the dendrites and/or axons of neurons in layers II/III. Next, we examined the relation between the sporadic reduction in PV immunoreactivity and neuronal activity. When the sporadic reduction was found, the neighboring section was stained with anti-Zif268 antibody, and a comparison was made by superimposing the section of PV immunostaining on that of Zif268. An immediate early gene, zif268, is regulated in an activity-dependent manner. We confirmed that the number of Zif268-immunopositive neurons and the expression of zif268 mRNA were apparently decreased in the VC contralateral to the enucleated eye. However, normal staining of Zif268 was found in the corresponding region, where the spontaneous activity was not altered.

We could not find any difference in PV immunoreactivity between the VC ipsilateral and contralateral to the enucleated eye during development. Therefore, monocular enucleation does not appear to affect the development of PV immunoreactivity in the rat VC.

**Regulation of the Expression of PV**

We have shown that monocular enucleation during the sensitive period did not affect the expression of PV in the rat primary VC. However, we also noted that a sporadic reduction of PV immunoreactivity occurred not only in the occipital part but also widely in other parts of the cerebral cortex. Disappearance of PV-immunoreactive neurons was often observed in the supragranular layers, and this was always accompanied by the loss of staining in layer I. It is therefore possible that the characteristic staining of fibers in layer I of the mature VC is derived from the dendrites and/or axons of neurons in layers II/III. Next, we examined the relation between the sporadic reduction in PV immunoreactivity and neuronal activity. When the sporadic reduction was found, the neighboring section was stained with anti-Zif268 antibody, and a comparison was made by superimposing the section of PV immunostaining on that of Zif268. An immediate early gene, zif268, is regulated in an activity-dependent manner. We confirmed that the number of Zif268-immunopositive neurons and the expression of zif268 mRNA were apparently decreased in the VC contralateral to the enucleated eye. However, normal staining of Zif268 was found in the corresponding region, where the spontaneous activity was not altered.

The number of PV-immunoreactive neurons increased until P20 and then markedly decreased, showing a nearly adult pattern by P27. These results correspond to previous reports in which PV-immunopositive neurons first appeared in layer V and showed a center-to-outside gradient. It also should be mentioned that the number of PV-immunopositive cells peaked at a relatively earlier phase of the sensitive period and decreased to the adult level in the middle of the sensitive period, suggesting that this molecule does not directly regulate ocular dominance plasticity. The sharp decline within a relatively short time window (1 week between P20 and P27) suggests that molecular switching takes place in a subpopulation of inhibitory neurons during this postnatal third week in the VC.
ous disappearance of PV immunoreactivity was observed in the neighboring section. In addition to the fact that no consistent change in PV immunoreactivity was induced in the Oc1M where Zif268 was significantly reduced after monocular enucleation, these results indicate that the sporadic reduction in PV immunoreactivity was not caused by a decrease in neuronal activity in a local region in the cerebral cortex, suggesting an activity-independent regulation of the PV-expression in the rat VC.

We showed that an increase in Ca$^{2+}$ concentrations (50 mM) in the perfusion solution induced no significant changes in the immunostaining of PV. This manipulation did not affect the frequency of the sporadic reduction in PV immunoreactivity, suggesting that a change in the local concentration of Ca$^{2+}$ was not the primary reason for the sporadic reduction of PV immunoreactivity.

A previous report$^{13}$ indicated that the selective loss of PV-immunopositive cells in the contralateral hippocampus is induced after unilateral kainate injections into the CA3 subfield. Damage to a distant subpopulation of nonpyramidal cells was proposed as a mechanism of cell death in human temporal lobe epilepsy. Our examination of this possibility in the rat VC, however, showed a slight reduction in staining only in the region surrounding the toxin injection site. No consistent change in PV immunoreactivity was found in the opposite hemisphere of the VC. It is therefore unlikely that the sporadic reduction was caused by selective cell death in a subpopulation of GABAergic cells in the VC.

Although we have concluded that neither neuronal activity nor local Ca$^{2+}$ concentration is the primary reason for the sporadic reduction in PV immunoreactivity, it is still unclear how the expression of PV is regulated in the rat VC. It is possible that multiple factors that interact with each other, including the two mentioned, may play an important role in the regulation of PV expression.

**Functional Implication**

It is well known that the GABAergic system plays an important role in the regulation of ocular dominance plasticity. Infusion of bicuculline a GABA$_{	ext{A}}$ receptor antagonist, into the kitten VC prevents the usual shift in the ocular dominance toward the normal opened eye after monocular deprivation (i.e., blockade of ocular dominance plasticity$^{39}$). The restoration of input from the deprived eye could occur after the blockade of GABA receptor function by the application of bicuculline.$^{40}$ It was also reported that monocular lid suturing or enucleation reduces the number of GABA and GAD immunoreactive neurons in deprived-eye dominance columns of adult monkeys.$^{41}$ A previous study reported that in the rat VC, monocular deprivation led to an increase in the number of GABA$_{	ext{A}}$ receptors in the deeper layers in the sensitive period.$^{42}$ This study suggested that binocular competition induces a reduction in the release of GABA in the rat VC (but see also Bear et al.$^{16}$).

It has been reported that approximately 37% of the GABAergic neurons in the cat VC contain PV.$^{30}$ Approximately 70% of GAD-immunopositive neurons in rat somatosensory cortex$^{43}$ and approximately 50.8% in rat VC$^{12}$ have been re-

### Table 2. Changes in the Number of PV-Immunopositive Neurons in the VC during Development after Monocular Enucleation at P14

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<th>P20</th>
<th>P27</th>
<th>P45</th>
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<tr>
<td>Ipsilateral</td>
<td>89.58 ± 20.72</td>
<td>63.80 ± 16.48$^*$</td>
<td>26.47 ± 5.57‡§</td>
<td>33.88 ± 12.26‡§</td>
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<tr>
<td>Contralateral</td>
<td>99.20 ± 19.84</td>
<td>79.85 ± 16.42‡</td>
<td>24.55 ± 8.39‡§</td>
<td>29.13 ± 9.07‡§</td>
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The mean ± SD in the ROI is shown in cells/0.5 square millimeters.

$^*$ $P < 0.05$ versus P17.

$^† P < 0.01$ versus P17.

$^‡ P < 0.001$ versus P17.

$^§ P < 0.0001$ versus P17.
ported to be PV immunopositive. These studies showed that PV-immunopositive cells occupy a relatively high proportion of GABAergic cells in the VC. Therefore, this correlation may imply that, as originally hypothesized by Cellerino et al.,15 PV plays an important role in the regulation of ocular dominance plasticity and that monocular deprivation during the sensitive period affects the PV expression selectively in the binocular region of the VC. However, our quantitative study showed that monocular enucleation or eyelid suturing during the sensitive period did not always affect the expression of PV in the rat VC. It is possible that the level of the PV is kept constant by strict regulatory mechanisms and/or that the involvement of the GABAergic system in the regulation of ocular dominance plasticity is mainly by a subpopulation of neurons that do not express PV.

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