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CRE-Mediated Gene Transcription in Neocortical Neuronal Plasticity during the Developmental Critical Period

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Summary

Neuronal activity–dependent processes are believed to mediate the formation of synaptic connections during neocortical development, but the underlying intracellular mechanisms are not known. In the visual system, altering the pattern of visually driven neuronal activity by monocular deprivation induces cortical synaptic rearrangement during a postnatal developmental window, the critical period. Here, using transgenic mice carrying a CRE–*lacZ* reporter, we demonstrate that a calcium- and cAMP-regulated signaling pathway is activated following monocular deprivation. We find that monocular deprivation leads to an induction of CRE-mediated *lacZ* expression in the visual cortex preceding the onset of physiologic plasticity, and this induction is dramatically downregulated following the end of the critical period. These results suggest that CRE-dependent coordinate regulation of a network of genes may control physiologic plasticity during postnatal neocortical development.

Introduction

The development of adaptive behaviors and sensory processing functions of the mammalian brain relies on the precise development of neural synaptic connections in the cerebral neocortex. Although the functional neuronal circuits within the brain are modified by experience throughout the life of the organism, it is during early postnatal life that intrinsic and experiential factors determine the basic organization of these synaptic connections (reviewed by Buonomano and Merzenich, 1998). The development of the mammalian central visual system reflects these general themes. In the kitten, the most well-studied system, rudimentary patterns of synaptic connections and visual processing circuits in the cortex initially arise independent of visual input, but their maintenance and further development are dependent on visual experience after birth (Hubel and Wiesel, 1963; Blakemore and Van Sluyters, 1975; Crair et al., 1998).

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In all mammals so far examined, there is a period early in life when changes in visual experience profoundly alter the patterns of neuronal connections in the visual cortex (reviewed by Katz and Shatz, 1996; Hubel and Wiesel, 1998). During this "critical period," deprivation of visual input from one eye of the animal leads to a rapid and dramatic shift in the responses of cortical neurons toward the nondeprived eye (Wiesel and Hubel, 1963; Blakemore et al., 1978; Drager, 1978; Gordon and Stryker, 1996). Similar deprivations in mature animals have no effect. A wealth of neurophysiologic data strongly suggests that this ocular dominance shift is dependent on correlated patterns of activity between thalamic and cortical neurons, and involves competition between inputs from the two eyes for cortical synaptic representation (reviewed by Miller, 1996).

The molecular mechanisms underlying this developmental neuronal plasticity remain unclear, nor is it known why neuronal plasticity in the cortex declines with maturation. By infusing pharmacologic substances into the visual cortex, it has been shown that various agents can block neuronal plasticity or prevent the normal development of ocular dominance columns. Substances that have been shown to interfere with visual neuronal plasticity include antagonists of the neuromodulators acetylcholine (Bear and Singer, 1986; Gu and Singer, 1993), norepinephrine (Kasamatsu and Pettigrew, 1976), and serotonin (Gu and Singer, 1995). Also, agents that block neuronal activity in the visual cortex prevent normal cortical plasticity (Rauschecker and Singer, 1979; Shaw and Cynader, 1984; Hata and Stryker, 1994). More recently, exogenous neurotrophic factors (brain-derived neurotrophic factor [BDNF] or neurotrophin 4 [NT-4]) infused into the cortex of kittens have been found to interfere with the development of ocular dominance columns and prevent plasticity during monocular deprivation (Cabelli et al., 1995; Galuske et al., 1996). Exogenous nerve growth factor (NGF) also has been reported to interfere with visual plasticity in rodents (Domenici et al., 1991).

The mechanisms through which these exogenous agents interfere with cortical development and plasticity are not known, but presumably they interfere with intracellular signaling pathways that convert changes in visual experience into long-lasting changes in patterns of synaptic strength. One possible molecular target of the signal transduction pathways that function during visual cortical plasticity is the transcription factor CREB (calcium/cAMP response element binding protein). CREB transcription factors are members of a family of ubiquitously expressed transcription factors (CREB/ATF family) that regulate transcription via their specific DNA target, the calcium/cAMP response element (CRE) (Brindle and Montminy, 1992; Sassone-Corsi, 1995). CREB activity is regulated by phosphorylation in response to diverse physiologic signals. Importantly, neuronal activity (via calcium influx) (Ghosh and Greenberg, 1995; Bito et al., 1996; Impey et al., 1996) and neurotrophic factors (Ginty et al., 1994; Finkbeiner et al., 1997) are major physiologic regulators of CREB function.

CREB-mediated transcription is thought to be critical

for a variety of adaptive neuronal responses. It has been shown to be important for memory and learning in a number of systems, including *Aplysia*, *Drosophila*, and mouse (reviewed by Martin and Kandel, 1996; Silva et al., 1998). Mice completely lacking CREB are not viable (Rudolph et al., 1998), but mutant mice with reduced CREB levels show a partial impairment of long-term memory (Bourtchuladze et al., 1994; Kogan et al., 1996). In addition, long-term potentiation (LTP) of synapses in hippocampal slices from these mice is reduced (Bourtchuladze et al., 1994). The important gene regulatory functions of CREB, together with its role in integrating important physiologic signals, suggest that gene expression via CREs might also be important for neuronal plasticity during development of the visual neocortex.

To examine the role of CRE-mediated gene transcription in visual cortical plasticity, we have utilized transgenic mice that carry a *lacZ* reporter construct driven by CREs. This artificial reporter construct contains multiple copies of consensus CRE elements placed in front of a minimal promoter fused to *lacZ*. Previous work using cultured neurons and brain slices from these mice has demonstrated that the inserted CRE–*lacZ* reporter is regulated by neuronal activity and by substances that enhance intracellular calcium and cAMP, and is blocked by voltage-sensitive calcium channel inhibitors (Impey et al., 1996). In the present study, using these mice, we have examined CRE-mediated transcription in vivo during neocortical plasticity. Following monocular visual deprivation (MD), a procedure that induces synaptic reorganization in the visual cortex during the critical period, we find robust activation of CRE–*lacZ* transcription in the visual cortex. Furthermore, we observe a dramatic downregulation of CRE–*lacZ* expression in the visual cortex following the end of the critical period. These results suggest that CRE-regulated transcription of gene networks plays a central role in physiologic neuronal plasticity during postnatal development of the visual cortex.

Results

Induction of CRE-Mediated Transcription in Visual Cortex following Monocular Visual Deprivation of Critical Period–Age Mice

Following MD, neurons in the primary visual cortex gradually shift their responsiveness toward the nondeprived eye. This process requires several days of continued MD for its effect. In mice, no change in neuronal ocular dominance is seen after 1 day of MD, while a partial shift is observed after 2 days, and at 4 days the effect is maximal (Gordon and Stryker, 1996). We expect that the signal transduction pathways that mediate the effects of MD would be activated well before the first physiologic effects are seen. Therefore, in the initial experiments, we deprived critical period–age CRE–*lacZ* mice for 24 hr and then assayed the effects on CRE-dependent transcription by detection of the *lacZ* reporter product.

The levels of *lacZ* expression were determined in histologic sections by indirect immunofluorescence. As shown in the confocal microscope images, in control CRE–*lacZ* mice there is a relatively small number of *lacZ*-immunopositive cells in the visual cortex, and they are distributed equally between the right and left hemispheres (Figures 1A and 1B). Following MD, there is a robust increase in the number of *lacZ*-immunopositive cells, especially strongly labeled cells, in the primary visual cortex (V1) contralateral to the open eye (Figure 1C). In comparison, in every section we find many fewer *lacZ*-immunoreactive cells in V1 ipsilateral to the open eye (Figure 1D). It is important to note that in the mouse V1 primarily receives projections from the contralateral eye, and that the medial 2/3 of V1 is driven exclusively by the contralateral eye. Even within the binocular zone, the responses through the contralateral eye predominate (Drager, 1978; Gordon and Stryker, 1996). The confocal images shown correspond to an area in the middle of the visual cortex that includes primarily monocular zone and possibly some binocular zone. Their locations in the visual cortex are indicated by boxes in Nissl-stained images of the same section (staining done after confocal imaging, Figures 1G and 1H). To determine the laminar distribution of the *lacZ*-immunopositive cells, the section shown in Figures 1C and 1D was stained with ethidium bromide (a fluorescent nucleic acid dye that labels every cell) to identify the cortical layers. As shown in Figures 1E and 1F, the cortical layers are clearly visible with ethidium bromide staining, and, when compared to the *lacZ* immunofluorescence pattern, show that the *lacZ*-immunoreactive cells are distributed in all of the layers with the exception of layer 1 (see also Figure 3). The heaviest concentration of *lacZ*-immunoreactive cells is in layer 4. Interestingly, only a subset of cortical cells show *lacZ* immunoreactivity, with a range of intensities, while many cells are not labeled.

There is considerable interanimal variability in the number of *lacZ*-immunoreactive cells, both in the control and in the deprived animals. Sections of visual cortex from the same animal, however, give highly consistent results using our *lacZ* immunostaining procedure. To quantitate this interanimal variability, we counted cells moderately to intensely labeled above background levels within a 450 μm wide (tangential) area of visual cortex in a coronal section. These results are shown in Figure 1I. There is an average 8-fold increase in the median number of moderately to strongly *lacZ*-immunoreactive cells with MD, when comparing between V1 contralateral to the open eye (of deprived animals) to V1 of control animals ($p < 0.04$, Mann–Whitney U test). In addition, we find that the hemisphere contralateral to the open eye has 4-fold more labeled cells compared to the ipsilateral hemisphere of the same sections ($p < 0.04$, Wilcoxon sign-rank test). The ipsilateral hemisphere receives primarily input from the closed eye and is labeled only slightly higher than controls.

In contrast to the effects of MD, binocular visual deprivation (BD), a condition that does not result in physiologic plasticity (Wiesel and Hubel, 1965; Gordon and Stryker, 1996), also does not activate CRE-mediated transcription in the visual cortex. These results are summarized in Figure 1I. The median *lacZ* cell count for the BD group mice is zero. These data indicate that

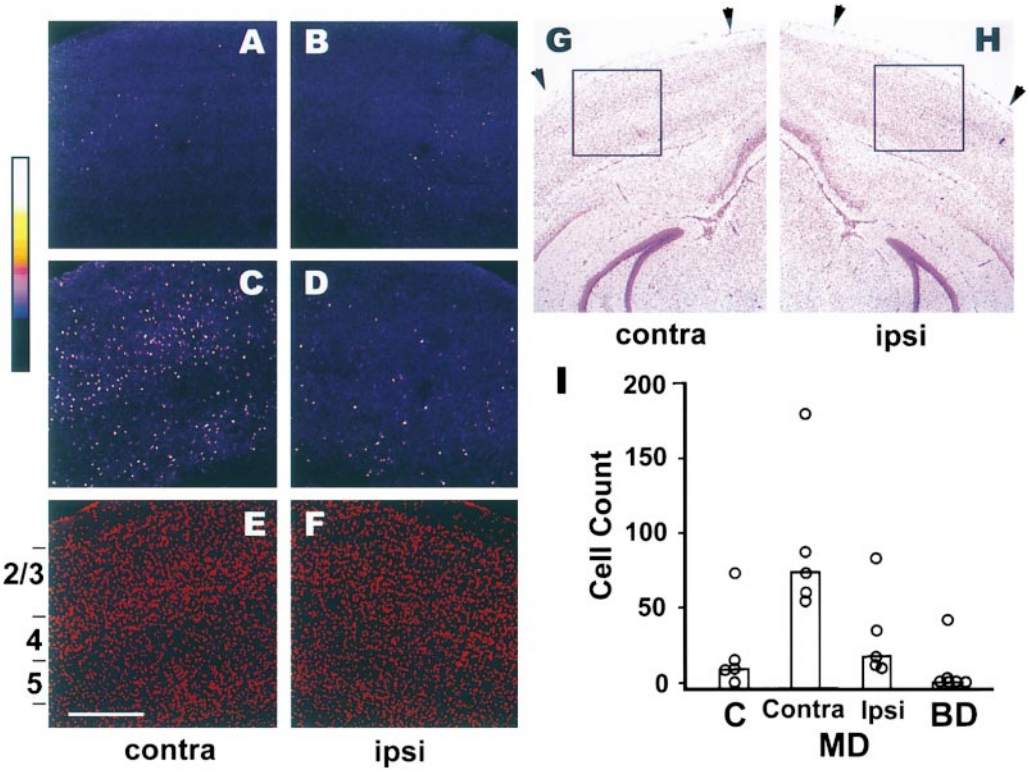

Figure 1. CRE–*lacZ* Transcription Is Induced in the Visual Cortex following Monocular Visual Deprivation

(A–D) Representative results of *lacZ* immunofluorescence in a P28 CRE–*lacZ* control mouse (A and B) and a mouse whose right eye had been sutured shut for 24 hr (C and D). Coronal histologic sections through the visual cortex were processed for *lacZ* immunofluorescence, imaged on a confocal microscope, and presented here in indexed color (as indicated by the color bar scale) to represent the relative fluorescence intensity. The images shown are for a set of sections processed in tandem and imaged under identical confocal microscope settings. The images shown are for an area in the middle of the visual cortex, ~2.25–2.5 mm from the midline. (A) and (C) show the right visual cortex (contralateral to the open eye in the MD animal), while (B) and (D) show the left visual cortex (ipsilateral to the open eye).

(E and F) Confocal microscope images (shown in false color) of the identical section shown in (C) and (D) stained with ethidium bromide to show the cellular density and cortical lamination. After imaging of *lacZ* immunofluorescence, the section was uncoverslipped and stained with ethidium bromide. (E) and (F) show the left and right visual cortex, respectively.

Scale bar, 0.3 mm for (A) through (F).

(G and H) Photomicrograph showing the same section shown in (C) and (D) stained with cresyl violet, subsequent to imaging of cells with ethidium bromide. The areas of (C) and (D) are marked by the square boxes. The boundaries of the primary visual cortex as determined by its cytoarchitectonics are marked by arrowheads.

(I) Quantitation of the interanimal variability in CRE–*lacZ* expression in V1 in deprived and nondeprived mice. For each animal, cells moderately to brightly labeled above background by *lacZ* immunofluorescence were counted. Counts were done blind to experimental condition on confocal microscope images. The cell number represents the number of cells counted in an area in the middle of V1 spanning layers 1 through 6 and 400 μm wide tangentially in a coronal section. Counts for control and BD animals are for the right V1. The sections used for comparative analysis were ~1.5–1.75 mm from the posterior end of the cortex. Each open circle represents the value for a single animal. The open bars denote the median cell count for each group. Abbreviations: C, control; MD, monocularly deprived (contralateral and ipsilateral to the open eye); BD, binocularly deprived.

activation of CRE–*lacZ* transcription following MD requires light experience through the nondeprived eye, and they are consistent with previous observations that competitive interactions between the two eyes are necessary to drive rapid changes in neuronal responses in the visual cortex.

CRE-Mediated Transcription Is Not Induced in the Lateral Geniculate Nucleus

Although neuronal plasticity following MD is reflected in cortical neuronal responses, it is possible that the ocular dominance shift is mediated by mechanisms presynaptic to layer 4 of V1. In the mouse, thalamic inputs to V1 originate from the dorsal lateral geniculate nucleus (dLGN) (Ribak and Peters, 1975). Therefore, we examined the dLGN for CRE-mediated transcriptional activity before and after MD. We find, however, that there are few *lacZ*-immunoreactive cells within the thalamus, and the dLGN shows very few cells that are clearly immunoreactive for *lacZ* (Figures 2A and 2B). There is also no significant consistent difference between deprived and control mice within the dLGN in the five animals examined for each group. In contrast, in the MD group, anterior portions of visual cortex contained within the same coronal sections that have dLGN show many intensely *lacZ*-immunolabeled cells (compare Figures 2B and 2C).

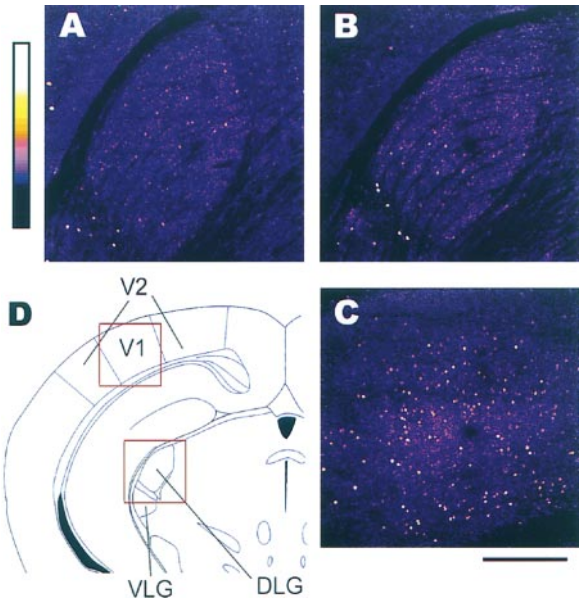

Figure 2. Few *lacZ* Immunopositive Cells Are Present in the Dorsal Lateral Geniculate Nucleus of Control or Monocularly Deprived CRE–*lacZ* Mice

Representative confocal images of the dorsal lateral geniculate nucleus (dLGN, the thalamic nucleus providing input to V1) after immunofluorescent labeling for *lacZ*. Confocal images are shown in indexed color (as represented by the color bar scale) to represent relative fluorescence intensity. The background was enhanced to show the LGN architecture. Consistent results were obtained in five control and five monocularly deprived mice.

(A) Confocal image of the right dLGN in a control mouse. Few clearly stained cells are seen within the dLGN itself, though some brightly stained cells can be seen in the ventral geniculate nucleus and within the boundary zone, the intergeniculate leaf.

(B) Confocal image of the right dLGN (contralateral to the open eye) in a mouse monocularly deprived for 24 hr.

(C) *lacZ* immunofluorescence within the right V1 in the same section shown in (B). Consistent with previous results, many brightly stained cells are seen in V1 contralateral to the open eye.

Scale bar, 0.3 mm for (A) through (C).

(D) A schematic diagram of the coronal section from which confocal images were taken. The areas of dLGN and V1 corresponding to the confocal images are marked by boxes. Abbreviations: VLG, ventral LGN; DLG, dorsal LGN.

These results indicate that if there are plastic effects on dLGN neurons following MD, they do not involve CRE-dependent signal transduction pathways.

Characterization of CRE-Mediated Transcription in the Cortex following MD

The above results indicate a robust response of the CRE–*lacZ* reporter following MD, but we were uncertain if we were seeing the full extent of the transcriptional response, or the tail end of the response if CRE-mediated transcription were an early (rapid onset) effect. For instance, CREB regulates the expression of immediate-early genes (such as *fos* and *jun*), which show a rapid burst of expression that peaks within 2 hr following an increase in neuronal activity. To determine whether CRE-mediated transcription following MD might show similar kinetics, we sacrificed CRE–*lacZ* mice at varying

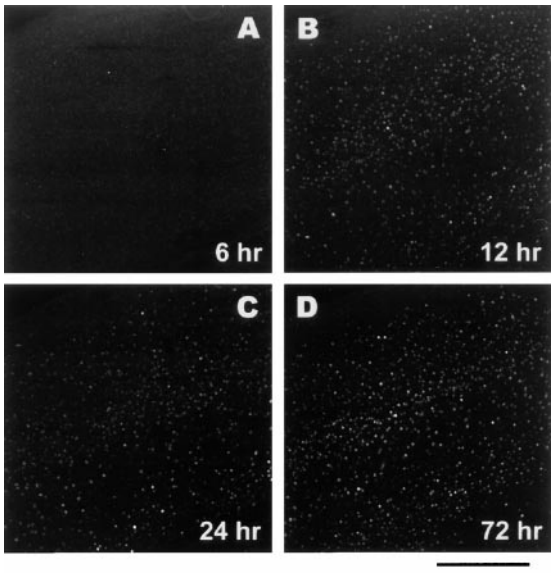

Figure 3. CRE–*lacZ* Transcription following Monocular Deprivation Is a Delayed Response

The results shown are for a set of animals of the same age (P27) that received monocular eyelid suture at the same time and were sacrificed at different times following MD. (A) through (D) show representative confocal microscope images of *lacZ* immunofluorescence in the left V1 (contralateral to the open eye) for mice deprived for 6, 12, 24, and 72 hr, respectively. Scale bar, 0.3 mm.

time points following monocular eyelid suture. Surprisingly, even after 6 hr of MD, we find no significant *lacZ* immunoreactivity above background in three mice examined (Figure 3A). We also do not see activation of CRE-mediated transcription after 4 hr of deprivation ($n = 3$ mice; data not shown). However, when the mice were deprived for 12 hr (Figure 3B), we find robust *lacZ* immunoreactivity ($n = 2$), similar to the result obtained for 24 hr of MD (Figure 3C). When we allowed the MD to continue for 3 days (72 hr, Figure 3D), we see a persistence of *lacZ* immunoreactivity in three out of four mice examined. Since the half-life of *lacZ* in neurons is $\sim$8 hr (S. I. and D. R. S., unpublished data), this result suggests that CRE-mediated transcription can persist for several days following the start of deprivation. Taken together, these results show that CRE-mediated transcription following MD is a delayed effect, requiring a prolonged period of deprivation for its expression.

In the neocortex, the cells are approximately equally divided between neurons and glial cells. To determine whether the *lacZ*-immunoreactive cells are neurons, we double labeled sections with anti-*lacZ* plus anti-NeuN, an antibody against a neuron-specific marker (Mullen et al., 1992). As shown in Figure 4, virtually all of the *lacZ*-immunoreactive cells (red fluorophore) also colabel with the neuron-specific marker (green fluorophore). Thus, very few (if any) glial cells show CRE-mediated transcriptional responses to monocular deprivation. Moreover, individual neurons exhibit widely differing levels of CRE–*lacZ* transcription, possibly reflecting differences in the pattern of synaptic inputs onto individual neurons.

To determine the precise distribution of CRE-mediated transcription following MD in the neocortex, we

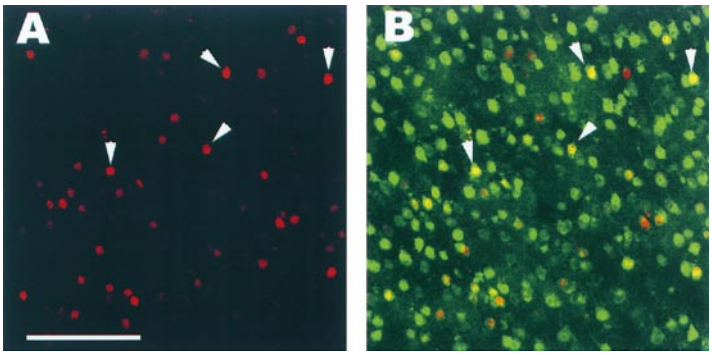

Figure 4. *lacZ* Immunoreactive Cells in Monocularly Deprived CRE–*lacZ* Mice Are Neurons

Sections from deprived animals were double labeled with anti-*lacZ* (red) and anti-NeuN (a neuron-specific marker, green), showing co-localization of *lacZ*-expressing cells with the neuronal phenotype.

(A) Higher power confocal image of *lacZ*-immunolabeled cells in layer 4. The section is from a mouse deprived for 12 hr. The cells marked by arrows here correspond to cells marked by arrows in (B).

(B) Fluorescent staining of neurons (green) superimposed on the image shown in (A). The double-labeled cells show yellow or orange labeling in this color-merged image. In a few cases, the *lacZ* (red) label is surrounded by the NeuN (green) label.

Scale bar, 0.125 mm for (A) and (B).

made composite images using overlapping individual confocal micrographs of sections labeled for *lacZ* immunofluorescence. Following confocal imaging, these same sections were stained with cresyl violet, allowing the boundaries of V1 to be determined by its distinctive cytoarchitecture in coronal sections. Compared to adjacent areas, V1 has a greater density of granule cells in layer 4 and greater thickness of the supragranular layers (Caviness, 1975). These characteristics allow us to mark the boundaries of V1 within the composite *lacZ* immunofluorescence image (Figure 5A). Consistent with the results shown in Figure 1, there is in general much greater *lacZ* immunoreactivity in visual cortex contralateral versus ipsilateral to the open eye. Surprisingly, we find *lacZ* immunoreactivity almost throughout the monocular zone, diminishing only in the most medial edge of V1. In contrast, ipsilateral to the open eye, *lacZ* immunoreactivity in V1 is concentrated in a comparatively narrow zone laterally that corresponds to the binocular zone. Medial to V1 (V2M or area 18b) bilaterally, there is a very low level of *lacZ* immunoreactivity. Although this area also receives visual input, it receives few projections from V1. Lateral to V1, in area V2L (18a) and areas still more lateral, there is a moderate degree of *lacZ* immunoreactivity. Area V2L receives direct thalamic input as well as abundant input from V1 (Simmons et al., 1982). In kittens, plastic changes are known to occur in area V2 following MD. CRE-mediated *lacZ* expression strongly declines in more ventral cortical areas.

To examine further the cortical distribution of CRE-mediated transcription following MD, we prepared sagittal sections. Figure 5B shows a composite confocal image from such a sagittal section, from a CRE–*lacZ* mouse monocularly deprived for 3 days. This section is from the hemisphere contralateral to the open eye; the corresponding ipsilateral hemisphere shows very little *lacZ* immunoreactivity in V1 (data not shown). The anterior boundary of V1 is determined from a mouse histology atlas (Franklin and Paxinos, 1997) and is ~2.7 mm from the posterior edge of the cortex. This analysis clearly shows that *lacZ* immunoreactivity is concentrated within V1, diminishes just anterior to V1, and is drastically reduced in the primary somatosensory cortex (most anterior part of section shown). Taken together, these results demonstrate that the activation of CRE–*lacZ* transcription following MD is primarily confined to areas of cortex receiving inputs from the nondeprived eye, and within V1 transcription is present in monocular as well as binocular zones.

Downregulation of CRE-Mediated Transcription in Visual Cortex following the End of the Critical Period

The robust effects of MD on cortical neurons diminish significantly by postnatal day 34 (P34) in mice and are virtually nonexistent by age 40 days (Gordon and Stryker, 1996). If CREB-dependent signaling pathways were fundamentally involved in visual neuronal plasticity, then CRE-mediated transcription might diminish as the animal matures and plasticity is lost. To test this possibility, we monocularly deprived CRE–*lacZ* mice of ages P40–P44 and looked for *lacZ* expression in the visual cortex. We found a dramatic reduction in *lacZ* immunoreactivity in the visual cortex of these more mature mice following MD, compared to critical period–age mice (P26–P28) (compare Figures 6A and 6B). Four mice of ages P40–P44 were examined, and sections from these animals all showed very weak *lacZ* immunofluorescence labeling in the visual cortex. When the results are quantified by cell counting as in Figure 1, we find a median *lacZ*-positive cell count of zero for this group. Analysis of nondeprived mice of similar age also showed little *lacZ* immunoreactivity ($n = 2$; data not shown). In addition, we examined six mice of ages P52–P56 (three control and three deprived) and found only background levels of fluorescence in visual cortical areas (data not shown).

In contrast to the downregulation of CRE-mediated gene transcription, the level of CREB family protein in the visual cortex appears constant over these ages. Figures 6C and 6D show comparable immunofluorescent staining of V1 with a polyclonal antibody (New England Biolabs) that recognizes most CREB subtype transcription factors. Therefore, the decline in CRE-mediated transcription is not likely to be secondary to changes in levels of CREB-related protein. It is striking also that CREB immunoreactivity is present in many

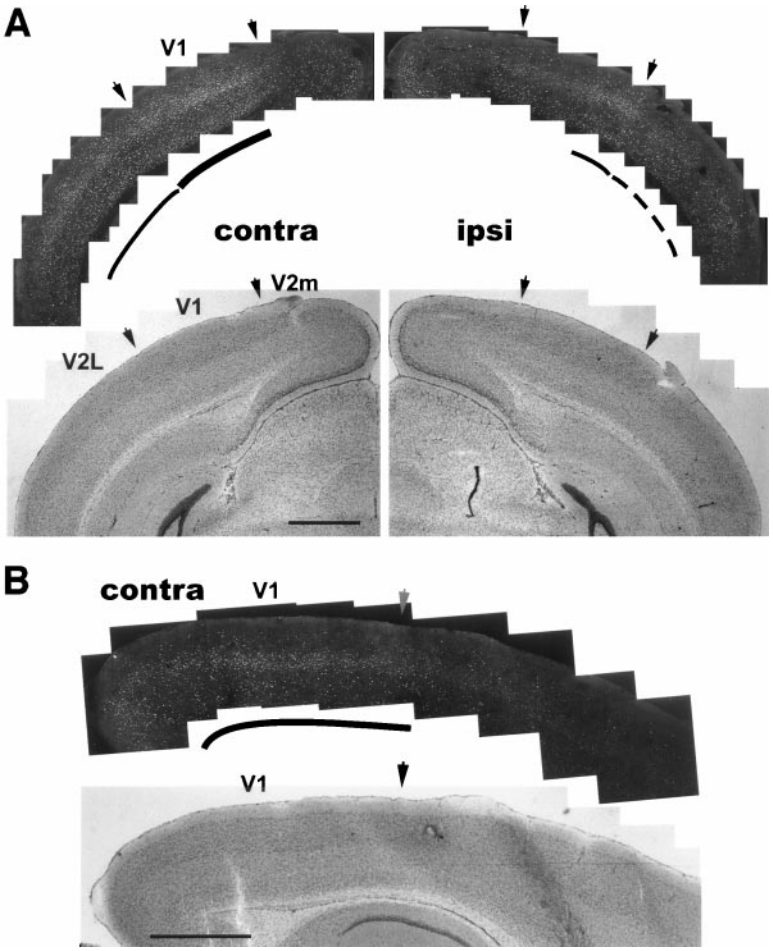

Figure 5. Distributions of *lacZ*-Immunoreactive Cells in Monocularly Deprived CRE–*lacZ* Mice Show that CRE–*lacZ* Transcription Is Concentrated in Cortical Areas Receiving Input from the Nondeprived Eye

In this experiment, CRE–*lacZ* mice were monocularly deprived for 3 days by right eyelid suture, and brain sections were processed for *lacZ* immunofluorescence. Overlapping confocal microscope images were taken and were assembled into a composite image using Adobe Photoshop image processing software. Individual images were adjusted only for slight variations in background brightness caused by fluctuations in laser intensity.

(A) Composite confocal microscope image of a coronal section is shown. The same section was later stained with cresyl violet (below). The boundaries of V1 were determined by cytoarchitectonics and marked by arrowheads. "Contra" and "ipsi" are in reference to the open eye.

(B) Composite confocal microscope image of a sagittal section and its accompanying cresyl violet staining. The anterior boundary of V1 (about 2.7 mm from the occipital end of the cortex) is marked by the arrowhead.

Scale bar, 1 mm for (A) and (B).

more neurons than those that are actually showing CRE-mediated transcription. These experiments suggest that the functional regulation of CREB family transcription factors, and not the absolute CREB level, likely determines the outcome of CRE signaling–dependent transcription in the postnatal neocortex.

Discussion

Early in postnatal life, the mammalian neocortex is characterized by a high degree of neuronal plasticity. During this period, a convergence of genetic and experiential instructions establish the basic framework of cortical neuronal connectivity that largely remains with the organism for its entire life. With maturation, much, but not all, of this plasticity is lost. The intracellular mechanisms that govern this neuronal activity–dependent developmental process, and its decline with maturation, are not known. In this report, using CRE–*lacZ* transgenic mice, we present evidence for the central involvement of a CRE-mediated transcriptional pathway. First, we show the activation of CRE-mediated transcription during conditions that result in synaptic reorganization in the visual cortex, with a delayed time course that precedes the onset of physiologic plasticity. Second, this activation of CRE-dependent transcription is limited to cortical areas receiving visual input. Third, CRE-dependent transcription following MD is observed only in immature (critical period) mice, reflecting physiologic plasticity.

CRE–Regulated Transcription during Physiologic Neuronal Plasticity in the Visual Cortex

Our results suggest an important role of CRE-mediated gene expression in critical period plasticity in the developing neocortex. These results are particularly compelling since CREB family transcription factors are critical mediators of neuronal activity–dependent gene expression and are important for memory and learning (Silva et al., 1998). In rodents, the CREB family is comprised of at least ten genes (reviewed by Brindle and Montminy, 1992; Sassone-Corsi, 1995). In addition to CREB, which itself is composed of several RNA splicing variants (Blendy et al., 1996), these include CREM (CRE modulatory factor) and its variants and ATF-related factors (activating transcription factor). All of these transcription factors can bind to the CRE sequence and can heterodimerize, leading to extensive cross-interaction by the different members. Within the brain, CREB subtype factors are the major CRE activators, while CREM factors may function primarily as negative modulators of CREB. CREB itself is inactive until it is phosphorylated at Ser-133, and various mechanisms dependent on calcium

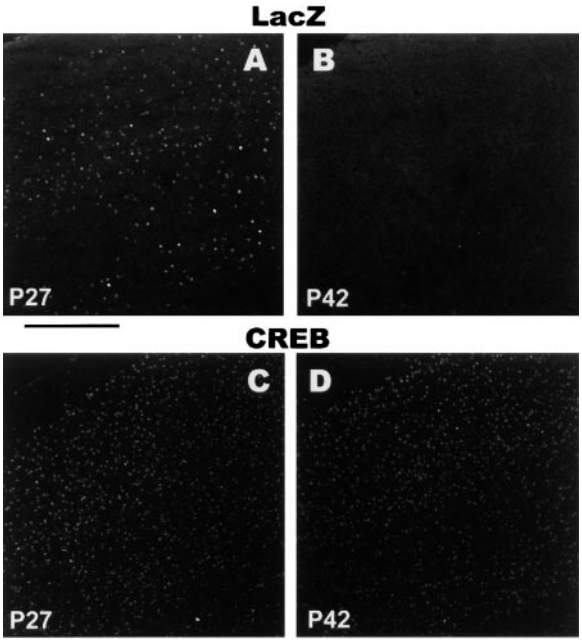

Figure 6. CRE–*lacZ* Transcription Is Strongly Downregulated in Post–Critical Period Mice, though CREB Immunoreactivity Is Unchanged

(A and B) Confocal microscope images of cryostat sections from animals of ages P27 (critical period peak) and P42 (post–critical period) that have been deprived for 24 hr and then processed in tandem for *lacZ* immunofluorescence.

(C and D) Confocal microscope images of sections from control mice of ages P27 and P42 that have been fluorescently labeled for CREB family immunoreactivity using a CREB polyclonal antibody. CREB family immunoreactivity for both age groups is present in virtually every cell and in all of the cortical layers. Immunoreactivity is unaffected by monocular deprivation (data not shown).

Scale bar, 0.3 mm for (A) through (D).

and cAMP regulate CREB phosphorylation (Gonzalez and Montminy, 1989).

Following monocular visual deprivation, CRE-dependent transcriptional machinery is engaged in the visual cortex, and CRE-mediated *lacZ* transcription is detected in areas receiving inputs from the nondeprived eye. No significant CRE-mediated transcription was observed in the dLGN (the thalamic nucleus projecting to V1) of control or deprived mice. This result is consistent with the lack of evidence for changes in the thalamocortical projections to V1 following prolonged MD ($>$20 days), as indicated by transneuronal labeling (Drager, 1978) or reconstruction of single axonal arbors (Antonini et al., 1997, Soc. Neurosci., abstract). In addition, in vivo electrophysiology experiments indicate that the magnitude of plasticity is much greater in the supragranular and infragranular layers of the cortex compared to layer 4, the primary thalamocortical recipient layer (Gordon and Stryker, 1996). Taken together, these observations suggest that deprivation-induced changes in the response properties of cortical neurons are mediated largely by events occurring within the cortical neurons.

Surprisingly, we find strong activation of CRE-mediated transcription within the monocular zone of the nondeprived eye as well as the binocular zone. Physiologic plasticity occuring within the monocular zone is not well understood, since higher mammals such as cats and primates (classic systems for studies of visual system function) are overwhelmingly binocular. Nonetheless, this CRE-mediated transcription may reflect an adaptive plastic response that compensates for the loss of visual field and binocular stereoscopic vision and that serves to enhance visual perception. Our results are consistent with those of Sherman and his colleagues that show significantly greater cortical responses in the nondeprived monocular zone compared to the deprived monocular zone of monocularly deprived cats (Wilson and Sherman, 1977; Watkins et al., 1978).

Mechanistic Considerations of CRE-Mediated Transcription in the Visual Cortex

Since we are only changing the pattern of neural activity during MD, we think it is likely that the induction of CRE–*lacZ* transcription is caused primarily by changes in the pattern and/or magnitude of neuronal activity in the visual cortex. However, this effect cannot be a simple direct result of deprivation since declines in neuronal firing rates do not activate CRE-dependent signaling mechanisms. We suggest instead that blocking visually driven neural inputs from one eye results in a decrement of tonic neuronal inhibition normally exerted on neurons that respond preferentially to the other eye. The net effect would be enhancement of activity in nondeprived cortical areas. Such interocular suppressive effect has been reported by Sengpiel and Blakemore (1994). The effect has been demonstrated even for apparently monocular neurons in strabismic animals (Sengpiel and Blakemore, 1994; Sengpiel et al., 1994; Walker et al., 1998). These results show that monocularly activated neurons commonly receive substantial subthreshold and inhibitory inputs from the nondominant eye that modulate their activity.

Interocular inhibitory effects in V1 could in principle be mediated by at least two mechanisms in the visual pathway: by local horizontal connections in the visual cortex, or by feedback projections to V1 from higher cortical centers (Figure 7A). Local cortical disinhibition could explain the enhancement in neuronal activity within the binocular zone, where inputs from the two eyes are intermixed. However, our finding of robust activation of the monocular zone suggests that this mechanism alone is not sufficient. Therefore, we suggest that removal of inhibition mediated by feedback projections from higher cortical centers may be involved also. The inhibition (and disinhibition) via these feedback projections could serve a homeostatic function to ensure overall constancy in neural activity in the cortex and to maintain stability within the neuronal circuit (in effect controlling the gain at the level of V1). In addition, diffuse inputs from neuromodulatory subcortical centers to the neocortex may play a role in the induction of CRE–*lacZ* transcription. Activity of cholinergic, noradrenergic, and serotonergic systems projecting to the neocortex is thought to be required for normal plasticity following deprivation in cats (Kasamatsu and Pettigrew, 1976; Bear and Singer, 1986; Gu and Singer, 1993, 1995). The involvement of multiple brain systems in gating plasticity at V1 has been proposed by Singer (1982).

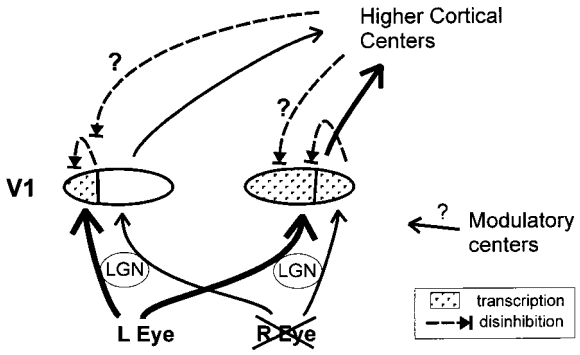

Figure 7. Schematic Representation of Visual Processing Circuitry and Physiologic Signals Affecting CRE Signaling

Neuronal disinhibition may enhance activity in V1 receiving input from the nondeprived eye and thus serve to drive CRE-regulated transcription in the primary visual cortex. This simplified diagram illustrates the neuronal pathways subserving vision. LGN and V1 receiving inputs from each eye are shown, with monocular (medial) and binocular (lateral) zones indicated for V1. The contralateral pathways project to all of V1, whereas the ipsilateral pathways project only to the binocular zones. With eyelid suture, pathways receiving direct input from the deprived eye show a reduction in neuronal activity. We hypothesize that there is a corresponding decrease in neuronal inhibition mediated by local horizontal connections and by feedback projections from higher cortical centers that receive inputs from V1 (see text). Modulatory centers refer to subcortical centers that send cholinergic, noradrenergic, and serotonergic projections to the cortex and that may facilitate cortical plasticity.

The delay between the beginning of MD and the observed CRE-mediated *lacZ* expression suggests that there may be intermediate steps required for the activation of CRE-mediated transcription. It is unlikely that this delay only reflects the lag time required for production of the *lacZ* protein, since in vitro depolarization of neurons in brain slices from CRE–*lacZ* mice results in robust accumulation of *lacZ* protein within 4 hr (Impey et al., 1996). The delayed response in vivo could be adaptive, since the synaptic rearrangements observed following MD are profound, and thus requires that mechanisms exist to ensure that the change in visual input is enduring and not transient. The time interval requirement for CRE-mediated transcription in the visual cortex is consistent with the delayed onset of physiologic ocular dominance shifts following MD. The delay in CRE-mediated transcription could be due to the presence of inhibitors of CREB, such as inhibitory transcription factors that dimerize with and inactivate CREB (Sassone-Corsi, 1995), or the presence of high levels of intracellular phosphatases that reverse CREB phosphorylation (Bito et al., 1996; Liu and Graybiel, 1996). These inhibitors would need to be downregulated before CREB can exert its effect. The presence of mechanisms that exert inhibitory effects on neuronal plasticity has been described in various systems of memory and learning (recently reviewed by Abel et al., 1998), and it remains to be determined whether similar mechanims occur in the neocortex during development.

Physiologic Role of CRE-Mediated Transcription in the Neocortex during Postnatal Development

How might CRE-mediated transcription be involved in changes in synaptic function following MD? We envision that the CRE-regulated gene targets could have modulatory effects on synaptic function. Thus, a major question that arises from this work is the identity of these endogenous gene targets. A large number of relevant neuronally expressed genes, many of which are known to function at synapses, that can be regulated by CREB have been described. These include *BDNF*, *calmodulin-dependent kinase IV*, *synapsin I*, *somatostatin*, a subtype of a voltage-gated potassium channel, and the *fos* and *jun* family of immediate-early genes (Sauerwald et al., 1990; Mori et al., 1993; Sassone-Corsi, 1995; Sun et al., 1995; Shieh et al., 1998; Tao et al., 1998). The possible involvement of neurotrophins is particularly interesting since they are possible modulators of cortical synapses (Domenici et al., 1991; Cabelli et al., 1995) and are known to regulate CREB function (Ginty et al., 1994; Finkbeiner et al., 1997). We don't know as yet whether these genes will show an expression pattern that correlates with that of the CRE–*lacZ* reporter. However, we expect that the level of induction of any single gene will be small compared to the CRE-regulated reporter because natural genes are driven by a variety of regulatory elements, constitutive as well as inducible. Therefore, the fundamental importance of our findings that show induction of CRE-mediated transcription is that these results imply the activation of a network of CRE-regulated genes in response to a physiologically relevant condition. Although the changes in expression of individual genes will likely be modest, their combined transcriptional enhancement could substantially synergize to produce major physiologic effects.

With maturation, we show that there is a dramatic downregulation of CRE-mediated transcription in response to MD, corresponding to the disappearance of the critical period for neuronal plasticity in the visual cortex. Our results are consistent with reports that describe declines in expression of several CRE-regulated genes with maturation and aging. These include *BDNF* (Hayashi et al., 1997), *somatostatin* (Hayashi et al., 1997), and c-*fos* and *zif268* (Mower, 1994; Kaplan et al., 1996). In all of these cases, the downregulations reported are modest, ranging from 40%–70% decline from the peak values. However, the coordinate downregulation of an entire network of genes regulated by CREB, as is suggested by our result, might be sufficient to explain the decline in cortical neuronal plasticity during maturation and aging. We do not know why CRE-mediated transcription declines with maturation, though our evidence suggests that it is unlikely to be caused by changes in levels of CREB-related transcription factors. There could be changes in calcium regulation and signal transduction with neocortical maturation. NMDA receptor function in the visual cortex appears to decline with maturation and thus may affect calcium signaling (Tsumoto et al., 1987; Fox et al., 1991; Carmignoto and Vicini, 1992).

Clearly, it will be important to determine the exact physiologic effects of deleting CREB-family transcription factors on neocortical plasticity. Unfortunately, a general deletion of CREB results in mice with neonatal lethality (Rudolph et al., 1998). Therefore, these experiments must necessarily await the development of mice

with a deficiency of CREB temporally restricted to postnatal development and spatially restricted to the occipital neocortex.

Experimental Procedures

CRE–*lacZ* Transgenic Mice

Mice containing an insertion of a CRE–*lacZ* promoter are the same as those described by Impey et al. (1996). Mice were bred by mating a male CRE–*lacZ* transgenic heterozygous animal with a C57/BL6 wild-type female obtained from a commercial vendor. Animals were genotyped by PCR.

Antibodies

Sources of antibodies and their dilutions used in experiments described are as follows: rabbit anti *lacZ*, ICN biomedical (1/2000); rabbit anti-CREB, New England Biolabs (1/1000); mouse anti-neuronal nuclei monoclonal (NeuN), Chemicon (1/1000); LRSC-conjugated goat anti-rabbit, Jackson (2 μg/ml); and Alexa 488 goat anti-mouse, Molecular Probes (5 μg/ml).

Visual Deprivation

CRE–*lacZ* mice were monocularly deprived by suturing the right eyelid as described previously (Gordon and Stryker, 1996). For binocular deprivation, animals either received right eyelid suture and then were placed in a dark chamber (five animals) or were bilaterally eyelid sutured (one animal). These two BD protocols gave similar results.

Immunofluorescence

The brain was rapidly dissected and then immediately frozen in OCT embedding media in a plastic mold. These were kept at −80°C until the time of sectioning. Fifteen micrometer sections were obtained using a cryostat and mounted on Superfrost slides (Fisher). The slides were typically stored at −80°C until further processing or used immediately. Slides were briefly air dried and then fixed in 4% paraformaldehyde for 30 min. After brief washes in phosphate buffered saline (PBS), the sections were blocked for 1 hr at room temperature using a solution of 5% BSA, 0.05% Triton X-100, and 4% normal goat serum in PBS. Reactions with primary antibodies were performed using appropriate dilutions of antibody (as indicated above) into the blocking solution. Sections were incubated overnight at 4°C (for *lacZ*) or at room temperature (CREB, NeuN/*lacZ* double labeling). After washing with PBS, the sections were incubated with secondary antibody in blocking buffer for 2 hr at room temperature.

Sections were imaged on a Bio-Rad laser scanning confocal microscope and images were recorded digitally. Image processing was done using NIH-image and Adobe photoshop software. For cell counting, representative images were adjusted slightly to equalize the background and then printed on a Phaser printer. The areas to be counted were marked, spanning the depth of all the cortical layers and 400 μm wide tangentially. Counts were done blind to experimental condition. Coronal sections used for cell counting were all ~1.5–1.75 mm from the posterior end of the cortex.

Histological Staining

After the *lacZ* immunofluorescence data were collected, the coverslips were gently removed from the slides. Sections were then washed briefly in PBS. Cresyl violet staining was performed immediately using standard procedures. For fluorescent cellular nucleic acid staining, the sections were incubated with a dilute solution (1 ng/ml) of ethidium bromide, rinsed, and coverslipped.

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