Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 5106–5110, June 1991
Neurobiology

Constitutive expression of *zif268* in neocortex is regulated by synaptic activity

(visual cortex/transcription factors/neural plasticity)

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Communicated by Torsten N. Wiesel, March 12, 1991

ABSTRACT **Transcription factors are rapidly and transiently induced in brain by excitatory stimuli and may be important in coordinating changes in gene expression underlying neuronal plasticity. In contrast to their transient induction after stimulation, certain transcription factors display stable, relatively high basal levels of expression in brain. Here we demonstrate that this "constitutive" expression of the transcription factor *zif268* in cortex is driven by natural synaptic activity. Blockade of afferent visual activity with intraocular injections of tetrodotoxin results in rapid, dramatic reductions of Zif268 mRNA and immunoreactivity in visual cortex. Moreover, dark-adaptation for several days lowers *zif268* expression in visual cortex, and expression rapidly returns to control levels upon subsequent light exposure. Several other transcription factors, which are induced in cortical neurons by excitatory stimuli, appear less responsive to changes in natural sensory input. These studies suggest that transcription factors play a role not only in responses to artificial stimuli but also in the normal maintenance of cortical physiology. Anatomic markers for *zif268* may be useful in mapping normal cortical activity in brain.**

Classical studies of visual cortex have established a critical role for afferent activity in normal cortical development (1–3). More recent studies have demonstrated that interference with sensory input in adults also affects the neurotransmitter and enzyme phenotype (4–7), as well as the synaptic connectivity (8), of cortical neurons. Attention has therefore been focused on identifying mechanisms mediating the trophic effects of naturally occurring afferent activity in adult neocortex. Transcription factors, rapidly induced by cell-surface receptor stimulation, are thought to mediate long-term effects of growth factors on cellular activity (9). It has been suggested that these factors may play an analogous role in stimulus-induced neuronal plasticity (10, 11). A variety of artificial and novel-physiologic stimuli produce rapid and transient activation of transcription factors in adult brain and spinal cord neurons (refs. 12–18; for review, see ref. 11). However, to assess a role for these factors in mediating trophic effects of naturally occurring synaptic activity in adult neocortex, it is essential to determine whether they are regulated by normal spontaneous synaptic activity. We therefore examined the possibility that the relatively high levels of expression of *zif268* and other transcription factor genes in cortex (14, 15, 19) are driven by naturally occurring afferent synaptic activity.

METHODS

Animal Preparation. Adult male Sprague–Dawley rats or Swiss ICK mice were used in all experiments. MK-801 (0.3, 1, or 3 mg/kg; in 50% ethanol) or CGS-19755 (10 mg/kg; in water) was administered by i.p. injection. For intraocular injections of tetrodotoxin (TTX), animals were briefly anesthetized with diethyl ether, and 10 μl of 0.1 mM TTX in saline was perfused into the vitreal space with a microsyringe. Dark-adaptation was performed by housing normally reared, adult rats in complete darkness for 1 day to 3 weeks. All animals were sacrificed by guillotine decapitation.

***In Situ* Hybridization and Northern (RNA) Analysis.** These procedures were done as described (14, 16). For *in situ* analysis, control and experimental tissues are frozen in the same tissue block to assure identical hybridization conditions.

Gel-Shift Assays. Nuclear extracts from cortex and hippocampus were prepared by a modified method of Dignam *et al.* (20) as described by Sonnenberg *et al.* (21). Gel-shift assays were done as described (22, 23). Probes used were a double-stranded oligonucleotide site for Zif268

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GATCCCCCTCGCCCACGCGA
    GGGGAGCGGGTGCGCTCTAG
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or a 38-base-pair (bp) fragment containing the AP-1 consensus binding site

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TCGACGGTATCGATAAGCTATGACTCATCCGGG
    GCCATAGCTATTCGATACTGAGTAGGCCCCCTAG.
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Immunohistochemistry. Affinity-purified rabbit polyclonal antisera selective for c-Jun and Zif268 were used. Ten-micron cryostat sections of mouse or rat brain were fixed in 1% paraformaldehyde, and immunoreactivity was detected by the ABC immunoperoxidase method (Vector Laboratories) as described (24). Omission of primary antibody resulted in complete loss of nuclear staining.

Tracer Studies. Rhodamine red-conjugated latex microspheres (Lumafluor, New York) were injected unilaterally into the lateral geniculate nucleus (0.1 μl) or the visual cortex (1 μl) of rats through a broken-tip, glass microelectrode connected to a microsyringe. Four days later, animals were sacrificed, and brain sections were prepared for immunohistochemistry as described. Microspheres were localized with standard fluorescence microscopy.

RESULTS

In our initial studies examining regulation of transcription factors in cortex, we administered agents that pharmacologically block excitatory synaptic transmission. Systemic administration of *N*-methyl-D-aspartate (NMDA) receptor antagonists MK-801 (25) or CGS-19755 (26) rapidly and dramatically reduced basal levels of Zif268 mRNA in cortex

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Abbreviations: TTX, tetrodotoxin; NMDA, *N*-methyl-D-aspartate.

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(Fig. 1). Similar results were also obtained in mice (n = 3). Reductions after MK-801 were dose dependent in the range of 0.3 mg/kg (n = 3) to 3 mg/kg (n = 4). Similar precipitous declines in Zif268 sequence-specific DNA-binding activity (Fig. 1*C*) and immunoreactivity (data not shown) indicate that the mRNA and protein respond in a parallel fashion. By contrast, c-Fos mRNA and AP-1-binding activity were not rapidly reduced by MK-801.

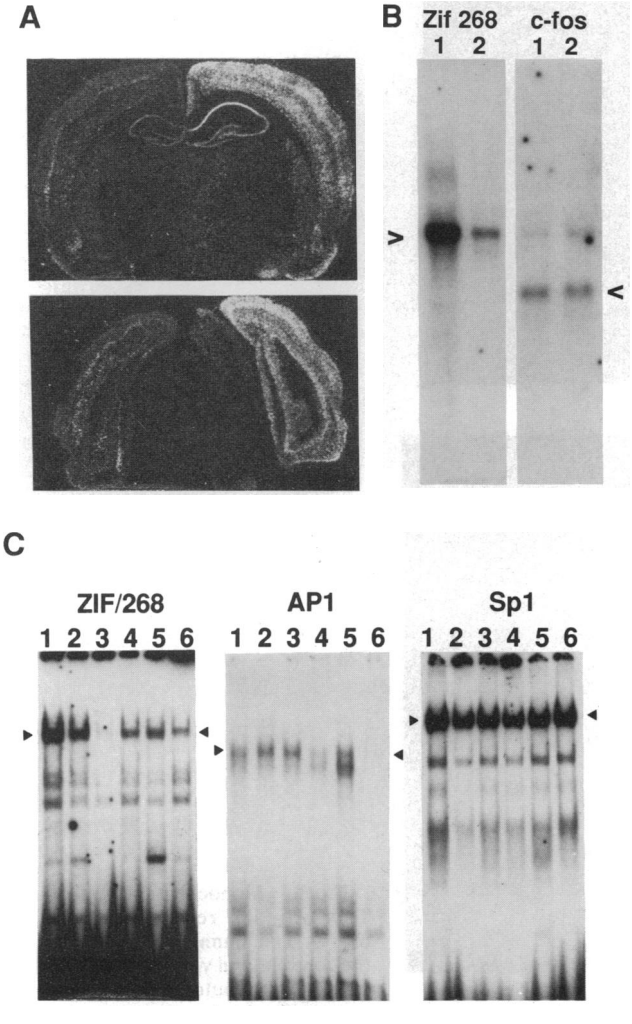

FIG. 1. NMDA-receptor antagonist selectively reduces levels of Zif268 mRNA and sequence-specific DNA-binding activity in rat cortex. (*A*) Zif268 *in situ* autoradiogram. The right half-brain of each autoradiogram is from a control animal, and the left half-brain is from an animal treated with the NMDA antagonist MK-801 (3 mg/kg) 4 hr before sacrifice. Zif268 mRNA levels are markedly reduced by MK-801 throughout the neocortex, pyriform cortex, and CA1 of hippocampus. (≈×3.) (*B*) Northern analysis of rat cortex RNA. RNA was isolated from cortex of control rats (lanes 1) and rats treated with MK-801 (3 mg/kg) for 4 hr (lanes 2). Levels of Zif268 and c-Fos mRNA were assayed by Northern analysis (10 μg of total RNA per lane) on duplicate blots. Consistent with results of *in situ* assays, levels of Zif268 mRNA are markedly reduced by MK-801, whereas c-Fos mRNA levels are unchanged. Arrowheads indicate *zif268* and c-*fos* bands. (*C*) Gel-shift assays of sequence-specific DNA-binding activity from rat cortex after MK-801. Nuclear extracts were prepared (20, 21) from control cortex (lanes 1) and cortex 2, 4, 20, and 44 hr after MK-801 (3 mg/kg; lanes 2–5). Lanes 6 are from control hippocampus. Gel-shift assays were done as described (22, 23). Zif268-specific antisera supershifted only one of the four bands (indicated by arrowheads). Krox-20 binds to a similar DNA sequence (27) and may correspond to one of the nonshifted bands. Note reduction in Zif268 binding activity 4 hr (lane 3) after MK-801, whereas AP-1 and Sp1 activities in the same extracts show little change. Similar results were obtained in an additional experiment that used different extract preparations.

We next focused on examining the regulation of *zif268* in visual cortex because afferent activity to this region can be readily manipulated. Rodent visual cortex receives a major input from the contralateral eye via the lateral geniculate nucleus (28). Intravitreal injection of TTX, which blocks retinal ganglion-cell activity (29) and visual evoked cortical responses (30), was used to induce acute visual deprivation. Monocular, intravitreal injection of TTX caused a rapid unilateral reduction of Zif268 mRNA in the contralateral primary visual cortex of the rat (n = 6; Fig. 2). Similar results were also obtained in mice (n = 3). Reductions were detected within 30 min of TTX injection, reached nadir levels by 2 hr, and remained reduced for at least 24 hr. Comparison of visual areas receiving afferents from the control and TTX-injected eyes demonstrates that reductions are associated with cortical layers II–VI. Within the deafferented rat visual cortex, the level of *zif268* hybridization was most markedly reduced in the region corresponding to monocular cortex and was somewhat higher in the adjacent binocular region (ref. 28; Fig. 2), consistent with anticipated levels of visual afferent activity in these regions. mRNA levels for several other known or putative transcription factors, including c-Fos, c-Jun, Jun-B, Krox-20, and Nur77, were not markedly affected by intravitreal TTX injections (n = 3, assayed 5 hr after injections).

To assess the response of Zif268 protein to changes in afferent activity, we performed immunohistochemistry. In control rat or mouse visual cortex, affinity-purified Zif268 antisera labels nuclei of cells in all cortical layers (Fig. 2). Staining is most prominent in layer IV, where ≈30% of cells appear immunoreactive. Monocular injection of TTX rapidly reduces Zif268 immunostaining; immunostaining was reduced within 2 hr and was maximally reduced by 4 hr in all cortical layers receiving input from the injected eye. Immunostaining with antisera selective for c-Jun was not reduced in visual areas in adjacent sections from these animals (Fig. 2*B*). Reductions of Zif268 mRNA and immunostaining were also induced by maintaining rats in complete darkness for 4 days (dark-adaptation; data not shown). These reductions were less prominent than those produced by intraocular TTX injections consistent with persistent retinal activity in the dark (31).

Zif268-immunoreactive cells in the visual cortex appear to be neurons, based on Nissel stains. To confirm this neuronal localization and to assess whether staining is associated with a particular neuronal subtype, we combined immunohistochemical and retrograde-labeling techniques (32). Rhodamine-conjugated latex microspheres injected into the lateral geniculate nucleus selectively labeled pyramidal neurons of layers V and VI, whereas injection into the cortex labeled neurons in layers II and III of the contralateral cortex, consistent with described (33) cortical circuitry. Zif268 immunostaining colocalizes with microspheres in many cells of these cortical layers (Fig. 3), confirming neuronal localization of Zif268 immunostaining.

The preceding experiments suggest that normal visually evoked synaptic activity maintains steady-state expression of *zif268* in cortical neurons. To directly examine the kinetics of Zif268 induction by visual afferent activity, we monitored Zif268 mRNA and immunoreactivity in visual cortex of animals exposed to light after dark-adaptation. Exposure of dark-adapted rats to ambient laboratory light (≈30 lux) rapidly increased Zif268 mRNA (Fig. 4) and immunoreactivity in visual cortex (data not shown). Levels of mRNA and immunostaining increased within 30 min of light exposure and rapidly returned to levels present in control visual cortex. No changes in c-Fos, Jun-B, c-Jun mRNA levels or in c-Jun immunostaining were detected in these animals.

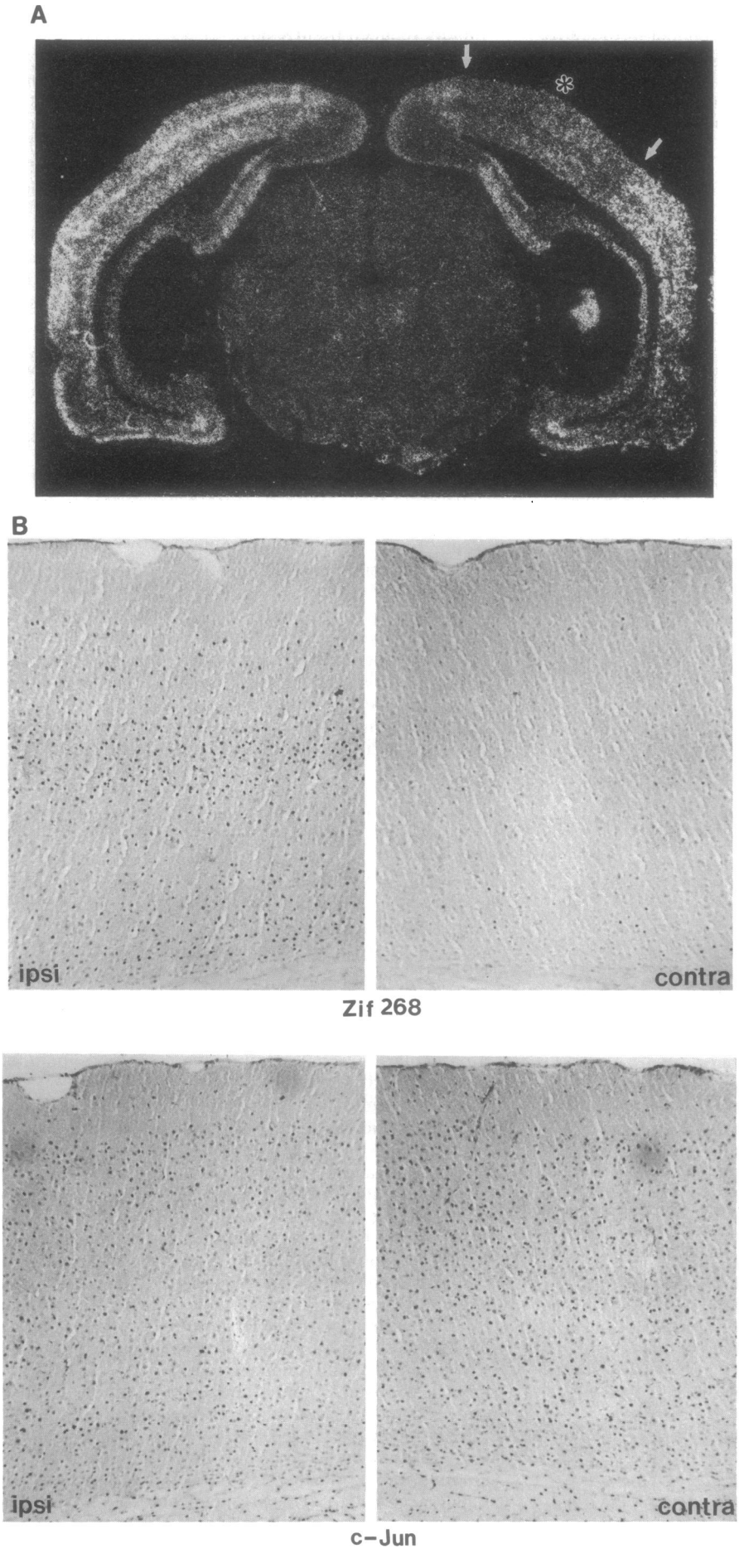

FIG. 2. Monocular injection of TTX rapidly reduces Zif268 mRNA and immunoreactivity in the contralateral visual cortex. (*A*) Zif268 *in situ* autoradiogram of a rat sacrificed 2 hr after a monocular, intravitreal injection of TTX. Zif268 mRNA is reduced in the contralateral primary visual cortex (between arrows). Cortex between the asterisk and the lateral arrow corresponds to the binocular region of rat visual cortex (28). Note that hybridization signal is higher in the binocular region than in the monocular region (medial arrow to asterisk). *In situ* hybridization was done as described (14, 16). (≈ ×8.) (*B*) Zif268 and c-Jun immunohistochemistry [ABC immunoperoxidase method (24)] of visual cortex from a mouse sacrificed 5 hr after monocular, intravitreal administration of TTX. Photomicrographs show full-thickness visual cortex ipsilateral (ipsi) and contralateral (contra) to TTX administration. Immunostaining is blocked by preadsorption of the respective antisera with either Zif268 or c-Jun bacterial protein. (≈ ×70.)

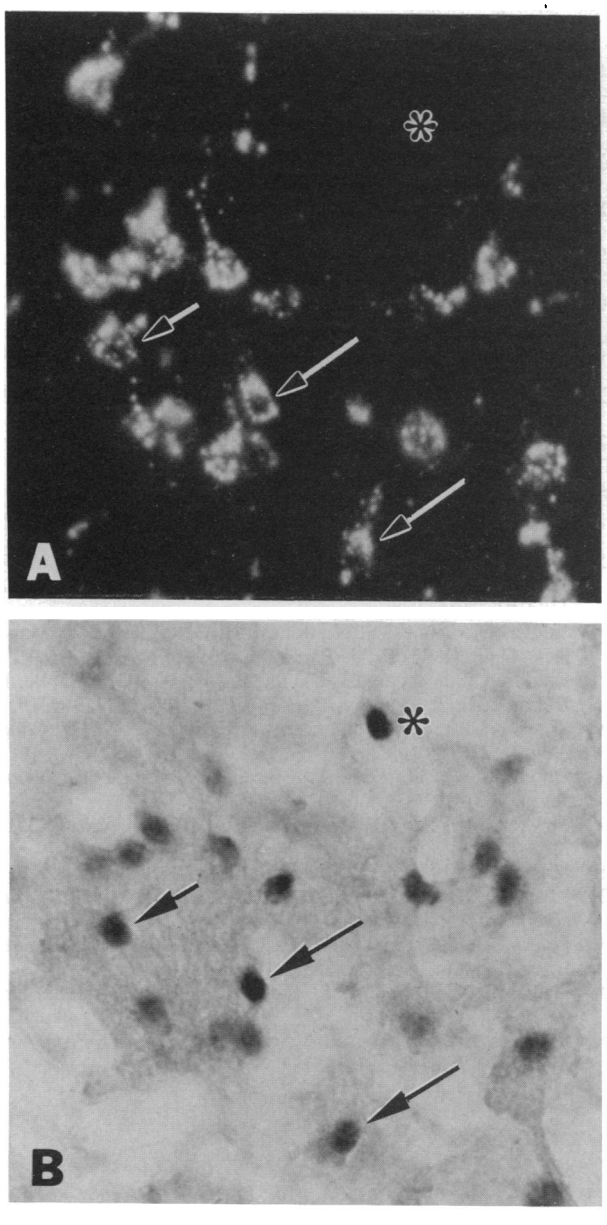

FIG. 3. Colocalization of Zif268 immunostaining and rhodamine microspheres in neurons of visual cortex. Projection neurons from cortical layers V and VI were retrogradely labeled by injecting the lateral geniculate nucleus with rhodamine-conjugated latex microspheres. Only neurons in layers V and VI were fluorescently labeled. (*A*) Fluorescence photomicrograph of layer V. (*B*) Bright-field photomicrograph of the same tissue section showing Zif268 immunostaining of nuclei. Note colocalization of fluorescence and immunostaining in many neurons (arrows); however, not all immunopositive cells were retrogradely labeled (asterisk). (×400.)

DISCUSSION

The major finding of this study is that natural synaptic activity regulates the "constitutive" expression of *zif268* in rat cortex. This conclusion is supported by the rapid reduction of Zif268 mRNA, sequence-specific DNA-binding activity, and immunoreactivity after systemic administration of NMDA-receptor antagonists. Similar rapid reductions in Zif268 mRNA and immunostaining follow intravitreal injections of TTX. We have also found that expression increases rapidly after light exposure in dark-adapted animals. Taken together, these findings suggest that the constitutive levels are maintained by tonic production of short-lived mRNA and protein in response to visually induced synaptic activity. The

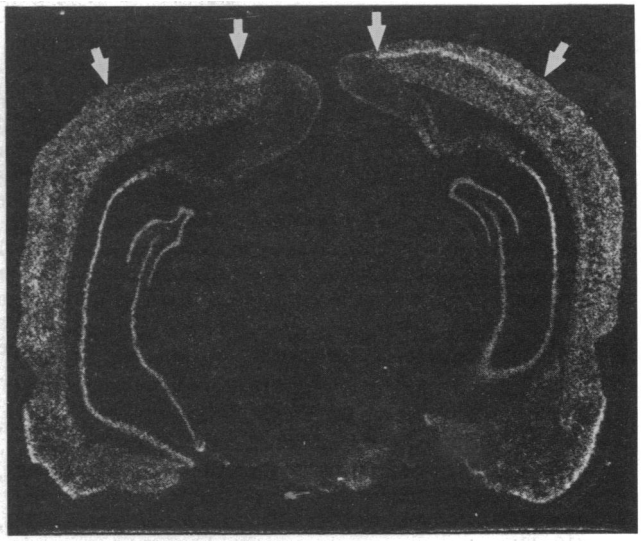

FIG. 4. Zif268 *in situ* autoradiogram demonstrating light-induced increases in Zif268 mRNA in visual cortex of dark-adapted rats. The left half-brain is from an animal maintained in complete darkness for 4 days before sacrifice, whereas the right half-brain is from an identically treated animal 30 min after exposure to light. Arrows delineate visual areas where Zif268 mRNA is light-responsive. Similar results were seen in three additional experiments. (≈ ×5.)

steady-state expression of *zif268* in normal cortex contrasts with its rapid and transient induction after seizures (14) or high-frequency synaptic stimuli (16). Nevertheless, the stable high level of expression in cortex appears to involve rapid turnover of Zif268 mRNA and protein.

Previous studies indicate a role for NMDA receptors in the synaptic regulation of Zif268 in hippocampus (16). In the present study, NMDA-receptor antagonists rapidly reduce basal expression of *zif268* in cortex. Mechanistic interpretation of this result is complicated by the fact that NMDA receptors are activated during normal excitatory transmission in cortex (34, 35). Accordingly, effects of NMDA-receptor antagonists in cortex cannot be easily distinguished from a general reduction of activity. Transmitter mechanisms involved in regulating *zif268* in normal cortex, therefore, remain to be identified.

Retrograde-tracer studies using rhodamine-conjugated latex microspheres demonstrate that Zif268 immunostaining is associated with pyramidal neurons of layers II–III and V–VI as well as with a dense population of cells in layer IV, which may be small pyramidal neurons (33). Intraocular TTX reduces Zif268 immunostaining in all cortical layers, indicating that activity regulates expression of Zif268 in several neuronal subtypes.

Markers for c-*fos* have proven valuable for monitoring neuronal activity and are induced by novel-physiologic stimuli in the spinal cord (12) and suprachiasmatic nucleus (17, 18). In the adult cortex, however, c-*fos* does not appear to be rapidly responsive to alterations of physiological activity. The selective responsiveness of *zif268* suggests that markers for this transcription factor may be especially useful for monitoring natural synaptic activity in cortical neurons.

Monocular deprivation in adult animals changes cytochrome oxidase (4–5), calcium/calmodulin-dependent protein kinase (6), and neurotransmitter expression (7) in neurons of the visual cortex, suggesting that activity is critical for maintaining normal neuronal phenotype. These changes typically occur within days of monocular deprivation and are relatively slow compared with changes in expression of *zif268*. The rapid and selective regulation of Zif268 mRNA and protein in cortex by natural activity suggests a special

role for this transcription factor in maintaining normal cortical physiology.

We are grateful to D. Nathans for advice and encouragement in conducting these studies and to D. Saffen and A. Lanahan for helpful comments and technical assistance. We thank D. Lawrence for expert secretarial assistance. This research was supported by grants from the Esther and Joseph A. Klingenstein Fund (P.F.W.), Lucille P. Markey Charitable Trust (J.M.B.), and Joseph P. Kennedy, Jr., Foundation (J.M.B.), by U.S. Public Health Service Grants 1R01 EY08900 (P.F.W.) and DA-00266 (J.M.B.) and Clinical Investigator Development Award K08-NS01360 (A.J.C.), and by an American Academy of Neurology Research Fellowship (A.J.C.). J.M.B. is a Lucille P. Markey Scholar. B.A.C. and Y.N. were supported by the Howard Hughes Medical Institute.

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