Egr3/Pilot, a Zinc Finger Transcription Factor, is Rapidly Regulated by Activity in Brain Neurons and Colocalizes with *Egr1/zif268*

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Abstract

Programs of gene activation may underlie long-term adaptive cellular responses to extracellular ligands. We have used a differential cDNA cloning strategy to identify genes that are strongly induced by excitatory stimuli in the adult rat hippocampus. Here, we report the rat cDNA sequence of a zinc-finger transcription factor, *Egr3/Pilot*, and characterize its regulated mRNA expression in brain. *Egr3* mRNA is rapidly and transiently induced in neurons of the hippocampus and cortex by electroconvulsive seizure. mRNA levels peak 2 hr after the seizure and remain elevated for as long as 8 hr. *Egr3* mRNA is also rapidly induced in granule cells of the dentate gyrus by synaptic NMDA receptor activation elicited by patterned stimulation of the perforant pathway and by drugs that alter dopamine neurotransmission in the striatum. Basal levels of *Egr3* mRNA in the cortex appear to be driven by natural synaptic activity because monocular deprivation rapidly decreases *Egr3* mRNA in the deafferented visual cortex. Aspects of the protein structure, sequence-specific

DNA binding, transcriptional activity, and regulation of *Egr3* are highly similar to another zinc-finger transcription factor, *Egr1/zif268*. Moreover, we demonstrate colocalization of *Egr3* and *zif268* mRNAs in neurons of normal and stimulated cortex. Our studies suggest that interactions between these coregulated transcription factors may be important in defining long-term, neuroplastic responses.

Introduction

Neurons exhibit the capacity to alter functional and anatomic properties in response to transmitters and growth factors. This capacity of neurons is believed to be essential for learning and memory and is prominently manifest during postnatal development of the neocortex where ocular dominance column formation is dependent on glutamatergic synaptic activity (for review, see Shatz 1990). Kandel and co-workers have demonstrated that long-term changes in synaptic connectivity and structure, that occur in response to transmitter stimulation, require new mRNA and protein synthesis during a relatively brief time window following the stimulus (Montarolo et al. 1986; Bailey et al. 1992). Insight into the cellular mechanisms underlying long-term plasticity has come from studies of growth factor signaling that indicate that receptor activation is linked to the rapid

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induction of regulatory genes, termed immediate-early genes (IEG) (for review, see Nathans et al. 1991). Several lines of evidence support the concept that coordinate and sequential gene regulation may also be relevant to understanding activity-dependent neuronal plasticity (Sheng and Greenberg 1990).

Studies using cultured invertebrate neurons demonstrate that interruption of a specific, IEG transcription factor blocks transmitter induced long-term synaptic plasticity (Alberini et al. 1994). In vertebrate systems, evidence linking IEGs with plasticity remains correlative but is compelling because of the close association between gene expression and the conditions that result in long-term plastic changes. The zinc-finger transcription factor termed *zif268* [(Christy et al. 1988) also termed *Egr1* (Sukhatme et al. 1988), *NGFI-A* (Milbrandt 1987), *Krox24* (Lemaire et al. 1988), and *TIS8* (Lim et al. 1987)] is a notable example of an IEG that is dynamically regulated in mammalian and avian brain in association with stimuli that induce long-term plastic changes. *zif268* is rapidly and transiently induced in neurons of the hippocampus by synaptic NMDA receptor-dependent stimuli in association with long-term potentiation (Cole et al. 1989; Worley et al. 1993 and refs. therein). *zif268* is also rapidly induced in the forebrain of songbirds upon presentation and learning of their species specific song (Mello et al. 1992). *zif268* is developmentally regulated, and peak expression in rodent and cat cortex occurs during the postnatal period when cortical architecture is maximally sensitive to environmental stimuli (Worley et al. 1990; McCormack et al. 1992). *zif268* is expressed at high basal levels in neurons of the cerebral cortex, and this expression is regulated by natural synaptic activity, which is known to be important for maintaining the phenotype of neurons in the adult cortex (Worley et al. 1991; Chaudhuri and Cynader 1993). These observations suggest that transcription factors, such as *zif268*, play a role in activity-dependent neuronal plasticity in the developing and adult vertebrate brain.

To further define the gene program induced in neurons of the mammalian brain by synaptic activity, we and others have used differential cDNA cloning techniques to identify mRNAs rapidly induced in the rodent hippocampus by excitatory stimuli (Nedivi et al. 1993; Qian et al. 1993; Yamagata et al. 1993). In our studies, one of the most abundant differential cDNAs (based on the

frequency of isolates) encodes the rat homolog of the transcription factor *Egr3*. *Egr3* is a 43-kD nuclear phosphoprotein with a zinc finger motif that is highly homologous to the zinc fingers of *zif268*/*Egr1* (Patwardhan et al. 1991). *Egr3* binds the DNA sequence 5'-GCGGGGCG-3' and activates transcription (Patwardhan et al. 1991). This DNA sequence is identical to the consensus sequence recognized by *zif268* (Christy and Nathans 1989; Cao et al. 1990). Despite these similarities, *zif268* and *Egr3* also exhibit several important differences. Outside of the zinc-finger domains, *zif268* and *Egr3* are only ~30% identical, suggesting that they may have distinct regulatory functions. *zif268* is widely expressed in adult tissues (Sukhatme et al. 1987, 1988; Christy et al. 1988) and, as noted, is induced by a variety of stimuli in brain. In contrast, initial studies of *Egr3* could not detect its mRNA expression in adult tissues (Patwardhan et al. 1991).

Because *Egr3* is abundantly represented in our differential cDNA analysis of stimulated brain and because of its many similarities to *zif268*, we undertook a more detailed analysis of its expression and regulation. Here, we report the sequence of the rat homolog of *Egr3* and characterize its mRNA expression and cellular distribution in brain. Our data indicate that *Egr3* mRNA is expressed at relatively high levels in neurons of normal brain and is rapidly regulated in parallel with *zif268*. Moreover, *zif268* and *Egr3* mRNAs have similar developmental and anatomic patterns of expression and are coexpressed in discrete populations of neurons of the normal and stimulated cortex. Because *zif268* and *Egr3* bind to the same consensus DNA sequence, our observations suggest that interactions between these transcription factors may be important in defining specific transcriptional responses in brain neurons.

Materials and Methods

CONSTRUCTION AND SCREENING OF THE SUBTRACTED cDNA LIBRARY

A subtracted cDNA library was constructed as described (Yamagata et al. 1993). The subtracted library was screened with ³²P-labeled cDNA prepared by reverse transcription of poly(A)⁺ RNA prepared from hippocampus of control or seizure stimulated rats pretreated with cycloheximide (20 mg/kg, i.p.) as described previously (Lanahan et al.

1992; Yamagata et al. 1993). cDNA clones of the rat homolog of *Egr3* represented ~30% of all differential cDNA isolates (14 of 47). A near full length cDNA of rat *Egr3* was isolated by iterative screening of an unsubtracted, oligo(dT)-primed cDNA library prepared from hippocampus 4 hr postseizure.

DNA SEQUENCING

Both strands of *Egr3* cDNA corresponding to the open reading frame were sequenced as double-stranded plasmids with synthetic, specific primers by the dideoxynucleotide chain termination method using deoxyadenosine 5'-[α - 35 S]thio triphosphate and Sequenase (U.S. Biochemical).

NORTHERN BLOT ANALYSIS

This procedure was performed as described (Linzer and Nathans 1983) with 10 μ g of total RNA per lane. The probe used for Northern analysis was a 1.7-kb 3' end fragment of rat *Egr3* cDNA that is entirely a noncoding sequence and is not homologous to other zinc finger genes. The cDNA fragment was labeled by the random priming technique (Pharmacia) using [α - 32 P]dCTP.

IN SITU HYBRIDIZATION

Freshly dissected brain tissue was frozen rapidly in plastic molds placed on a dry-ice/ethanol slurry as described previously (Cole et al. 1990). Control and experimental tissues were frozen in the same tissue block to assure identical conditions during tissue sectioning, subsequent storage, and in situ hybridization. 35 S-Labeled *Egr3* antisense riboprobe was prepared from an appropriately restricted pBS plasmid containing a 1.7-kb *Egr3* 3' cDNA sequence that, as noted above, is unique for *Egr3*. Digoxigenin-labeled *zif268* antisense cRNA was prepared from a full-length cDNA cloned in pBS according to the manufacturer's protocol (Boehringer Mannheim). In situ hybridization was performed as described (Saffen et al. 1988). The concentration of riboprobes was 20–40 $\times 10^6$ cpm/ml of hybridization buffer (50% formamide, 600 mM NaCl, 1 $\times$ Denhardt's solution, 10 mM Tris-Cl at pH 7.4, 1 mM EDTA, 0.2 mg/ml of yeast tRNA, 10 mM DTT, 10% dextran sulfate, 100 μ g/ml heat-denatured, sheared salmon sperm

DNA) for *Egr3* and ~1 μ g/ml for *zif268*. Tissue sections were incubated under glass coverslips overnight at 56°C. Coverslips were removed in 2 $\times$ SSC (10 min at room temperature), and tissue sections were washed in 2 $\times$ SSC (2 $\times$ 10 min at room temperature) followed by incubation in 2 $\times$ SSC containing 10 mM β -mercaptoethanol and 10 μ g/ml of RNase A (Worthington) for 30 min at 37°C and subsequently washed 20 min in 2 $\times$ SSC. For experiments examining *Egr3* mRNA alone, tissue sections were briefly dipped in dH₂O, dehydrated through ascending ethanols, and exposed to XAR5 film for 12–72 hr. For studies of combined localizations of *Egr3* and *zif268*, the digoxigenin-labeled riboprobe was first detected using an alkaline phosphatase-linked, antidigoxigenin antiserum (Boehringer Mannheim) according to the manufacturer's directions. Typically, the alkaline phosphatase reaction was continued for ~18 hr. Tissue sections were then dehydrated and dipped in a 2% collodion solution (Electron Microscopy Sciences) to prevent reactivity between the alkaline phosphatase product and the photographic emulsion (Young 1989). Selected slides were treated with photographic emulsion (Kodak NTB2) and counterstained as described previously (Jordan 1990). Operationally, positive alkaline phosphatase reaction was defined as staining sufficiently strongly to clearly delineate cellular borders. In addition, staining in the relatively hypocellular molecular layer served as an internal background control for layers II–VI. The validity of our double-labeling analysis was confirmed by simultaneous trials in adjacent sections, using radiolabeled probes for both genes, which displayed similar relative proportions of mRNA labeling.

ELECTROPHYSIOLOGY

Maximal electroconvulsive seizures (MECS) were induced in adult rats using a constant current signal generator (ECT unit, Ugo Basil) as described previously (Cole et al. 1990). For long-term potentiation (LTP) studies, Fischer-344 rats were implanted bilaterally with stimulating and recording electrodes in the perforant path and hilus of the dentate gyrus as described previously (Worley et al. 1993). Rats were allowed to recover for at least 3 weeks before any recordings were performed.

To examine the regulation of *Egr3* mRNA by synaptic stimuli, 12 chronically implanted rats received high-frequency (HF) stimulation in one

hemisphere and low-frequency (LF) stimulation in the other hemisphere. Electrical stimuli consisted of 200 msec diphasic, constant current pulses given at a stimulus intensity of 500 mA. The LF test stimulation was delivered at 0.1 Hz, and the HF stimulation parameters consisted of 50 repetitions of a 20-msec train (i.e., 8 pulses) delivered at 400 Hz (400 total pulses). The HF parameters reliably induce LTP (Dragunow et al. 1989; Jeffrey et al. 1990; Abraham et al. 1992; Worley et al. 1993). Following this treatment, the rats were sacrificed at either 30 min ($n=6$), 1 hr ($n=2$), 2 hr ($n=2$), or 4 hr ($n=2$). Three additional animals were pre-treated with MK-801 (1 mg/kg, i.p.) 1 hr prior to the delivery of the HF stimulus. The intensity of the stimulus was adjusted such that the postsynaptic population response was identical to that prior to MK-801 administration. Animals were sacrificed 30 min after the HF stimulus and processed for in situ hybridization.

VISUAL DEAFFERENTATION AND ADMINISTRATION OF DOPAMINERGIC AGENTS

Visual deafferentation was performed in adult Sprague–Dawley rats as described previously (Worley et al. 1991) using monocular, intravitreal injections of the sodium channel antagonist tetrodotoxin (TTX). Dopaminergic agents were admin-

istered by intraperitoneal injection as described previously (Cole et al. 1992) and included cocaine (15 mg/kg), the selective D₁ receptor antagonist SCH 23390 (Hyttel 1983; 0.5 mg/kg), and the D₂ receptor antagonist haloperidol (1 mg/kg).

Results

NUCLEOTIDE AND AMINO ACID SEQUENCE OF *Egr3*

We cloned and sequenced a nearly full-length cDNA of rat *Egr3*/Pilot cDNA. This cDNA encodes a single open reading frame of 387 amino acids and a calculated molecular weight of 42,579. Its amino acid sequence is 98% identical to human *Egr3*/Pilot (Patwardhan et al. 1991; Mages et al. 1993). Figure 1 shows a comparison of amino acid sequences of rat *Egr3*, human *Egr3* (Patwardhan et al. 1991), and rat NGFI-A. Figure 2 is a schematic representation of the zinc-finger domains of rat *Egr3* and NGFI-A (identical to zif268) underscoring their high homology.

EXPRESSION OF *Egr3* IN BRAIN NEURONS AND INDUCTION BY SEIZURE

Egr3 mRNA was detected in control hippocampus, using Northern blots of total RNA, and

Figure 1: Comparison of the predicted rat (r) *Egr3* protein, human (h) *Egr3* protein (Patwardhan et al. 1991), and rat (r) NGFI-A (Milbrandt 1987). The conserved cysteine and histidine residues of the zinc fingers are marked with an asterisk (*). The amino acids that are conserved in two or more members are boxed. The nucleotide sequence has been entered into GenBank under accession number U12428.

Figure 2: Diagrammatic representation of the three rat (r)Egr3 zinc fingers predicted from the crystal structure of NGFI-A (Pavletich and Pabo 1991). Amino acid loops or fingers are produced by cysteine and histidine pairs that chelate a zinc ion. Each zinc finger contains two β -sheets (thick black lines) and one α -helix domain (thick gray line). Two residues in each zinc finger (star) contact guanine nucleotides in the major groove of the DNA. rEgr3 and rNGFI-A conserved residues are encircled, whereas nonconserved are contained in squares (rNGFI-A and zif268 zinc-finger domain sequences are identical). Below the diagram, rEgr3 and rNGFI-A zinc-finger sequences are compared. β -Sheet residues are in bold, α -helix domains appear underlined, and DNA contacts are outlined.

was strongly induced by a single MECS (Fig. 3A). The message is ~ 4.4 kb and is very similar in size to the human transcript (Patwardhan et al. 1991; Mages et al. 1993). In situ hybridization was performed to examine the time course and anatomical

distribution of basal and induced *Egr3* mRNA in hippocampus. *Egr3* mRNA was strongly induced in granule cells within 30 min after MECS, peaked within 1–2 hr, and remained elevated as long as 8 hr after the seizure (Fig. 3B). The time course of

Figure 3: Regulation of *Egr3* mRNA expression by seizure. (A) Northern analysis. Total RNA was prepared from hippocampus of control rats and rats pretreated for 30 min with cycloheximide (20 mg/kg) and sacrificed 4 hr after MECS. Ten micrograms was loaded per lane. Arrows indicate the positions of the 18S and 28S ribosomal bands. (B,C) Time course of *Egr3* (B) and *zif268* (C) mRNAs in hippocampus after seizure. Hippocampi were dissected from control rats and from rats 0.5, 1, 2, 4, 6, 8, and 24 hr after MECS and 6 hr after MECS from an animal pretreated with cycloheximide. The hippocampi from the different time points were processed for in situ hybridization together in a single tissue block to assure identical hybridization conditions. Magnification, $\sim 4\times$. The time of sacrifice after MECS is indicated by the number above each individual hippocampus. MECS induces an increase in *Egr3* (B) and *zif268* (C) mRNAs in the granule cell layer of the hippocampus, which is indicated by the arrowhead in the control (c) hippocampus. Note that *Egr3* mRNA remains elevated at 8 hr whereas *zif268* mRNA returns to basal levels by 6 hr (*).

the increase in *Egr3* mRNA following MECS was somewhat prolonged compared with that of *zif268*, which returned to basal levels by 6 hr. Both *Egr3* and *zif268* mRNAs were strongly induced in animals pretreated with cycloheximide, suggesting that they are regulated as IEG in brain (Qian et al. 1993).

Egr3 and *zif268* mRNA localizations in forebrain were examined and compared in control and MECS stimulated rats. In control rat brain, highest levels of *Egr3* mRNA were detected in cerebral cortex, caudate-putamen, amygdala and hippocampal granule, and pyramidal cell layers (Fig. 4A). Two hours after a single MECS, *Egr3* mRNA increased dramatically in the hippocampus, superficial and deep layers of the cerebral cortex, piriform cortex, amygdala, and hypothalamus. The an-

Figure 4: Comparison of anatomic localizations of *Egr3* and *zif268* mRNAs in forebrain assayed by in situ hybridization. Composite figure showing coronal sections of unstimulated (left half-brain) and MECS-stimulated (right half-brain) rat brain. *Egr3* (A) and *zif268* (B) basal mRNA levels are detected in cerebral cortex, hippocampus, basal ganglia, and amygdaloid complex. After MECS, there is a marked increase in both mRNAs in these same regions. Note the overall similarity in the pattern of basal and induced expression of *Egr3* and *zif268*. Magnification, $\sim 5\times$.

atomic pattern of *Egr3* mRNA is very similar to that of *zif268* in both the control and MECS stimulated forebrain (Fig. 4B).

Photographic emulsion was used to determine the cellular localization of *Egr3* mRNA by in situ hybridization. In control and MECS stimulated forebrain, clusters of autoradiographic grains were associated with neurons based on the size, cytological features, and staining properties of the cells. In control brain, double in situ localizations of *Egr3* and *zif268* (detected using digoxigenin-labeled riboprobe) demonstrated, in addition to well-defined digoxigenin-labeled profiles and independent clusters of grains, double-labeled cells that represented coexpression of these mRNAs in a subset of neurons in layer 2–3 of the neocortex (Fig. 5A). Coexpression of *Egr3* and *zif268* mRNAs was also detected in subsets of neurons in layers 5–6 of neocortex, layer 2 of piriform cortex, and pyramidal cells of hippocampus in control animals (not shown). Although we did not quantitate the number of neurons coexpressing *Egr3* and *zif268* relative to the numbers expressing either mRNA alone, cells expressing both mRNAs were abundant in all microscopic fields. Two hours after MECS, there was a large increase in the number of neurons expressing either *Egr3* or *zif268* (increase in cell number and intensity of cell staining) and many cells coexpressed the mRNAs (Fig. 5B).

STIMULATION OF *Egr3* mRNA BY NMDA RECEPTOR ACTIVATION

The chronic in vivo LTP paradigm was used to deliver monosynaptic stimuli of defined intensities and frequencies to granule cells of the hippocampus in alert, behaving rats (McNaughton et al. 1986; Worley et al. 1993). *Egr3* mRNA was strongly induced in the hippocampal granule cells ipsilateral to the HF stimuli (Fig. 6) in each of six animals sacrificed 30 min after the stimulus, as well as in each of the animals sacrificed after 1 hr ($n = 2$), 2 hr ($n = 2$), or 4 hr ($n = 2$). No induction was detected in granule cells that received the LF stimulation ($n = 12$) or in hippocampus that had been implanted with electrodes but not electrically stimulated ($n = 2$).

Consistent with previous studies (McNaughton et al. 1986; Worley et al. 1993), this pattern of HF stimulation induced LTP in each of these animals. LTP induced by HF stimulation is dependent

Figure 5: Localization of *zif268* and *Egr3* mRNAs in cortex of control (A) and seizure-stimulated (B) rats. *zif268* mRNA is detected using digoxigenin-labeled riboprobe (alkaline phosphatase reaction product that fills the cell soma), and *Egr3* is detected with ^{35}S -labeled riboprobe (autoradiographic silver grains) as described in Materials and Methods. Photomicrographs are from superficial layers of somatosensory cortex. *zif268* and *Egr3* mRNAs are expressed in neurons of lamina 2–3 (ml = molecular layer). A subpopulation of neurons coexpresses *zif268* and *Egr3* (arrowheads). One hour after MECS, there is a large increase in the number of neurons expressing either *zif268* or *Egr3* and both mRNAs. Bar, 100 μm .

on activation of NMDA-type glutamate receptors (Abraham and Mason 1988). To examine whether the induction of *Egr3* mRNA is mediated by NMDA receptor activation, we pretreated rats with the noncompetitive NMDA receptor antagonist MK-801 (1 mg/kg) 1 hr prior to delivery of the standard HF stimulus and sacrificed animals 30 min after the stimulus. In animals pretreated with MK-801 ($n = 3$), there was no detectable induction of

Egr3 mRNA in the hippocampus by the HF stimulus (not shown).

ACTIVATION OF *Egr3* mRNA BY COCAINE AND HALOPERIDOL

To determine whether dopaminergic stimulation can activate *Egr3* mRNA in adult rat brain, we examined the effect of cocaine administration on *Egr3* mRNA levels using in situ hybridization. Cocaine (15 mg/kg) produced a rapid increase in *Egr3* mRNA levels in the striatum (Fig. 7A). *Egr3* mRNA was induced within 1 hr and remained elevated 4 hr after cocaine administration. Induction of *Egr3* by cocaine was blocked by prior treatment with the selective D_1 receptor antagonist SCH

Figure 6: Induction of *Egr3* mRNA in hippocampal granule cells by HF stimulation of the perforant pathway. Rats were chronically implanted for in vivo recording and were stimulated as described in Materials and Methods. Stimulated brains were analyzed by in situ hybridization together with half-brains from naive control (C) and MECS-stimulated (S) rats. *Egr3* mRNA is increased in the granule cell layer of the hippocampus 30 min after the HF but not the LF stimulus. Note that the magnitude of *Egr3* mRNA induction by the HF stimulus is comparable with that of MECS. Identical results were obtained in each of five additional animals killed 30 min after onset of HF stimulation.

Figure 7: *Egr3* mRNA is rapidly induced in the striatum following acute administration of cocaine (15 mg/kg) or haloperidol (1 mg/kg). Half-brains from control rats (C) and rats sacrificed at the indicated times (1, 2, and 4 hr) after drug administration were processed together for in situ hybridization. Magnification, $\sim 4\times$. Note that cocaine and haloperidol induce *Egr3* mRNA selectively in the striatum and persist to 4 hr.

23390 (0.5 mg/kg administered 30 min before cocaine; data not shown). In contrast, administration of the D₂ antagonist haloperidol (1 mg/kg) resulted in a rapid increase in *Egr3* mRNA in the striatum (Fig. 7B). These responses of *Egr3* mRNA are similar to those of *zif268* reported previously (Cole et al. 1992; Nguyen et al. 1992).

REGULATION OF BASAL EXPRESSION BY NATURAL SYNAPTIC ACTIVITY

We examined the hypothesis that basal *Egr3* expression in the cortex is regulated by natural synaptic activity. Interruption of afferent synaptic activity to the visual cortex, by infusing tetrodotoxin into one eye, results in a rapid and anatomically discrete decrease in *zif268* mRNA in the deprived visual cortex of rats (Worley et al. 1991). Consistent with previous studies, *zif268* mRNA was reduced in the primary visual cortex of rats that received an intravitreal injection of tetrodotoxin 18 hr prior to sacrifice (Fig. 8B). *Egr3* was similarly reduced in the primary visual cortex of these rats (Fig. 8A, $n = 3$).

DEVELOPMENTAL REGULATION OF *Egr3* mRNA

In situ analysis of newborn (day 1) pups demonstrates *Egr3* mRNA is enriched in the caudate-

Figure 8: *Egr3* and *zif268* mRNAs are regulated by afferent visual activity. Adult rats received monocular, intravitreal injections of TTX and were sacrificed 18 hr later. In situ hybridization of *Egr3* (A) and *zif268* (B) mRNAs is reduced in primary visual cortex contralateral to the ocular TTX injection. Arrowheads at lower left of each photograph indicate the region of reduced hybridization in the visual cortex. Upper panels correspond to control brains hybridized, for each probe, simultaneously to TTX-injected subjects. Magnification, $\sim 4\times$.

Figure 9: Developmental regulation of *Egr3* mRNA in rat brain. In situ localizations at postnatal days 1 (A), 5 (B), 12 (C), and 21 (D). Magnification, $\sim 3\times$. *Egr3* mRNA is prominent in the caudate putamen (CP), septum (S), hippocampus (H), amygdala (A), and piriform cortex (Pi) at day 1. By day 5, levels are also prominent in the neocortex. The pattern of expression at day 12 and 21 is similar to the adult.

putamen, septum, piriform cortex, and hippocampal CA1–CA4 regions (Fig. 9A). By postnatal day 5, *Egr3* mRNA is also enriched in neocortical regions (Fig. 9B). Day 12 and 21 forebrains demonstrate a qualitatively similar pattern of expression to that of adult brain (Fig. 9C,D).

Discussion

CLONING OF RAT *Egr3*

We are using a differential cloning strategy to identify genes that are rapidly regulated in brain

neurons in response to transmitter stimulation as approach to understand the molecular basis of neuronal plasticity (Yamagata et al. 1993). The rat homolog of *Egr3* was identified and characterized on the basis of its rapid induction in brain by depolarizing stimuli. The nucleotide and predicted amino acid sequences of rat *Egr3* are highly homologous to the human sequences. The predicted rat *Egr3* amino acid sequence is 98% identical to the human sequence, and all substitutions are conservative.

Aspects of the protein structure and transcriptional activity of *Egr3* are discussed with the initial characterization of this gene (Patwardhan et al. 1991; Mages et al. 1993) and are reviewed here only briefly. *Egr3* contains three tandem zinc finger structures near its carboxyl terminus. Within this region, *Egr3* is highly homologous to other members of the family of Cys₂-His₂ zinc-finger transcription factors that includes *zif268*, *Egr2* (Chavrier et al. 1988; Joseph et al. 1988), *NGFI-C*, and the Wilms' tumor gene product (Rauscher et al. 1990). The crystal structure of the *zif268*-DNA complex indicates that each zinc finger contains a β -sheet domain in the amino-terminal region of the finger and a α -helical domain in the carboxyl half of the finger (Pavletich and Pabo 1991). A zinc ion is chelated by two histidines in the α -helical domain and two cysteines in the β -sheet. Amino acid residues from the amino-terminal portion of the α -helix contact base side chains of the G-rich strand in the major groove. Consistent with the high degree of conservation within their finger regions, each of the members of this group of zinc finger transcription factors binds to the same consensus DNA sequence (Christy and Nathans 1989; Cao et al. 1990; Chavrier et al. 1990; Lemaire et al. 1990; Rauscher et al. 1990; Crosby et al. 1991; Patwardhan et al. 1991; Mages et al. 1993).

Analysis of the *Egr3* amino acid sequence in the nonfinger domain shows an abundance of proline, serine, and alanine. The homologous regions of *zif268*, *Egr2*, and *NGFI-C* are also rich in these amino acids, and like these other zinc finger transcription factors, *Egr3* demonstrates sequence-specific transcriptional activation (Patwardhan et al. 1991). Proline rich regions have been described as activator domains of transcription factors (Mermod et al. 1989), including *zif268* (Gashler et al. 1993), and this general conservation may reflect an underlying common functional role. However, there is only ~30% identity in this region between *zif268* and *Egr3*, and domains that

might confer distinct functions on *Egr3* have not yet been identified.

REGULATION OF *Egr3* mRNA IN BRAIN

Initial studies characterizing *Egr3* demonstrated its expression in mitogen-stimulated fibroblasts and T cells but suggested that its expression may be highly restricted in other tissues (Patwardhan et al. 1991; Mages et al. 1993). Our data provide the first demonstration of the expression and regulation of *Egr3* in the whole animal. *Egr3* mRNA is detected in many neuronal populations in normal brain and is strongly induced in the adult brain by depolarizing stimuli. After a single MECS, *Egr3* mRNA increases markedly in hippocampal granule cells, neurons of the piriform cortex, amygdala, and neurons of layers 2–3 and 5–6 of the neocortex. The time course of *Egr3* mRNA induction after MECS is rapid and transient and similar to its time course of expression in fibroblasts following serum stimulation (Patwardhan et al. 1991).

zif268, *NGFI-C*, and *Krox20* are also rapidly and transiently induced in hippocampal neurons by MECS (Cole et al. 1990; Bhat et al. 1992; Crosby et al. 1992). A direct comparison of the time courses of *Egr3* and *zif268* mRNA responses in hippocampal granule cells following MECS indicates that their initial inductions are similarly rapid but that *Egr3* mRNA remains elevated several hours longer than *zif268*. Differences in the time courses of these highly related proteins could be important in effecting specific responses to a particular stimulus. For example, at early time points, *Egr3* and *zif268* might compete for the same enhancer regions, whereas at later points, *Egr3* could act alone. Accordingly, it will be important to determine if this difference in time course between *zif268* and *Egr3* mRNAs is also manifest at the level of the protein.

Egr3 mRNA is induced in hippocampal granule cells by NMDA-dependent synaptic mechanisms. Using the chronic in vivo LTP paradigm, HF, but not LF, stimulation of perforant-pathway afferents results in a robust induction of *Egr3* mRNA. The time course of this response appears similar to that induced by MECS. Consistent with previous studies (Abraham and Mason 1988; Reed and Robinson 1991; Worley et al. 1993), systemic administration of an NMDA-type glutamate receptor antagonist blocked synaptic enhancement in our

preparations and also blocked the induction of *Egr3* mRNA. These data indicate that *Egr3* is induced by NMDA-dependent synaptic mechanisms in association with LTP.

Because *Egr3* mRNA is expressed at relatively high levels in neocortex of normal, behaving rats, we have examined the hypothesis that basal *Egr3* expression is regulated by natural synaptic activity. Blockade of visual activity, by infusing TTX into one eye, results in a rapid reduction of *Egr3* mRNA in the deafferented visual cortex. This observation is consistent with the notion that afferent visual activity continuously activates the expression of specific IEG transcription factors in neurons of the visual cortex and thereby regulates their basal expression (Worley et al. 1991; Chaudhuri and Cynader 1993). Visual deprivation in the adult animal results in time-dependent, phenotypic changes in neurons of the visual cortex, including reductions in cytochrome-oxidase and GABA staining as well as increases in type II calcium/calmodulin-dependent kinase (Hendry and Carder 1992). These changes are observed typically several days following continuous visual deafferentation and presumably underlie adaptive cortical plasticity. The rapid time course of changes in *Egr3* mRNA with visual deprivation suggests that it may play a regulatory role in long-term changes of neuronal phenotype.

Developmental studies indicate that *Egr3* mRNA expression is high in the newborn striatum, septum, piriform cortex, and hippocampus. During the first postnatal week, expression increases markedly in the neocortex. By postnatal day 12, *Egr3* expression is very similar to the adult pattern and level of expression. This developmental and anatomic pattern of expression is similar to that of *zif268* (Worley et al. 1990) with the exception of the high *Egr3* mRNA levels in the neonatal striatum. The developmental profile together with observations indicating that physiological activity regulates expression of *Egr3* suggest a role for *Egr3* in activity-dependent development of brain.

Previous studies have demonstrated that dopaminergic stimulation elicits robust IEG expression in the striatum (Robertson et al. 1989; Graybiel et al. 1990; Cole et al. 1992). Pharmacological agents that modify dopamine-dependent signaling also induce *Egr3* in the brain. Cocaine administration results in a rapid increase in *Egr3* mRNA in the striatum that is blocked by a selective D₁ antagonist. Additionally, haloperidol, a D₂ antagonist, induces a rapid increase in *Egr3* mRNA in

the striatum. This pharmacological profile is similar to that of *c-fos* and *zif268*, which are also induced in the striatum by D₁ agonists and D₂ antagonists (Moratalla et al. 1992; Nguyen et al. 1992; Robertson et al. 1992). A role for cocaine- or haloperidol-induced gene expression has been proposed in chronic adaptive responses to these drugs (Hope et al. 1992; Nguyen et al. 1992).

COEXPRESSION OF ZINC FINGER FAMILY TRANSCRIPTION FACTORS IN BRAIN

Double in situ localizations indicate that *Egr3* and *zif268* mRNAs are coexpressed in neurons of normal, as well as MECS-stimulated, cortex. *zif268* and *Egr3* are also coregulated in granule cells of the hippocampus in the LTP paradigm, in the adult visual cortex in our monocular deafferentation experiments, and in the striatum following cocaine or haloperidol administration. Because *zif268* and *Egr3* bind to the same consensus DNA sequence, our observations suggest that interactions between these transcription factors may be important in defining transcription factor-specific effects. The potential complexity of interactions between transcription factors is further underscored by observations that *Egr2* and *NGFI-C* are also induced in brain by seizures (Bhat et al. 1992; Crosby et al. 1992) and that *Egr2* is induced with LTP (Worley et al. 1993). A similar situation is evident for the AP1 family of transcription factors, which are coinduced in brain by seizures (Saffen et al. 1988; Sonnenberg et al. 1989). It should be noted that evidence of coexpression of these transcription factors in the brain is largely limited to conditions of nonnatural stimulation such as LTP or seizures, and it remains a possibility that under natural conditions, different transcription factors are expressed in distinct populations of cells. Accordingly, we were interested in demonstrating that *Egr3* and *zif268* are coexpressed in neurons of control cortex.

Several mechanisms have been identified that may be important in determining a specific transcriptional response under conditions in which multiple transcription factors are induced that can bind to the same DNA consensus sequence. Differences in the time course of expression of potentially competing transcription factors may provide for functional distinction. This may be the case for *zif268* and *Egr3* and for different AP-1 complexes (Sonnenberg et al. 1989). Additionally, transcrip-

tion factor family members may exhibit differential binding affinities to their shared core consensus sequence or binding may be selectively altered by DNA sequences flanking the core region (Ryseck and Bravo 1991). Transcription factors are also known to interact with other proteins that modulate DNA binding or transcription efficiency (Russo et al. 1993) and undergo post-translation modifications that can alter these functions (Fahrner et al. 1990). *zif268* has gained prominence as being one of the most sensitive and specific markers of synaptic activity and plasticity and has focused attention on target genes that may be regulated by *zif268*. Evidence of coexpression of *Egr3* and *zif268* mRNAs in neurons of control brain suggests that a detailed analysis of mechanisms regulating transcription from their shared core consensus sequence will be important in understanding the molecular basis of natural plasticity.

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Egr3/Pilot, a zinc finger transcription factor, is rapidly regulated by activity in brain neurons and colocalizes with Egr1/zif268.

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