The Neuroplasticity-Associated Arc Gene Is a Direct Transcriptional Target of Early Growth Response (Egr) Transcription Factors

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Early growth response (Egr) transcription factors (Egr1 to Egr4) are synaptic activity-inducible immediate early genes (IEGs) that regulate some aspects of synaptic plasticity-related to learning and memory, yet the target genes regulated by them are unknown. In particular, Egr1 is essential for persistence of late-phase long-term potentiation (L-LTP), for hippocampus-dependent long-term memory formation, and for reconsolidation of previously established memories. Here, we show that Egr1 and Egr3 directly regulate the plasticity-associated activity-regulated cytoskeletal-related (Arc) gene, a synaptic activity-induced effector molecule which is also required for L-LTP and hippocampus-dependent learning and memory processing. Moreover, Egr1-deficient and Egr3-deficient mice lack Arc protein in a subpopulation of neurons, while mice lacking both Egr1 and Egr3 lack Arc in all neurons. Thus, Egr1 and Egr3 can indirectly modulate synaptic plasticity by directly regulating Arc and the plasticity mechanisms it mediates in recently activated synapses.

Neurons process and retain information by establishing networks of synaptic connections that are modified by repeated activation. These “plasticity” changes are mediated by synaptic activity-regulated intraneuronal signal transduction pathways, some of which induce new gene expression and protein synthesis. Immediate early genes (IEGs) are rapidly induced as the earliest genomic response to synaptic activity (6). IEGs encode both transcriptional regulators and direct effector molecules, such as structural proteins, signaling enzymes, and growth factors that participate in synaptic and structural alterations made by neurons in response to their activity-dependent history (8). Transcriptional regulatory IEGs further diversify neuron responses to activation by broadening the regulatory control over long-term plasticity changes involved in complex processes, such as learning and memory. However, the precise molecular mechanisms mediated by activity-induced transcriptional regulators are poorly defined because the target genes they regulate are unknown.

The early growth response (Egr) family of activity induced IEGs encodes four zinc-finger transcription factors that are designated Egr1 (also known as zif268, NGFI-A, Krox24, Tis8, and ZENK), Egr2 (also known as Krox20), Egr3 (also known as Pilot), and Egr4 (also known as NGFI-C and PAT133) (20). They have nearly identical zinc finger DNA binding domains near their C terminus, they are often coregulated by similar stimuli in many cell types (including neurons), and they can transactivate gene expression by binding a GC-rich motif (Egr response element [ERE]) located in the promoter regions of target genes (31). In neurons, Egr genes are robustly and transiently expressed by elevated cytosolic calcium produced by synaptic activity (7, 18), and their expression is coupled with N-methyl d-aspartate (NMDA) receptor activation and intracellular mitogen-activated protein kinase (MAPK) signaling in excitation glutamatergic synapses, by either abnormal (seizure induced) or physiologic synaptic activity. A critical role for the archetypal family member Egr1 (zif268) in regulating some neuroplasticity responses has been clearly established. For example, early studies demonstrated a strong correlation between Egr1 gene expression and stabilization of long-term potentiation (LTP) (a particular form of synaptic plasticity) in response to appropriate-patterned synaptic activity (1, 40). Recently, studies using Egr1-deficient mice and antisense oligonucleotides have confirmed a critical role for Egr1 in LTP persistence (late-phase LTP [L-LTP]), long-term memory in hippocampus-dependent tasks (12) and in reconsolidation of previously memorized tasks (5, 13). Thus, despite the fact that Egr1 has a clear role in mediating gene expression required for some learning and memory processes, the specific molecular mechanisms involved are poorly defined because the target genes that Egr1 regulates in the brain have not yet been identified. Moreover, comparatively little is known about the potential role of other Egr transcription factors in plasticity-related mechanisms, although they are regulated by similar intracellular signal transduction pathways and they may also mediate some forms of neuroplasticity, such as stabilization of LTP (38, 41).

In a previous study, we identified the activity-regulated cytoskeletal associated (Arc) gene as a potential target gene regulated by Egr transcription factors (2). Arc (also known as Arg3.1) is a particularly interesting effector plasticity-associated molecule because, like Egr transcription factors, it is rapidly induced by synaptic activity (15, 17), its expression depends upon excitatory synaptic NMDA receptor activation (28) and intracellular MAPK signaling (36), and it has a critical role in maintaining LTP and long-term, but not short-term, memory formation (9). In activated neurons, Arc mRNA is rapidly
distributed into dendrites, where it is targeted to recently activated synapses and locally translated into protein, suggesting that it has a direct role in the plasticity responses made within synapses after activation (26, 27). Moreover, similar to Egr transcription factors, the Arc gene is regulated as an immediate early gene that is rapidly upregulated without the need for new protein synthesis. However, the Arc gene is also regulated as a delayed target gene by a poorly understood protein synthesis-dependent mechanism that appears to modulate its expression beyond the brief transient immediate early phase (35).

Based upon evidence that the Arc gene may be regulated as a delayed target gene and that there are numerous similarities between Egr and Arc gene regulation and function related to memory processing, we examined whether Egr transcription factors are necessary for regulating Arc during its protein synthesis-dependent phase of expression. We found that enforced expression of Egr1 and Egr3 in myotubes and primary hippocampal and cortical neurons was sufficient to induce Arc expression by directly binding and transactivating the Arc promoter through a species-conserved high-affinity ERE in its proximal promoter. Accordingly, when Egr1 and Egr3 proteins were coinduced by seizure-related synaptic activity in vivo, the protein synthesis-dependent phase of Arc expression required Egr3. However, during physiologic synaptic activity induced by a novel environment, both Egr1 and Egr3 were required to induce normal levels of Arc expression in the brain and in mice lacking both Egr1 and Egr3 proteins, Arc protein was absent in activated neurons.

Egr1 and potentially Egr3 are important transcriptional regulators of some plasticity changes associated with learning and memory but to what extent they regulate genes capable of modifying synaptic function or influencing the structural arrangement of synapses is not known. These results identify the Arc gene as the first known direct Egr target gene that has a clear role in mediating some forms of synaptic plasticity during learning and memory processing. Hence, Egr1 and Egr3 can indirectly regulate the physiologic properties of recently activated synapses by directly modulating Arc gene expression.

MATeRIALS AND METHODS

Animals and experimental treatments. (i) Animals. Egr1-deficient and Egr3-deficient mice were generated and genotyped as previously described (14, 32, 34, 37). Egr1-deficient mice were backcrossed 10 generations to C57BL/6J mice and Egr3-deficient mice were backcrossed four generations to C57BL/6J mice. Littermate wild-type mice were used as controls for all experiments in which comparisons were made between wild-type and Egr gene-deficient mice. All experimental procedures complied with protocols approved by the Northwestern University Institutional Animal Care and Use Committee.

(ii) Kainic acid-mediated seizure induction. Seizures were induced by kainic acid (KA) (20 mg/kg; Sigma, St. Louis, MO) intraperitoneal injection (i.p.) in adult mice (2-15 weeks of age). Control mice received phosphate-buffered saline (PBS) injections, and some experimental mice received PBS and some received cycloheximide (CHX) (120 mg/kg; Sigma, St. Louis, MO) 15 min prior to KA administration to inhibit protein synthesis. Seizures were induced in all KA treated mice which were sacrificed 2 or 4 h after seizure or PBS treatment. No differences in the intensity or duration of the seizures were noted between wild-type and mutant mice.

(iii) Exposure to novel environment. To examine the role of Egr genes in regulating Arc expression during physiologic synaptic activity, 21-day-old (P21) wild-type, Egr1-deficient, Egr3-deficient, and Egr1/Egr3 double-deficient (Egr1/3 dKO) mice were subjected to a novel environment exploration paradigm to induce Egr and Arc gene expression by increasing physiologic synaptic activity. The exploration paradigm was similar to that previously reported to induce Arc gene expression and consisted of an open square box measuring 61 by 61 cm with 20-cm-high walls (23). The box was partitioned into a three by three grid with seven identical three-dimensional objects fixed in place. The box was located in a room with several landmarks on the walls and ceiling. Wild-type and knockout mice were removed from their litermates and allowed to explore the environment for 5 min. The mice were placed in a new grid every 15 s to maintain a novel environment and to facilitate exploration. Each grid was visited two or three times during the exploration session, and there was no obvious difference in the exploratory behaviors exhibited by wild-type or mutant mice. Following the exploration session each mouse was returned to a cage in isolation. Four hours after exposure to a novel environment, the mice were anesthetized and the brains were processed as indicated (below) for in situ hybridization analysis and immunohistochemistry.

Tissue preparation. Anesthetized (ketamine, 150 mg/kg; xylazine 10 mg/kg; i.p.) P21 and adult wild-type and Egr gene-deficient mice were examined. In some mice, brains were removed fresh, microdissected to obtain dorsal hippocampus (coordinates were as follows: interaural, 2.34 mm; bregma, 1.46 mm) (22) and trunk/barrel somatosensory cortex (coordinates were as follows: interaural, 2.34 mm, bregma, 1.46 mm) (22), snap frozen in liquid nitrogen, and stored at −80°C for subsequent nuclear protein, total protein, or RNA preparation. For in situ hybridization and immunohistochemical analysis, anesthetized mice received intracardiac phosphate-buffered saline (0.1 M, pH 7.2) followed by aldehyde perfusion (4% paraformaldehyde, 0.1 M phosphate buffer [PB], pH 7.4). The brains were removed, mid sagitally hemisected, cryoprotected in 30% sucrose–PBS overnight, frozen in OCT embedding media (VWR, Westchester, PA), and stored at −80°C. The brains were cut on a freezing microtome into four parallel series of 8- to 15-μm-thick sections.

In situ hybridization. In situ hybridization was performed using digoxigenin-labeled riboprobes with slight modification to previously published protocols (2). The probes were generated by PCR, and the amplicons were polished (End-it; Epicenter, Madison, WI), subcloned into the EcoRV site of Bluescript (Stratagene, La Jolla, CA) and sequence verified. Sense and antisense riboprobes were synthesized using in vitro transcription and digoxigenin-labeled UTP. The sense probe was used on parallel tissue sections as a control for nonspecific labeling. For some experiments and in all the cases the sense probes showed no hybridization signal. The probes used for in situ hybridization spanned coding sequences as follows: for the Egr3 gene (GenBank NM_007913), nucleotides [nt] 890 to 1389; Egr1 gene (GenBank NM_007913), nt 1589 to 2088; and Arc gene (GenBank NM_018790), nt 577 to 1082. In all cases in which gene expression comparisons were made between treatment conditions or genotypes, a complete set of tissue sections was processed together and the chromogen reactions were terminated simultaneously.

All experiments were repeated three or four times to ensure consistency and accurate interpretation. Representative photographs were obtained with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Nikon E600 microscope and by using the Spot image acquisition software. The digital images were acquired using the internal light meter to determine camera shutter speed and no additional manipulations were applied to the acquired images.

Immunohistochemistry. Immunohistochemistry was performed on paraformaldehyde-fixed and frozen tissue sections. Tissue sections were incubated with blocking buffer (3% normal serum–0.1% Triton X-100 in PBS) for 1 h at room temperature. The sections were incubated in primary antibody diluted in blocking buffer overnight at 4°C. The sections were washed in PBS/s0.1% Triton X-100 and incubated with appropriate fluorescent secondary antibodies (Cy3 or Cy5, Jackson ImmunoResearch) for 1 h at room temperature. The following antibodies were used: rabbit anti-Egr3 antibody (sc-191 1:1000), rabbit anti-Egr1 antibody (sc-110, 1:1000), rabbit anti-Arc (sc-15325, 1:1000), mouse anti-Arc (sc-17839, 1:100) (all from Santa Cruz Biotechnology, Santa Cruz, CA), Cy3-conjugated goat anti-rabbit and Cy5-conjugated goat anti-rabbit antibody (both from Jackson ImmunoResearch, West Grove, PA). Fluorescent images were captured with a Zeiss LSM510 confocal microscope with identical aperture and photomultiplier tube voltage settings used for all of the acquired images. In some cases unmanipulated images were assembled into montages with Photoshop (Adobe) to use for neuron counting.

Western blotting. Microdissected hippocampi from adult wild type, Egr1-deficient and Egr3-deficient mice receiving either KA to induce seizures or PBS were homogenized in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 10 mM NaF and 1× Complete protease inhibitors (Roche, Alameda, CA). Eighty micrograms of total cellular protein was resolved by SDS-PAGE on a 10% acrylamide gel and transferred to a nitrocellulose membrane. Blots were probed with anti-Egr1 (sc-110), anti-Egr3 (sc-191), anti-Arc (sc-17839), or antiactin (sc-1616) antibo-
gies, all from Santa Cruz Biotechnology, Santa Cruz, CA, and then with an horseradish peroxidase-conjugated goat-antirabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) and visualized using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Electrophoretic mobility shift assays (EMSA). DNA binding proteins were extracted from nuclei by using a slight modification of previously described procedures (3). Briefly, mice were injected with KA to induce seizures (20 mg/kg, i.p.) or with PBS, and hippocampi were dissected 2 h later. Hippocampi were dissociated by passage though a 26-gauge needle and resuspended in 400 μl ice-cold lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM NaCl, 0.5 mM dithiothreitol [DTT], 1 mM Na3VO4, 10 mM NaF, 20 mM 2-phenethylphosphorylcholine, Complete protease inhibitors [Roche, Alameda, CA]). Dissociated cells were allowed to swell on ice for 15 min, after which 25 μl of 10% NP-40 was added, and the tubes were vortexed for 10 s. After centrifuga- tion and supernatant removal, the nuclear pellet was resuspended in 100 μl ice-cold extraction buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM Na3VO4, 10 mM NaF, 20 mM 2-phenethylphosphorylcholine, Complete protease inhibitors [Roche, Alameda, CA]) for 60 min. Binding reactions using the nuclear protein extracts were carried out at room temperature for 20 min with 15 μg of nuclear extract, 0.5 μg32P-labeled oligonucleotide probe, 1 mg poly(dI-dC) in a binding buffer consisting of 10 mM Tris (pH 7.5), 25 mM KCl, 1 mM MgCl2, 0.5 mM ZnCl2, 1 mM EDTA, 5 mM DTT, and 5% (vol/vol) glycerol. For supershift experiments, nuclear extracts were preincubated with 2 μg of anti-Egr1 (sc-1898a), anti-Egr2 (sc-1908a), anti-Egr3 (sc-1911a), or anti-Egr4 (sc-19688) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min on ice before initiating the binding reaction. The sequence of the wild-type sequence in bold, are as follows: E1 (5′-CTGGGCGCCGCCAATGGGAG-3′), E2m1 (5′-GCTCCAGGCTGCC-3′), E2m2 (5′-CTGGGCGCCGCCAATGGGAG-3′), E3 (5′-G31-3′), and E2m3 (5′-GCTCCAGGCTGCC-3′). All probes were 5′ end labeled with T4-polynucleotide kinase and γ[32P]ATP (Perkin Elmer, Boston, MA).

Luciferase assays and reporter constructs. Plasmids for luciferase experiments were constructed using mouse genomic DNA as the template. The amplified promoter fragments were cloned into pGL3 Basic luciferase reporter vector (Promega, Madison, WI) and sequence verified. Luciferase reporter plasmids (0.5 μg) were cotransfected into SH-SY5Y-trkA cells (a generous gift of Sven Påhlman, Lund University, Sweden) with pRL-TK (Promega, Madison, WI) and 1 μg of expression plasmid consisting of full-length rat Egr1, Egr3, or Nalb2 cloned into pCDNA (Invitrogen, Carlsbad, CA) or pCDNA alone. Twenty-four hours after transfection, lysates were processed for luciferase activity using the Dual Luciferase Assay (Promega, Madison, WI) according to the manufacturer’s specifications.

Adenovirus preparation. Recombinant adenoviruses were generated using homologous recombination in Escherichia coli as previously described (10). The generation, characterization, and amplification of the enhanced green fluores- cent protein (EGFP) and transcriptional activative Egr3 (Egr3A) adenovi- ruses were previously described (2). The Egr1-expressing adenovirus was generated for this study by using a full-length N-terminal hemagglutinin-tagged rat Egr1 cDNA (GenBank NM_012551) which was cloned into 5′AdTrackCMV. The recombinant Egr1 5′AdTrack shuttle vector was recombined by homologous recombination into the adenoviral genomic (5′AdEasy) in E. coli B5183 to generate a recombinant replication-deficient adenoviral genomic plasmid. The replication-deficient EGFP, Egr1, Egr3, and Egr3A expression viruses were packaged and amplified in transfected HEK-293 cells (ATCC, Manassas, VA), purified/concentrated on cesium chloride gradients, and titered by using EGFP fluorescence and 50% culture infective doses.

Primary cell culture and adenoviral infection. (i) Myotubes. Primary myo- blasts were isolated and differentiated into myotubes for 10 days in vitro (DIV) as previously described (2).

(ii) Neurons. Primary neuron cultures were prepared from hippocampal or cerebral cortices of mouse embryos as described previously (4). Briefly, dorsal hippocampi (coordinates were as follows: interaural, 2.34 mm; bregma, 1.46 mm) (22) and trunk/barrel somatosensory cortices (coordinates were as follows: inter- aural, 2.34 mm; bregma, 1.46 mm) (22) were dissected from embryonic day 16.5 (E16.5) C57BL/6J mice, treated with 0.25% trypsin for 15 min at 37°C, and dissociated by trituration with 0.25% DNase I (Sigma, St. Louis, MO). Dissoci- ated cell suspensions were plated at 1.25 × 106 cells per well on six-well tissue culture plates or at 3 × 106 cells per dish on 60-mm tissue culture dishes coated with poly-l-lysine, in minimum essential medium (MEM, Mediatech, Inc., VA) supplemented with 10% horse serum (Harlan, Indianapolis, IN), 100 U/ml pen- ticillin, and 0.1 mg/ml streptomycin. After 4 h, neurons were maintained in glia-conditioned MEM containing N2 supplements plus 0.1% ovalbumin (Sigma, St. Louis, MO) and 0.1 mM sodium pyruvate (Sigma, St. Louis, MO). Cytosine-β-β-arabinofuranoside (2.0 μM; Sigma, St. Louis, MO) was added on the third day after plating (3 DIV) to inhibit cellular mitosis of contaminating glial cells.

Adenoviral infections. Myotubes were infected with Egr3 and Egr3A adenovirus with a multiplicity of infection of 100 to obtain 100% infection efficiency without visible toxicity. Cultured mouse hippocampal and cortical neurons were infected with EGFp, Egr1, or Egr3 adenovirus at 6 DIV at a multiplicity of infection of 1.000. The neuron cultures were >95% free of glial contamination, and the infections were 60 to 70% efficient as determined by averaging several random fields of the number of green (infected) neurons relative to the number of neurons (DAPI [4′,6-diamidino-2-phenylindole] stained) present (data not shown). There was no morphological evidence of cytotoxicity and the neurons were harvested in Trizol (Invitrogen, Carlsbad, CA) 24 h following infection.

RNA preparation, qRT-PCR, and semiquantitative gene expression analysis. Total RNA was isolated from adenovirus-infected primary myotubes (7 DIV), differentiated hippocampal neurons (6 DIV), and cortical neurons (6 DIV) with Trizol (Invitrogen, Carlsbad, CA). Total RNA (0.5 to 1.0 μg) was reverse trans-cribed using random octamer primer and Powerscript reverse transcriptase according to the manufacturer’s specifications (BD Biosciences Clontech, Palo Alto, CA). Quantitative reverse transcription-PCR (qRT-PCR) was performed on an SDS7500 sequence detector (Applied Biosystems, Foster City, CA) by using SYBR green (Molecular Probes, Eugene, OR) fluorescence chemistry. For expression analysis studies, non-intron-spanning primers that amplified coding sequence were designed for each target gene. The RNA was treated with RNase- free DNase (Promega, Madison, WI) and parallel reverse transcription reaction efficiencies were measured using SYBR green fluorescence. For each cDNA or ChIP sample, the relative level of target and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence was determined by the comparative Ct method (36). For each cDNA or ChIP sample, the relative level of target and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence was determined by the comparative Ct method (36). For each cDNA or ChIP sample, the relative level of target and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence was determined by the comparative Ct method (36). For each cDNA or ChIP sample, the relative level of target and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence was determined by the comparative Ct method (36). For each cDNA or ChIP sample, the relative level of target and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence was determined by the comparative Ct method (36).

Chromatin immunoprecipitation. Mice were injected with PBS or KA to induce seizures (20 mg/kg, i.p.), and hippocampi were dissected 2 h later. Hip- pocampi were dissociated by passage through a 26-gauge needle and DNA/ protein complexes were cross-linked in PBS containing 1% formaldehyde for 10 min at room temperature. After being washed twice in PBS, the tissue fragments were lysed in 500 μl nucleic lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, Complete protease inhibitors [Roche, Alameda, CA]). The lysates were incubated on ice for 15 min, and the chromatin was sonicated 10 times for 15 s using a cone sonicator (Sonic Dismembrator, model 300; Fisher Scientific, Pitts- burg, PA) at 30% power. Insoluble material was cleared by centrifugation, the sheared chromatin was diluted 10-fold in dilution buffer (16.7 mM Tris, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl), and incubated overnight in the presence of 5 μg of rabbit anti-Egr1, rabbit anti-Egr3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Immunocomplexes were captured using 50 μl of protein A/g-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) which had been previously blocked with sheared salmon sperm DNA. The beads were sequentially washed with 5 times binding buffer (20 mM Tris-HCl, pH 8.1, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), 5 times Tris-HCl, pH 8.1, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl), LiCl buffer (10 mM Tris, pH 8.1, 0.25 M LiCl, 1% sodium deoxycholic acid, 1 mM EDTA), and then twice in 10 mM Tris (pH 7.2)–1 mM EDTA. The immu- nocomplexes were eluted in 0.1 M NaHCO3 (pH 9.0)–1% SDS while shaking for...
30 min. Cross-links between protein and DNA were reversed at 65°C for 5 h in the presence of 10 μg RNase–0.3 M NaCl and the chromatin was ethanol precipitated. The pellets were resuspended in proteinase K buffer (10 mM Tris, pH 8.5, 1 mM EDTA, 0.25% SDS) and incubated for 3 h at 4°C with 15 μg/ml of proteinase K (Invitrogen, Carlsbad, CA) and the remaining DNA was purified using QIAquick spin columns (QIAGEN, Valencia, CA). Quantitative PCR (qPCR) was performed as described above using the following primers (numbers according to previously published mouse Arc promoter sequence [36]): to amplify the distal Arc promoter (nt −1842 to −1352), Pr1 (5′-GGTACGACA GCCAGGTTCCGAT-3′) and Pr2 (5′-ACACACAGGGGTTCCATCC-3′), and to amplify the proximal Arc promoter (nt −518 to −210), Pr3 (5′-AC ACACACAGGGGTTCCATCC-3′) and Pr4 (5′-GAACATGCTGCTGCGG CGG-3′). To control for nonspecific DNA that was captured in the ChIP library, a region of the GAPDH coding sequence was amplified using primers 5′-ACGGCCAAATCCACCGCAGCTACA-3′ and 5′-GCTTTCAGAAGGG CCATCACCAG-3′, and relative enrichment of Arc sequences in the ChIP DNA were normalized to GAPDH. Alternately, the ChIP DNA was used as a template in a standard PCR and the products were electrophoresed on a 2% agarose gel.

Neuron counts. Neurons were counted in 21-day-old mice that had been exposed to a novel environment 4 h prior to sacrifice using images of random 0.1-mm² high power fields (HPFs) obtained by confocal microscopy. Immunofluorescence histochemistry was performed for Arc and Egr1, Arc and Egr3, or Arc counterstained with SYTOX green (Invitrogen, Carlsbad, CA) to label DNA in all neurons. For colocalization studies in somatosensory cortex, the percent of Arc-positive neurons that were either Egr1 or Egr3 positive were averaged from a total of six HPFs from two wild-type mice and three HPFs per mouse. For quantification of Arc in wild-type and Egr gene-deficient neurons, Arc-immunopositive neurons were counted as a percent of the total SYTOX green staining neurons in each of six total HPF from three mice of each genotype and two closely related tissue sections per mouse. For all of the mice, the neuroanatomical regions analyzed corresponded to the dorsal hippocampus (coordinates were as follows: interaural, 2.34 mm; bregma, 1.46 mm [22]) and truncal/barrel somatosensory cortex (coordinates were as follows: interaural, 2.34 mm; bregma, 1.46 mm [22]).

RESULTS

Egr1 and Egr3 induce Arc gene expression in multiple cell types. The Arc gene was identified as a potential Egr target gene from an Affymetrix microarray analysis intended to characterize Egr3 target genes that control intrafusal muscle fiber morphogenesis in muscle spindle stretch receptors (2). The microarray results were confirmed by comparing Arc expression in primary murine myotubes infected with an adenovirus that expressed EGF and Egr3, and to that in myotubes infected with an adenovirus that expressed EGFP and transcriptionally active Egr3 (Egr3). Arc was upregulated 11.3-fold in primary myotubes expressing Egr3 relative to Egr3 (Fig. 1A). In the hippocampus, Egr1 and Egr3 are the major Egr-related proteins upregulated after seizure-induced synaptic activity (21). Therefore, we examined whether Egr1 and/or Egr3 could regulate Arc gene expression in a neuronal context in differentiating murine primary hippocampal and cortical neurons using adenoviruses to express EGFP, EGFP and Egr1, or EGFP and Egr3. Arc gene expression was upregulated by Egr1 and Egr3 250- and 168-fold, respectively, in hippocampal neurons (Fig. 1B) and by 8- and 15.5-fold, respectively, in cortical neurons (Fig. 1C). The difference in the relative magnitudes of induction of hippocampal and cortical neurons was apparently due to the fact that hippocampal neurons had very low basal levels of Arc expression, whereas cortical neurons had higher levels under these culture conditions (data not shown). Nevertheless, either Egr1 or Egr3 significantly induced Arc gene expression in primary myotubes and neurons in vitro.

Egr1, Egr3, and Arc proteins are coordinately expressed in the hippocampus after kainic acid-induced synaptic activation. Egr proteins might regulate the protein synthesis-dependent phase of Arc gene expression since previous results indicate that Egr and Arc genes are regulated by neuronal activity, their expression is coupled with NMDA and MAPK signaling within hippocampal and cortical neurons, and Egr1 and Arc are both necessary for hippocampal L-LTP and normal memory processing. Consistent with this hypothesis, Egr1, Egr3, and Arc proteins were all transiently upregulated in the hippocampus after KA seizure-induced synaptic activation (Fig. 1D). Moreover, Egr1, Egr3, and Arc were coexpressed in most neurons in vivo (hippocampal dentate gyrus neurons are shown) after KA seizure-induced synaptic activation (Fig. 1E and F).

The proximal promoter region of the Arc gene contains a high-affinity ERE that is necessary and sufficient for Egr-mediated Arc transcription. Since Egr proteins regulate gene transcription through an incompletely defined mechanism that involves obligate binding to a GC-rich sequence (ERE) in target gene promoters, we examined whether Egr1 and/or Egr3 could directly bind Arc promoter sequences in vivo and hence whether they might directly regulate Arc gene expression. Chromatin from the hippocampus of unseized control mice and mice subjected to KA induced seizures 2 h prior to hippocampal dissection was sheared into fragments averaging 0.5 to 1 kb in length. Genomic DNA bound to either Egr1 or Egr3 was isolated by ChIP, and PCR primers were used to amplify precipitated DNA representing either a distal segment of the Arc promoter (primers Pr1 and Pr2) (Fig. 2A) or a proximal segment near the transcription start site (primers Pr3 and Pr4) (Fig. 2A) (36). In ChIP samples from unseized and seized hippocampus, neither Egr1 nor Egr3 was bound to distal (Pr1 and Pr2) regions of the Arc promoter. However, when proximal primers were used for PCR (Pr3 and Pr4), Arc genomic DNA was amplified from seized hippocampus but not unseized hippocampus, indicating that both Egr1 and Egr3 were bound to a region of genomic DNA near the Arc transcription start site after they were induced by seizures.

To examine whether the Egr proteins bind to a functional ERE capable of transactivating the Arc gene promoter, a series of Arc promoter-luciferase reporter constructs were generated. A fragment of the 5′ regulatory sequence of the Arc gene, −775 nucleotides upstream of the transcription start site (−775Arc), was fused to a luciferase reporter construct to test the ability of Egr1 and Egr3 to transactivate luciferase expression in vitro. To isolate the region of the promoter that conferred Egr-dependent transcriptional activity, serial 5′ deletion mutants of the promoter fragment were tested. However, Egr1 and Egr3 were capable of significantly transactivating all of the promoter deletion constructs, and there was no appreciable change in the ability of either Egr1 or Egr3 to transactivate the promoter constructs down to −87 nucleotides upstream of transcription start site, suggesting that a functional ERE is located within this proximal segment of the promoter (Fig. 2B).

To more precisely define the ERE within the Arc promoter, genomic sequence consisting of the −87 nucleotide regulatory fragment from mouse, rat, and human was aligned to the TATA box upstream of the transcription start site, and two GC-rich domains (E1 and E2) that could contain Egr protein binding sites were identified (Fig. 3A). To examine whether...
either of these regions is capable of binding to Egr1 and/or Egr3 proteins, nuclear extracts from seized and unseized microdissected hippocampus and radiolabeled oligonucleotide probes representing the E1 and E2 domains were used in EMSA. By contrast to the E1 probe, which formed no specific protein-DNA complexes with nuclear extracts from unseized hippocampus, the E2 probe generated multiple complexes, indicating that specific proteins within the hippocampal extracts were capable of binding this small region of the Arc gene promoter in vitro (Fig. 3B). Interestingly, in unseized hippocampus, no complexes were formed, indicating that all of the E2 DNA binding proteins were induced by seizure. To examine whether any of these complexes contained Egr proteins, Egr protein-specific antibodies were incubated with the radiolabeled oligos and nuclear extracts. Whereas antibodies that cross-reacted with Egr2 and Egr4 had no effect on the formation of the protein-DNA complexes, the Egr1 antibody specifically supershifted the high-molecular-weight complex and the Egr3 antibody supershifted the lower-molecular-weight complexes (Fig. 3B, asterisk). The supershifting that occurred for multiple low-molecular-weight complexes by the Egr3 antibody is consistent with previous results demonstrating the presence of multiple Egr3 isoforms produced by alternative translation start sites (19) and that all of these Egr3 isoforms are recognized by the C-terminal-epitope-specific Egr3 antibody. Moreover, Egr1 and Egr3 are the only Egr-related proteins that bind to the E2 domain, consistent with previous results indicating that they are the major Egr proteins upregu-
We next examined whether this newly identified ERE was responsible for binding Egr1 and Egr3 within the E2 domain, a mutation was made to the E2 probe within the putative core binding domain (E2m1) known to abrogate Egr protein-DNA binding within the consensus ERE. A second probe was also generated with a mutation outside of the putative core binding domain (E2m2) that would not be expected to interfere with Egr protein binding to the core ERE (39). As expected, when these oligos were bound to nuclear extracts from seized hippocampus in EMSA, E2m1 was not capable of binding any proteins, whereas E2m2 was capable of binding both Egr1 and Egr3 similar to the wild-type E2 sequence (Fig. 3D). Thus, Egr proteins bind to the Arc promoter through a precisely defined high-affinity and species-conserved binding domain proximal to the transcription start site.

We next examined whether this newly identified ERE was necessary for Egr-mediated Arc gene expression. Indeed, when the E2m1 Egr protein-binding mutation was introduced into the −775Arc luciferase reporter construct, transactivation of the proximal Arc promoter by either Egr1 or Egr3 was completely abrogated in SH-SYSY human neuroblastoma cells (Fig. 3E, left panel). Egr3 consistently activated the −775Arc promoter better than Egr1 when an equal mass of cytomegalovirus (CMV) expression vector was used. Similar results were also observed when a minimal 4×ERE reporter construct was used (Fig. 3E, center panel), consistent with previous results demonstrating increased expression/stability of Egr3 relative to Egr1 when expressed in vitro from these CMV expression constructs (24). Finally, in NIH 3T3 cells, both Egr1 and Egr3 significantly activated the −775Arc promoter, and this activation was completely abrogated by coexpression of the Egr coregulator Nab2 (29) (Fig. 3E, right panel).

**Egr3 mediates the protein synthesis-dependent phase of Arc gene expression in the hippocampus after seizure.** In the rat hippocampal dentate gyrus granule neurons, the Arc gene appears to be regulated as an immediate early gene that is rapidly induced after synaptic activation in the absence of new protein synthesis. However, it is also regulated by a protein synthesis-dependent mechanism that modulates its expression beyond the transient immediate early phase (35). To examine whether Egr1 and/or Egr3 may mediate protein synthesis-dependent Arc gene expression in mouse hippocampus, we first identified a time point after seizure when Arc expression is completely dependent upon new protein synthesis. Adult wild-type littermate mice were injected either with phosphate-buffered saline or with KA, and they were sacrificed 2 and 4 h after KA-induced seizure or PBS injection. In addition, the mice received either PBS or CHX 15 min prior to the PBS or KA treatment to inhibit protein synthesis. Compared to the low
basal levels of Egr1 (not shown), Egr3 (Fig. 4A), and Arc (Fig. 4A′) expression in caged adult mice treated with PBS, mice receiving KA-induced seizures 2 h prior to analysis, but without CHX pretreatment, showed marked induction of Egr1 (Fig. 5A′, inset), Egr3 (Fig. 4B), and Arc expression (Fig. 4B′). Unlike with Egr1 and Egr3, where the RNA was confined largely to the neuronal somata (Fig. 4B′, arrow), consistent with previous results in rats (17). Similarly, after KA-induced seizure and CHX pretreatment, Egr1 (data not shown), Egr3 (Fig. 4C), and Arc (Fig. 4C′) genes were highly induced, consistent with their regulation as immediate early genes that do not require synaptic activation induced protein synthesis. By 4 h after KA-induced seizure in mice not receiving CHX pretreatment, Egr1 expression had returned to baseline (Fig. 5D′, inset), and increased Egr3 (Fig. 4D) and Arc gene expression (Fig. 4D′) was still present in the hippocampus, consistent with the results from the kinetic analysis of protein induction in the hippocampus using Western blotting (Fig. 1D). However, 4 h after KA-induced seizure in mice receiving CHX pretreatment, both Egr1 (not shown) and Egr3 (Fig. 4E) were superinduced, but Arc expression was completely abrogated (Fig. 4E′). These results identify two time points after KA-induced synaptic activation in mice when the protein synthesis-independent (2 h postseizure) and protein synthesis-dependent (4 h postseizure) Arc expression levels can be clearly distinguished in hippocampal dentate gyrus granule neurons (compare Fig. 4C′ and E′).
FIG. 4. Arc is transiently expressed as a protein synthesis-independent immediate early gene and its expression is modulated by a protein synthesis-dependent mechanism. In caged wild-type adult mice treated with PBS, Egr3 (A) and Arc (A’) expression were very low in the hippocampus. Two hours after KA-induced seizure, Egr3 (B) and Arc (B’) were markedly upregulated. Unlike Egr3 mRNA (B), which was localized within the neuron somata and nuclei, Arc mRNA (B’, arrow) was also characteristically transported into the dendrites of hippocampal dentate gyrus neurons. When mice were treated with CHX 15 min prior to seizure to inhibit protein synthesis, there was no detectable difference in Egr3 (C) or Arc (C’) expression compared to mice not receiving CHX. By 4 h after KA-induced seizure in the absence of CHX treatment, Egr3 (D) and Arc (D’) expression were still elevated above basal (PBS treatment) levels. In CHX-pretreated mice 4 h after KA-induced seizure, Egr3 (E) expression was superinduced, consistent with its regulation as an immediate early gene; however Arc (E’) expression was completely abrogated. Thus, Arc expression is independent of protein synthesis within at least 2 h after KA-induced seizure but by 4 h after seizure, Arc expression is completely regulated by a protein synthesis-dependent mechanism. Scale = 0.5 μm.
We next asked whether Egr1 and/or Egr3 regulate the protein synthesis-dependent phase of Arc gene expression in vivo. Whereas wild-type mice subjected to KA-induced seizure 2 h prior to analysis showed the characteristic pattern of Arc (A), Egr3 (A'), and Egr1 (A', inset), which were markedly upregulated in wild-type mice independent of new protein synthesis 2 h after seizure, Arc (B) and Egr3 (B') were appropriately induced in Egr1-deficient mice and Arc (C) and Egr1 (C') were appropriately induced in Egr3-deficient mice, indicating that similar seizure induced activity was generated the Egr gene-deficient mice to induce qualitatively equivalent levels of protein synthesis-independent Arc expression. However 4 h after seizure, when Arc expression was dependent upon new protein synthesis, Arc (D) and Egr3 (D') were still upregulated and (D', inset) Egr1 had returned to basal levels. In Egr1-deficient mice, Arc (E) and Egr3 (E') were also elevated similar to wild-type mice. However, in Egr3-deficient mice, Arc (F) expression was markedly diminished and Egr1 (F') was slightly elevated above basal levels. Thus, protein synthesis-dependent Arc expression requires Egr3 after seizures in the hippocampus. Scale = 0.5 μm.

### FIG. 5. The protein synthesis-dependent phase of Arc gene expression requires Egr3 after seizure-induced synaptic activation.

Similar to Arc (A), Egr3 (A'), and Egr1 (A', inset), which were markedly upregulated in wild-type mice independent of new protein synthesis 2 h after seizure, Arc (B) and Egr3 (B') were appropriately induced in Egr1-deficient mice and Arc (C) and Egr1 (C') were appropriately induced in Egr3-deficient mice, indicating that similar seizure induced activity was generated the Egr gene-deficient mice to induce qualitatively equivalent levels of protein synthesis-independent Arc expression. However 4 h after seizure, when Arc expression was dependent upon new protein synthesis, Arc (D) and Egr3 (D') were still upregulated and (D', inset) Egr1 had returned to basal levels. In Egr1-deficient mice, Arc (E) and Egr3 (E') were also elevated similar to wild-type mice. However, in Egr3-deficient mice, Arc (F) expression was markedly diminished and Egr1 (F') was slightly elevated above basal levels. Thus, protein synthesis-dependent Arc expression requires Egr3 after seizures in the hippocampus. Scale = 0.5 μm.

We next asked whether Egr1 and/or Egr3 regulate the protein synthesis-dependent phase of Arc gene expression in vivo. Whereas wild-type mice subjected to KA-induced seizure 2 h prior to analysis showed the characteristic pattern of Arc (Fig. 5A), Egr3 (Fig. 5A'), and Egr1 (Fig. 5A', inset) induction, there was no substantial difference in the patterns or intensities of Arc (Fig. 5B) and Egr3 (Fig. 5B') induction in Egr1-deficient mice or of Arc (Fig. 5C) and Egr1 (Fig. 5C') induction in Egr3-deficient mice. Thus, as expected, Arc expression is not dependent upon Egr1 or Egr3 during the immediate early protein synthesis-independent phase of its expression in the hippocampus. However, when mice were examined 4 h after KA-induced seizure, wild-type mice showed elevation of Arc (Fig. 5D) and Egr3 (Fig. 5D') expression but basal levels of Egr1 expression (Fig. 5D', inset), consistent with the relative protein levels observed in hippocampal lysates between PBS-treated mice at 0 h and 4 h after KA-induced seizure (Fig. 1D). Similarly, Arc (Fig. 5E) and Egr3 (Fig. 5E') expression levels
Arc expression after seizure in vivo, because the kinetics of both Egr1 or Egr3 can bind the Arc ERE and transactivate in situ hybridization results (Fig. 5F). Thus, despite the fact the situation with wild-type mice (Fig. 6). Moreover, Arc protein was present at a very low level, similar to observed in wild-type mice 4 h after seizure (Fig. 1D), we observed kinetics of Egr1, Egr3, and Arc protein induction that was dent upon new protein synthesis (4 h postseizure). Similar to deficient mice after seizure when Arc expression was depen-

remained elevated 4 h after seizure in Egr1-deficient mice. By contrast, in Egr3-deficient mice, Arc expression (Fig. 5F) was markedly diminished and Egr1 expression (Fig. 5F’) was similar to the low basal levels observed in wild-type mice 4 h after KA-induced seizure in the hippocampus (Fig. 5D’, inset). Thus, Egr3, but not Egr1, is required to mediate the protein synthesis-dependent phase of Arc gene expression in this seizure paradigm. In Egr1-deficient mice, there are high levels of Egr3 which are sufficient to regulate Arc expression whereas in Egr3-deficient mice there is an insufficient amount of Egr1 protein to compensate for Egr3 loss leading to corre-

FIG. 6. Prolonged elevation of Egr3 protein in the hippocampus after seizure explains why Egr3, but not Egr1, regulates the protein synthesis-dependent phase of Arc expression. Hippocampal lysates obtained four hours after seizure, when Arc expression depends upon protein synthesis in wild-type hippocampus, contain very low levels of Egr1 and high levels of Egr3 protein because of the difference in kinetics between the two proteins after seizure induction. Similarly, Egr3 protein levels remain high in the hippocampus of Egr1-deficient mice, and consequently, Arc protein levels are comparable to wild-type levels. By contrast, in the hippocampus of Egr3-deficient mice, Egr1 protein levels are very low, as they are in wild-type mice 4 h after seizure, and without Egr1 or Egr3, Arc protein levels are very low compared to those in wild-type or Egr1-deficient mice. These results indicate that Egr1 is dispensable for regulating the protein synthesis-dependent phase of Arc expression in this seizure paradigm. In Egr1-deficient mice, there are high levels of Egr3 which are sufficient to regulate Arc expression whereas in Egr3-deficient mice there is an insufficient amount of Egr1 protein to compensate for Egr3 loss leading to corre-

reversibly return to the basal level observed when Arc expression is not induced (Fig. 1D). Thus, Egr3, but not Egr1, is required to mediate the protein synthesis-dependent phase of Arc gene expression in this seizure paradigm. However, during physiologic synaptic activity, when neurons are asynchronously active, Egr1 may have an important role in regulating Arc expression, particularly if Egr1 and Egr3 are differentially regulated within individual hippocampal and cortical neurons. Twenty-one day old mice were examined 4 h after exposure to a novel environment to standardize the level of stimulation they received and to induce Arc expression (see Materials and Methods) (23). In wild-type mice, Arc was expressed in a fraction of hippocampal dentate gyrus granule neurons (Fig. 7B) and cortical neurons (Fig. 7D and G) as previously reported for rats (17). Egr1 and Egr3 were also expressed in a similar pattern in hippocampal dentate gyrus neurons. Some Arc-expressing neurons (Fig. 7B) coexpressed Egr1 (Fig. 7A and B), Egr3 (Fig. 7B and C), or both Egr1 and Egr3 (Fig. 7A through C), suggesting that Egr1 and Egr3 may be differentially regulated in some neurons to regulate Arc expression during physiologic synaptic activity. Similarly, in the somatosensory cortex, Arc protein (Fig. 7D) was highly colocalized with Egr1 protein (Fig. 7E) such that 94.6 ± 4.1% of Arc protein-containing neurons also contained Egr1 protein (Fig. 7F). Moreover, Arc protein (Fig. 7G) was highly colocalized with Egr3 protein (Fig. 7H) such that 96.6 ± 2.4% of Arc protein-containing neurons also contained Egr3 protein (Fig. 7I). Thus, >95% of cortical neurons that express Arc protein also express Egr1, Egr3, or both Egr1 and Egr3 proteins during physiologic synaptic activity.

To examine whether Egr1 and/or Egr3 regulates Arc expression during physiologic synaptic activity, we examined 21-day-old wild-type, Egr1-deficient, Egr3-deficient, and Egr1/3 dKO mice 4 h after exposure to a novel environment. Wild-type mice showed a characteristic expression of Arc in the frontal cortex and hippocampus, with less expression in subcortical areas (Fig. 8A). High levels of Arc expression were noted in a subpopulation of dentate gyrus granule neurons (Fig. 8E) and frontal cortex (somatosensory cortex shown) (Fig. 8I). Tissue sections processed in parallel from Egr1-deficient and Egr3-deficient mice showed a clear decrease in Arc expression throughout the brain (Fig. 8B and C) that appeared to reflect a decrease in the number of Arc expressing neurons in hippocampal dentate gyrus (Fig. 8F and G) and a decrease in number and intensity of Arc expressing neurons in frontal cortex (Fig. 8J and K). Normal levels of Arc expression clearly
depended upon both Egr1 and Egr3 since Egr1/3 dKO mice expressed barely detectable levels of Arc throughout the brain (Fig. 8D). In the hippocampus, Arc-expressing neurons were entirely absent (Fig. 8H), and in frontal cortical neurons, Arc expression was barely detectable (Fig. 8L).

The in situ hybridization results were quantitatively validated using immunohistochemistry to detect Arc protein in wild-type and Egr gene-deficient mice 4 h after exposure to novel environmental stimuli. In somatosensory cortex from wild-type mice, Arc protein was identified in 17.5% of wild-type neurons (Fig. 9A and E), 2.2% of Egr1-deficient neurons, and 1.3% of Egr3-deficient neurons (Fig. 9E), and despite the fact that highly sensitive digoxigenin in situ hybridization detected very low levels of Arc mRNA, Arc protein was not detected in cortical neurons (0%) in Egr1/3 dKO brains (Fig. 9B and E). These results were not explained by a generalized decrease in cortical neuron activity, since c-fos immunoreactivity (an unrelated immediate early gene that is also regulated by neuronal activity) was not apparently different between wild-type and Egr1/3 dKO mice (Fig. 9C and D). Similarly, in wild-type hippocampus, Arc was expressed in 1.6% of hippocampal dentate gyrus neurons and in 0.8% and 0.2% of dentate gyrus neurons in Egr1-deficient and Egr3-deficient mice, respectively, consistent with the intermediate expression levels observed by in situ hybridization (Fig. 9F). In Egr1/3 dKO mice, Arc protein was absent (0%) in hippocampal dentate gyrus neurons (Fig. 9F). However, c-fos expression also appeared to be decreased in the hippocampus of Egr1/3 dKO mice (data not shown) making it impossible to definitively ascribe the loss of Arc expression in the hippocampus to the loss of Egr-mediated regulation in this region of the brain.

**DISCUSSION**

Egr1, potentially along with other Egr transcriptional regulators, influences synaptic plasticity and learning/memory pro-
cesses presumably by regulating target genes, the identities of which are currently unknown. Here, we present in vitro and in vivo evidence linking Egr1 and Egr3 to direct regulation of the plasticity-associated Arc gene. Arc is rapidly and transiently regulated without the need for new protein synthesis, and we showed that its expression is also modulated by a protein synthesis-dependent mechanism that requires Egr1 and Egr3.

Many studies have focused on the role of Egr1 in synaptic plasticity and learning/memory processes, while the role of other Egr transcriptional regulators in neurons has been largely ignored. Whether Egr2, Egr3, or Egr4 has any role in learning and memory mechanisms has not been determined, as mice that constitutively lack Egr2 are postnatally lethal (25, 30) and mice lacking Egr3 or Egr4 have not been studied (32, 33).

In primary myotubes, and hippocampal and cortical neurons, enforced expression of either Egr1 or Egr3 was capable of significantly upregulating Arc gene expression. Moreover, after seizure-induced synaptic activation, Egr1 and Egr3 were bound in vivo to a species-conserved high-affinity response element in the proximal Arc promoter that was necessary and sufficient for Arc transactivation in vitro. Transcriptional activation of Arc by either Egr1 or Egr3 was completely repressed by coexpression of the Egr coregulatory molecule Nab2, suggesting that Egr-mediated Arc regulation may be subject to

FIG. 8. Arc expression requires Egr1 and Egr3 in the context of physiologic synaptic activity. (A) In 21-day-old mice, four hours after exposure to a novel environment, Arc was expressed at high levels by many cortical and hippocampal neurons. In the hippocampus, a subpopulation of neurons (arrow) expressed high levels of Arc (E), and in the frontal cortex (somatosensory cortex shown), pyramidal neurons in superficial and deep layers of cortex preferentially expressed Arc (I). In Egr1-deficient mice (B) and Egr3-deficient mice (C), Arc expression was decreased compared to that in wild-type mice, which was reflected by a decreased number of Arc-expressing hippocampal dentate gyrus granule neurons (F and G, arrows) and a decrease in the number and intensity of Arc-expressing neurons in cortex (I and K). (D) In Egr1/3 dKO mice, Arc expression was very low compared to that in wild-type mice. In the hippocampal dentate gyrus, no Arc-expressing neurons were identified (H) and similarly in cortex, Arc expression was barely detectable (L). Boxes in panels A through D orient the magnified regions shown in panels E through L. (A through D) Scale = 1 mm. (E through H) Scale = 100 μm. (I through L) Scale = 50 μm.
complex control in vivo (29). Considering that Egr1 and Egr3 were both bound to the Arc ERE after their induction, it was surprising to find that only Egr3 was required for the protein synthesis-dependent phase of Arc expression after synchronous synaptic activation by seizure in vivo. The results were not explained by differences in seizure response to kainic acid treatment between Egr1-deficient and Egr3-deficient mice, since the animals displayed highly reproducible seizures that were subjectively equivalent in intensity and duration, and the immediate early components of Arc expression within the first two hours after seizure were identical between wild-type and Egr gene-deficient mice. Rather, the protein synthesis-dependent phase of Arc expression depended upon Egr3 after synchronous synaptic activation by seizure most likely because it was expressed with prolonged kinetics relative to Egr1 (21). Thus, after a single-seizure epoch, Egr3 was essential for regulating the protein synthesis-dependent phase of Arc expression presumably because it was the only Egr protein available to regulate Arc expression 4 h after seizure (Fig. 1D).

During physiologic synaptic activity, when neurons were asynchronously activated by exposure to a novel environment, both Egr1 and Egr3 were required for normal levels of Arc expression in cortical and hippocampal neurons. Egr1 and Egr3 were differentially expressed in some neurons after physiologic synaptic activity, with Egr3 being expressed at higher levels than Egr1 in some neurons (29). Considering that Egr1 and Egr3 were both bound to the Arc ERE after their induction, it was surprising to find that only Egr3 was required for the protein synthesis-dependent phase of Arc expression after synchronous synaptic activation by seizure in vivo. The results were not explained by differences in seizure response to kainic acid treatment between Egr1-deficient and Egr3-deficient mice, since the animals displayed highly reproducible seizures that were subjectively equivalent in intensity and duration, and the immediate early components of Arc expression within the first two hours after seizure were identical between wild-type and Egr gene-deficient mice. Rather, the protein synthesis-dependent phase of Arc expression depended upon Egr3 after synchronous synaptic activation by seizure most likely because it was expressed with prolonged kinetics relative to Egr1 (21). Thus, after a single-seizure epoch, Egr3 was essential for regulating the protein synthesis-dependent phase of Arc expression presumably because it was the only Egr protein available to regulate Arc expression 4 h after seizure (Fig. 1D).

During physiologic synaptic activity, when neurons were asynchronously activated by exposure to a novel environment, both Egr1 and Egr3 were required for normal levels of Arc expression in cortical and hippocampal neurons. Egr1 and Egr3 were differentially expressed in some neurons after phys-
logic activation since they expressed Egr1, Egr3, or both Egr1 and Egr3. After seizure, Egr1 and Egr3 are coactivated and transiently expressed with differing stereotypical kinetics (Fig. 1D). If the differential pattern of Egr1 and Egr3 expression after physiologic synaptic activity was a result of coactivation and differing kinetics of expression then all neurons that expressed Egr1 should have also expressed Egr3, which was not the case (compare Fig. 7A and C). Moreover, while most neurons that expressed Arc also expressed Egr1, Egr3, or both Egr1 and Egr3, some Egr-expressing neurons did not express Arc. This suggests that additional regulatory mechanisms might exist to prevent Arc expression in some circumstances despite the presence of Egr1 and/or Egr3. One possibility is that coregulatory factors, such as Nab2, which can inhibit Egr-mediated Arc gene transactivation (Fig. 1E, right panel), may be differentially regulated in these neurons to provide an additional level of control over Arc transactivation (29). Similarly, the majority of Arc-expressing cortical neurons also expressed either Egr1 or Egr3 but the extent to which these neuron populations overlapped was not determined because antibodies that permit simultaneous labeling of Egr1, Egr3, and Arc were not available. Nevertheless, the data indicate that Egr1 and Egr3 are differentially regulated by physiologic synaptic activation to express Egr1, Egr3, or both Egr1 and Egr3 proteins in particular neurons at a specific time. Consistent with this hypothesis, we found that the frequency of Arc-expressing cortical and hippocampal dentate gyrus neurons, after similar exposure to a novel environment, was decreased in Egr1- and Egr3-deficient mice relative to wild-type mice. In addition, Arc mRNA was barely detectable and Arc protein was completely absent in cortical and hippocampal dentate gyrus neurons from Egr1/3 dKO mice after exposure to a novel environment. While these results seem to confirm the fact that Arc expression depends upon Egr-mediated transcription, they could be explained by decreased neuronal activity in the mutant mouse brains. Similar levels of expression of the activity-regulated immediate early gene c-fos in cortical neurons between wild-type and Egr1/3 dKO mice strongly argues against this possibility, although c-fos expression was decreased in hippocampal dentate gyrus neurons. Thus, in the hippocampal dentate gyrus neurons from Egr 1/3 dKO mice, it is not clear whether the lack of Arc-containing neurons can be definitively attributable to loss of Egr-dependent regulation.

Arc is an unusual plasticity-associated molecule that is induced by NMDA receptor activation in glutamatergic neurons (28). Arc mRNA is transported to recently activated synapses where it is locally translated into protein and integrated into the NMDA multigene complex (11), thereby potentially altering the response properties of recently activated synapse associated receptors. Indeed, Arc appears to have an important role in activity-induced synaptic plasticity related to LTP stabilization and consolidation of hippocampal-dependent memory (9), but a more thorough characterization of Arc function will require analysis of mice with neuron-specific loss of Arc, since germ line mutations are embryonic lethal (16). By directly regulating Arc in a subpopulation of neurons, Egr1 would be expected to indirectly influence the properties of recently activated synapses. Thus, we propose that the learning and memory defects that have been defined in Egr1-deficient mice are, at least in part, explained by deregulation of Arc expression in a subpopulation of neurons. Whether Egr3-deficient mice have any defects in LTP stabilization and learning/memory behavior or whether Egr1/3 dKO mice have exaggerated defects is currently under investigation. Ultimately, Arc regulation by Egr transcription factors may be complex, considering the fact that Egr1 and Egr3 appear to have overlapping regulatory control of Arc expression in some neurons and distinct regulatory control in subpopulations of neurons where only a single Egr protein is expressed at any given time.

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