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Brief Communication

Experience-Dependent Coincident Expression of the Effector Immediate-Early Genes *Arc* and *Homer 1a* in Hippocampal and Neocortical Neuronal Networks

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The transcription of the immediate-early genes *Arc* and *Homer 1a* (*H1a*) is dynamically regulated in response to synaptic activity; their protein products function at the postsynaptic sites of excitatory synapses. Previous studies demonstrate a role for *Arc* in the maintenance of long-term potentiation and in memory consolidation processes and indicate a role for *H1a* in modifying glutamatergic signaling pathways. Using double-label fluorescence *in situ* hybridization, we demonstrate that *Arc* and *H1a* RNA expression is induced strongly in the same neurons of rat hippocampus and neocortex after exploration of a novel environment. These findings support the view that novel experience activates a cell-specific genomic program and that *Arc* and *H1a* may function in concert in the structural and functional modifications of dendrites that lead to long-term changes in synaptic efficacy.

Key words: memory; learning; transcription; hippocampus; cortex; immediate-early; gene; Arc*;* Homer 1*; dendrite; plasticity*

Research over the past few years has identified several proteins localized to the neuronal soma and dendrites that are rapidly and dynamically regulated by synaptic activity. These "effector" immediate-early genes (IEGs) have a number of cellular functions capable of modifying synaptic function (Lanahan and Worley, 1998). Two such effector IEGs are *Arc* (activity regulated cytoskeletal-associated protein, also known as *Arg3.1*) (Link et al., 1995; Lyford et al., 1995) and *Homer 1a* (*H1a*) (Brakeman et al., 1997). *Arc* mRNA and protein can be selectively targeted to active regions of the dendritic arbor (Steward and Worley, 2001). Moreover, disrupting *Arc* protein expression in the hippocampus impairs the maintenance of long-term potentiation (LTP) and the consolidation of memory for spatial experience (Guzowski et al., 2000). *H1a* interacts with several proteins within the postsynaptic density (PSD) and may play an important role in modifying glutamatergic signaling pathways (Xiao et al., 2000).

As more is learned about the individual functions of different IEGs, it is becoming increasingly important to determine which genes function in concert, as part of the same plasticity mechanisms, within the same neurons. Here, we use fluorescence *in situ* hybridization (FISH) to demonstrate that the transcription of *Arc* and *H1a* is dramatically upregulated in the same hippocampal and neocortical neurons of rats after exploration of a novel environment. These findings raise the possibility that *Arc* and *H1a* might function in concert as part of an activity-dependent genomic program to induce and stabilize long-term changes in synaptic efficacy in neural networks encoding memory for specific experiences.

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MATERIALS AND METHODS

Subjects, apparatus, and behavior. The subjects were male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), weighing 250–275 gm at arrival. Maximal electroconvulsive shock (MECS) was induced using a constant-current generator (ECT unit; Ugo Basile, Comerio, Italy) (Cole et al., 1990). For behavioral experiments, the rats were handled daily for 1 week before training to habituate them to the handling procedures. For the *Arc*/*H1a* behavioral time course experiment (see Figs. 2, 3), each rat ($n = 3$ per time point) sampled a novel environment for 5 min. The environment was a box divided into nine 400 cm^2 grids surrounded by 30-cm-high walls. The box was positioned on a table in a room with ambient lighting, thus allowing the rats access to both local and distant visual cues. Each rat was picked up and released into the center of a different grid square every 15 sec, on a semirandom schedule. This procedure was used to ensure that the rats sampled the entire environment. At the end of the 5 min session, the rat was returned to its home cage until it was killed at the assigned time after exploration. Because rats have a strong tendency to explore novel environments, the rats ($n = 6$) used to establish the correlation between *Arc* cytoplasmic and *H1a* intranuclear foci (INF) staining (see Fig. 4) were allowed to explore freely for 6 min a novel open box (61 × 61 cm with 24-cm-high sides), returned to their cages in the colony room, and killed 26 min later. All rats explored the novel environment completely, as evidenced by their multiple crossings of each floor grid (data not shown).

FISH. After decapitation with a rodent guillotine, the brains were removed rapidly, quick-frozen in isopentane (approximately −50°C), and then stored at −70°C until being sectioned on a cryostat. Twenty-micrometer-thick sections were mounted on slides such that all groups were represented on each slide. Digoxigenin- or fluorescein-labeled riboprobes were generated using commercial transcription kits (MaxiScript; Ambion, Austin, TX) and RNA labeling mixes (Roche Products, Hertforshire, UK). The plasmid used to generate the *Arc* antisense and sense riboprobes contained a full-length cDNA (3.0 kbp) of the *Arc* transcript (Lyford et al., 1995). The *H1a* antisense riboprobe was generated using an *H1a* cDNA clone and was directed to the 4.4 kb 3′ untranslated region (UTR) of the *H1a* mRNA (Brakeman et al., 1997).

Single- or double-label FISH was performed as described in detail previously (Guzowski et al., 1999; Guzowski and Worley, 2001; Bottai et al., 2002). In *Arc*/*H1a* double-labeling studies, digoxigenin-labeled *Arc* riboprobe was detected with anti-digoxigenin–HRP (Roche Products) and a cyanine-3 substrate kit (CY3 DirectFISH; PerkinElmer Life Sciences, Emeryville, CA). After detection of the *Arc* riboprobe, the slides were treated with 2% H_2O_2 to quench residual HRP activity. Fluorescein-labeled *H1a* probe was then detected with anti-fluorescein HRP (Roche Products) and a cyanine-5 substrate kit (CY5 DirectFISH; PerkinElmer Life Sciences). Nuclei were counterstained with either 4′,6′-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) (see Fig. 1) or YOYO-1 (quinolinium,1,1′-[1,3-propanediylbis [(dimethyliminio)-3,1-propanediyl]]bis[4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]]-tetraiodide; Molecular Probes, Eugene, OR). The specificity of the labeling was confirmed by two control conditions. Some slides were hybridized with *Arc* and *H1a* sense riboprobes; on other slides the riboprobe was omitted. For both control conditions, the remaining detection steps were performed without modification from the standard procedure.

Image acquisition and analysis. Images were acquired using a Leica (Nussloch, Germany) TCS-4D confocal microscope equipped with a krypton–argon laser. Photomultiplier tube (PMT) assignments, pinhole size, and contrast values were kept constant for each brain region within a slide. Depending on the required analysis, the settings were adjusted to optimize either the appearance of cytoplasmic labeling or that of INF (see Fig. 2*a*). Optimizing for INF was achieved by increasing the offset of the PMT. Figure 2*a*, *iii*, shows an image optimized for the appearance of *Arc* cytoplasmic signal and *H1a* INF. Z-stacks of either 1- or 2-μm-thick optical sections were acquired with either a 40× oil or 20× objective lens, respectively. Images for the behavioral time course analysis of *Arc* and *H1a* INF were collected at 20× magnification (one Z-stack per slide in two different slides, totaling 104–170 cells per area per slide). For the *Arc*/*H1a* correlation study (see Fig. 4), cell counts for each rat varied between 79 and 124 for CA1, 43 and 54 for CA3, and 73 and 127 for the parietal cortex.

Only putative neurons were included in the analyses. Putative glial-cell nuclei were identified based on their small size (~5 μm in diameter) and bright, uniform nuclear counterstaining (see Fig. 2*a*, *iii*). Furthermore, these cells did not express *Arc* or *H1a*, consistent with a previous report that *Arc* was expressed predominantly, if not exclusively, in excitatory neurons (Cirelli and Tononi, 2000). Z-stacks were analyzed using MetaMorph software (Universal Imaging Corporation, West Chester, PA). First, neuronal nuclei present in the median planes (representing 20% of the stack thickness) were identified and outlined. Then nuclei were characterized for the presence of *Arc* and *H1a* INF (see Fig. 3) or *Arc* cytoplasmic labeling and *H1a* INF (see Fig. 4). The results were expressed as a percentage of the total neuronal nuclei analyzed per stack. To prevent bias, the experimenter was unaware of the relationship between the images and the behavioral conditions they represented. The median planes criterion reduced the likelihood of analyzing partial nuclei, which could yield false negative results. This approach is essentially an optical dissector technique that minimizes sampling errors attributable to partial cells and stereological concerns, because variations in cell volumes do not influence sampling frequencies (West, 1993).

Statistical analyses. The main effect of treatment (e.g., caged/exploration or time after the end of behavioral testing) was evaluated by ANOVA. When the main effect was significant at the $\alpha = 0.05$ level, additional comparisons between groups were conducted with Fisher *post hoc* tests (Statview software; Statview, Berkeley, CA).

RESULTS

Previous characterization of *Homer 1* transcriptional regulation in mice demonstrated that the appearance of the *Homer 1* RNA signal after MECS was dependent on the location of the riboprobe along the primary transcript (Bottai et al., 2002). Here, we examined the time course of the appearance of the signal from an antisense riboprobe specific to the 3′UTR of rat *H1a*. This probe distinguishes synaptic activity-dependent *H1a* transcripts from the constitutive *Homer 1* forms (Xiao et al., 2000). These results were compared with those obtained using an antisense riboprobe generated from the entire *Arc* cDNA. Rats received MECS to activate IEG expression in the hippocampus and cortex. As reported previously (Guzowski et al., 1999), the *Arc* RNA signal

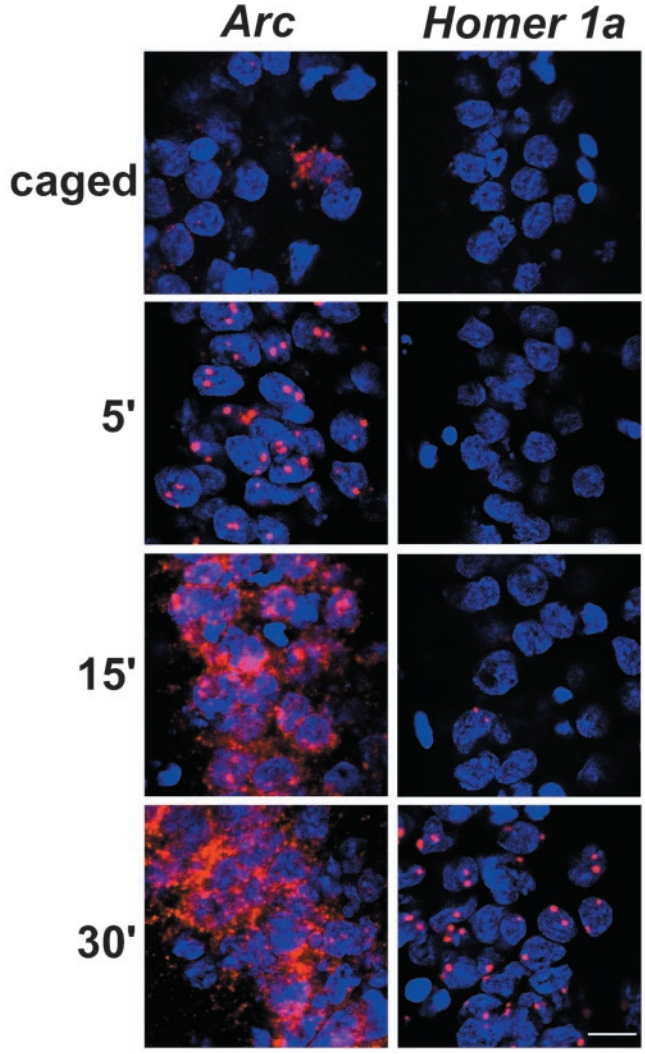

Figure 1. Distinct temporal profiles of *Arc* and *H1a* RNA appearance in CA1 neurons after MECS. Rats were killed at the indicated time (in minutes) after MECS. *Arc* and *H1a* RNAs were detected with digoxigenin-label antisense riboprobes as described in Materials and Methods. IEG RNAs were detected with CY3 (*red*), and nuclei were counterstained with DAPI (*blue*). Scale bar, 20 μm.

was detected in INF in ~95% of the neurons (see Materials and Methods) in the hippocampus (Fig. 1, *left*) and the parietal cortex within 5 min of MECS. IEG INF appear as either one or two (usually two) distinct areas of intense fluorescent staining within neuronal nuclei (Fig. 1). INF indicate the genomic sites of IEG RNA synthesis (Guzowski et al., 1999; Bottai et al., 2002). By 30 min, the *Arc* signal was prominent in the cytoplasm, and the proportion of INF-positive cells was similar to baseline (Fig. 1, *left*). *H1a* RNA was also detected in most hippocampal (Fig. 1, *right*) and parietal cortical neurons. However, *H1a* INF did not appear until 30 min after MECS (Fig. 1, *right*). The delayed appearance of the *H1a* INF is explained by the relative position of the 3′UTR riboprobe along the primary transcript (~40 kb from the transcript start site) (Bottai et al., 2002) and the limited elongation rate of RNA polymerase II (~1.4 kb/min) (Femino et al., 1998). These data show that with a strong stimulus, both *Arc* and *H1a* can be induced in the majority of the hippocampal and cortical neurons.

Next, we examined the kinetics of *Arc* and *H1a* RNA appear-

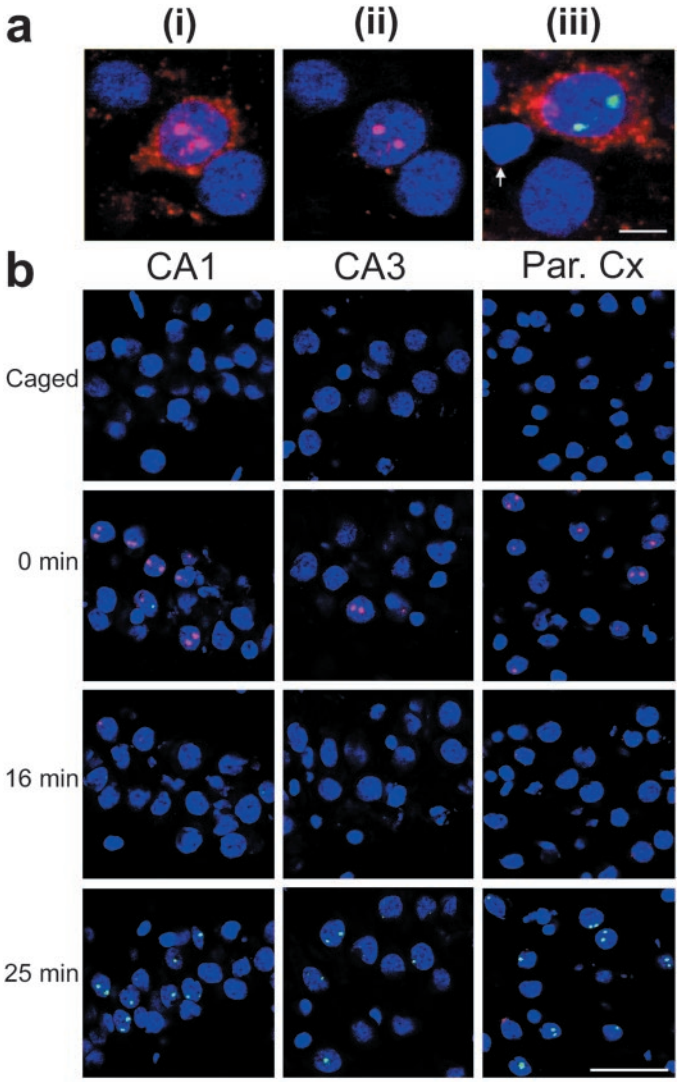

Figure 2. Dynamic appearance of *Arc* and *H1a* INF in CA1, CA3, and parietal cortical neurons after behavioral experience. *a*, Optimizing confocal microscope settings for the detection of either *Arc* cytoplasmic RNA labeling or *Arc* INF. *i* and *ii* represent the same field imaged with different confocal settings: *i*, Clear *Arc* cytoplasmic RNA labeling is achieved when the PMT offset is low; *ii*, unobstructed image of *Arc* INF is achieved by increasing the PMT offset; *iii*, an image optimized for detection of both *Arc* cytoplasmic labeling (*red*) and *H1a* INF (*green*). The *white arrow* points to a putative glial cell nuclei. *b*, Rats were exposed to a novel environment for 5 min and killed at the indicated time (in minutes) after removal (n = 3/group). Double-label FISH for *Arc* and *H1a* was performed on coronal brain sections as described in Materials and Methods. In these images, the confocal microscope settings were optimized for the detection of INF; the *Arc* signal is indicated in *red*, the *H1a* signal is indicated in *green*, and nuclei are indicated in *blue*. Note the predominance of the *Arc* INF at 0 min and *H1a* INF at 25 min in each brain region. *Par. Cx,* Parietal cortex. Scale bar, 50 μm.

ance after open field exploration (a behavior that induces place-cell activity in hippocampal neurons). Rats were exposed to a novel environment for 5 min and then killed at 0, 8, 16, 25, or 35 min after exploration (n = 3/group). Rats that were killed after a delay were returned to their home cage in the colony room between exploration and being killed. The brains were processed for *Arc* and *H1a* double-label FISH, and confocal images were acquired for qualitative (Fig. 2) or quantitative (Fig. 3) analysis. The dynamics of *Arc* and *H1a* transcription were similar in the

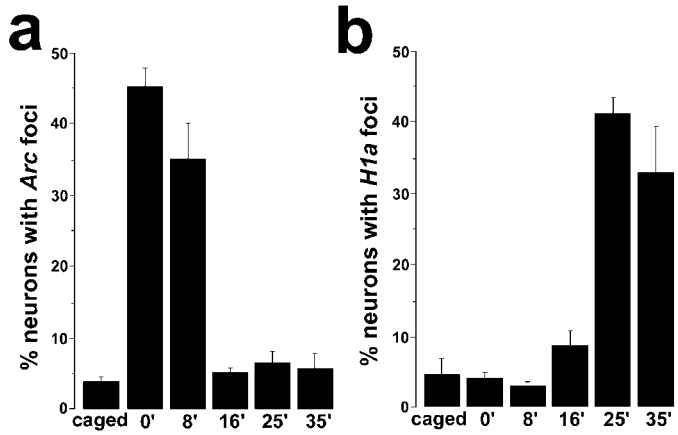

Figure 3. Experience-dependent appearance of *Arc* INF in CA1 neurons is rapid, transient, and does not coincide in time with the delayed appearance of *H1a* INF. Confocal Z-stacks of the CA1 region were collected from *Arc*/*H1a* double-label slides from rats killed at different delays after behavioral exploration (see Results and Fig. 2). The percentage of counted nuclei positive for *Arc* (*a*) or *H1a* (*b*) INF is indicated for each time point. Note that compared with the caged control group, the percentage of neurons with *Arc* INF was significantly higher only in the 0 and 8 min groups, whereas the percentage of neurons with *H1a* INF was significantly higher only in the 25 and 35 min groups.

hippocampal CA1 and CA3 regions and in the parietal cortex (Fig. 2*b*). In each region, the greatest proportion of *Arc* INF-positive cells was seen in the group killed immediately after exploration. Furthermore, the proportion of *Arc* INF-positive cells returned to control levels within 16 min after exploration. In contrast, an increase in the proportion of *H1a* INF-positive cells above control levels was not seen until 25 min after exploration.

Quantitative analysis of Z-stacks from CA1 confirmed the above observations for both *Arc* (Fig. 3*a*) and *H1a* (Fig. 3*b*). For *Arc* cell counts, there was a significant effect of time of death (overall ANOVA; $F_{(5,12)} = 51.34$; $p < 0.0001$), and *post hoc* comparisons revealed significant differences between the caged and 0 min groups, caged and 8 min groups, and 8 min and 16 min groups ($p < 0.0001$ for all three comparisons). The proportions of *Arc* INF-positive cells in the 16, 25, and 35 min groups were not statistically different from the caged group. In contrast, the percentage of *H1a* INF-positive CA1 neurons was comparable with that of caged controls until 25 min after exploration (Fig. 3*b*) (overall ANOVA; $F_{(5,12)} = 26.97$; $p < 0.0001$). *Post hoc* comparisons revealed significant differences between the caged and 25 min groups and the caged and 35 min groups ($p < 0.0001$), whereas those between the caged and 0, 8, and 16 min groups did not reveal a significant effect. In addition, the percentage of *H1a* INF-positive neurons in the 25 min postexploration group was significantly higher than that of the 16 min group ($p < 0.0001$).

The finding that *Arc* and *H1a* were expressed in a similar proportion of hippocampal and parietal cortical neurons of rats that had explored a novel environment is consistent with the idea that these genes might be dynamically regulated in the same neurons. To test this possibility directly, the correspondence of neurons containing *Arc* cytoplasmic labeling and *H1a* INF in the hippocampus and cortex of caged control rats was compared with rats that had explored a novel environment 30 min before being killed. Neurons were scored as containing *Arc* cytoplasmic labeling only, *H1a* INF only, or both *Arc* cytoplasmic labeling and *H1a* INF (Fig. 2*a*, *iii*). In the hippocampus (CA1 and CA3 regions)

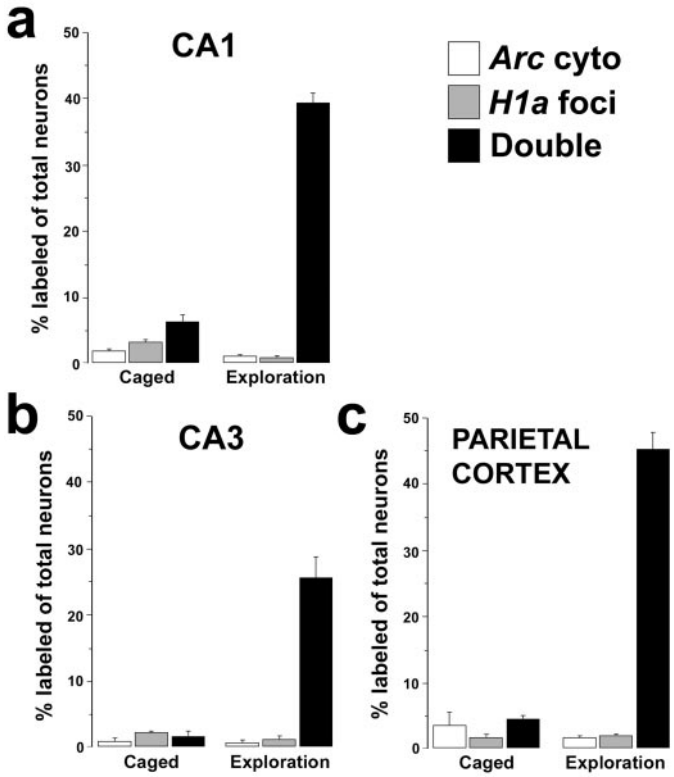

Figure 4. Exploration of a novel environment induces the coincident expression of *Arc* and *H1a* in single neurons of the rat hippocampus and parietal cortex. One group of rats was exposed to a novel environment for 6 min, returned to their home cages in the colony room for 26 min, and then killed (exploration group; $n = 6$). A separate group of rats was killed directly from their home cages (caged control group; $n = 3$). Double-label FISH for *Arc* and *H1a* was performed on coronal brain sections, and image stacks were collected and analyzed from CA1, CA3, and parietal cortical regions. The percentage of neurons in a respective brain region expressing *Arc* cytoplasmic RNA staining only (*white bars*), *H1a* INF only (*gray bars*), or both (*black bars*) is shown for rats from both groups. Note that the only staining class population that changed with exploration (relative to the caged controls) was the *Arc* cytoplasmic+/*H1a* INF+ double-label class (*black bars*). *Arc cyto*, *Arc* cytoplasmic.

and the parietal cortex of the rats from the exploration group, the correspondence of *Arc* cytoplasmic labeling and *H1a* INF was >90% (Fig. 4) (95% for CA1, 94% for CA3, and 93% for the parietal cortex).

DISCUSSION

The current findings demonstrate that transcription of the IEGs *Arc* and *H1a* is dynamically regulated by physiological activity in the same hippocampal and cortical neurons. Double-label FISH revealed distinct temporal and spatial patterns of *Arc* and *H1a* RNA appearance after MECS (Fig. 1) and behavioral experience (Figs. 2–4). The highest proportion of *Arc* INF-positive cells was observed in the rats killed immediately after exploration (Figs. 2, 3), indicating rapid induction of *Arc* transcription. This period of transcription is very brief, because the proportion of *Arc* INF-positive cells returned to control levels within 16 min after exploration, and *Arc* mRNA was prominent within the cytoplasm by 30 min (Figs. 2–4) (Guzowski et al., 1999). In contrast, the proportion of *H1a* INF-positive cells detected with the 3′UTR riboprobe did not change until 25 min after exploration. At that time, the proportion of *H1a* INF-positive cells was highest and was similar to that of *Arc* INF-positive neurons from the rats killed immediately after exploration (Figs. 2, 3). Double-label FISH performed on brain sections of rats that had explored a novel environment 30 min before being killed revealed >90% correspondence of neurons positive for cytoplasmic *Arc* mRNA and *H1a* INF (Fig. 4). Thus, with a discrete behavioral stimulus of known onset and limited duration, transcriptional activation of *Arc* and *H1a* is coincident in single neurons of the hippocampus and parietal cortex.

The coincident transcription of *Arc* and *H1a* may be regulated by the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) cascade. The finding that a MAPK/ERK inhibitor blocked forskolin-induced *Arc* transcription in cultured hippocampal neurons (Waltereit et al., 2001) is consistent with this hypothesis. In addition, pretreatment with MAPK kinase (MEK) inhibitors blocked LTP and prevented increases in ERK2 and cAMP response element-binding protein phosphorylation as well as increases in *Arc* RNA expression caused by the local infusion of BDNF in the dentate gyrus of intact rats (Ying et al., 2002). Similarly, MEK inhibitors blocked glutamate-induced increases in *H1a* transcription in cultured cerebellar granule cells (Sato et al., 2001). Thus, the coincident transcription of *Arc* and *H1a* demonstrated here could result from the activation of the MAPK/ERK pathway rather than from the activation of separate, parallel pathways acting on the promoters of these genes. However, it must be noted that although *Arc* and *H1a* may use the same mechanisms for transcriptional activation, *H1a* is subject to additional levels of activity-dependent regulation, including the use of an alternative transcription termination signal and conversion of an intronic sequence (in constitutive forms) to an exonic sequence (in IEG forms) (Bottai et al., 2002).

The distinct temporal patterns of *Arc* and *H1a* INF are explained by the different architectures of the transcription units for these genes (*Arc* mRNA is derived from a limited primary transcript; ~3.5 kb with two small introns; GenBank accession number AF177701), whereas *H1a* mRNA is derived from a primary transcript spanning ~50 kb (Bottai et al., 2002). Thus, the time course of appearance of *H1a* INF is dependent on the position of the riboprobe along the primary transcript: Riboprobes to either exon 1 or intron 1 of *Homer 1* detect *H1a* INF with induction kinetics indistinguishable from that of *Arc* INF (Bottai et al., 2002), whereas those to the 3′UTR detect *H1a* INF with much delayed kinetics (Figs. 1–4) (Bottai et al., 2002).

The coincident expression of *Arc* and *H1a* seen after experience (Fig. 4) and the temporally offset appearance/disappearance of *Arc* and *H1a* INF (Fig. 3) enable an important modification of the cellular analysis of temporal activity by FISH (catFISH) brain imaging method (Guzowski et al., 1999, 2001). The power of catFISH is its ability to identify, at a single-cell level, neuronal ensembles activated by two distinct behavioral experiences within an animal, a property that distinguishes catFISH from other imaging methods. In the original method, *Arc* INF indicate cells active in the 5–10 min preceding death, whereas the cytoplasmic *Arc* signal indicates cells active ~30 min before death. Although catFISH has been used successfully to determine neural activity for two experiences (Guzowski et al., 1999), quantifying the cytoplasmic IEG RNA signal can be difficult when many cells are activated in a region with a high cell density, such as the pyramidal cell layers of the hippocampus. However, the combined use of *Arc* and 3′UTR *H1a* riboprobes in double-label FISH circumvents this problem by exploiting the transcription rate of RNA polymerase II (~1.4 kb/min) (Femino et al., 1998) and the natural "genomic timers" of cellular activation afforded by the dissimilar

gene structures of *Arc* and *H1a* (Bottai et al., 2002). With this approach (*Arc*/*H1a* catFISH), the activity history of many neurons can be distinguished based solely on strong intranuclear signals: *Arc* INF indicate cells activated by an experience shortly before (<15 min) death, whereas *H1a* INF indicate cells activated at least 25 min earlier. The exclusive use of intranuclear signals greatly facilitates manual analysis and makes catFISH amenable to computer automation for large-scale investigations of neural population interactions during learning and memory.

Several lines of evidence suggest that both *Arc* and *H1a* are involved in synaptic plasticity. First, the expression of both genes is increased dramatically in the dentate gyrus after the induction of perforant-path LTP (Link et al., 1995; Lyford et al., 1995; Brakeman et al., 1997) and in the insular cortex after the LTP-inducing stimulation of the amygdala (Jones et al., 1999). Moreover, inhibiting hippocampal *Arc* protein expression disrupts the maintenance of perforant-path LTP and impairs memory consolidation (Guzowski et al., 2000). Biochemical evidence indicates that *Arc* may be a component of NMDA receptor complexes (Plath et al., 2001). The *Homer 1* gene is alternatively spliced from a large primary transcript to form two constitutively expressed forms (*Homer 1b* and *Homer 1c*) and two synaptic activity-regulated IEG forms (*H1a* and *ania3*) (Xiao et al., 1998, 2000; Bottai et al., 2002). Homer 1b/c proteins are implicated in coupling group 1 metabotropic glutamate receptors (mGluR) with IP_3 and ryanodine receptors and in coupling either of these intracellular receptors with the NMDA receptor-associated PSD-95 complex (Xiao et al., 2000). It has been hypothesized that *Homer 1* IEG forms, which lack the C-terminal coiled-coil domain of the constitutive forms, act as natural antagonists to Homer 1b/c forms to modify glutamatergic signaling pathways (Xiao et al., 2000). Consistent with this hypothesis, *H1a* transgene expression in Purkinje neurons alters mGluR-induced Ca^{2+} release from intracellular stores (Tu et al., 1998).

Arc transcription has been shown previously to be activated in CA1 neurons in an environmental context-specific manner (Guzowski et al., 1999), as observed for place-cell firing activity in hippocampal neurons. The striking concordance of *Arc* and *H1a* coincident expression shown here indicates that, like *Arc*, *H1a* is induced in the neural networks engaged in information processing. The fact that both *Arc* and *H1a* are dynamically expressed in the same neurons by experience and are localized to the postsynaptic density suggests that these genes may function in concert to modify synaptic efficacy in the hippocampal and neocortical networks responsible for encoding the memory of discrete experiences.

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