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Environment-specific expression of the immediate-early gene *Arc* in hippocampal neuronal ensembles

John F. Guzowski[1], Bruce L. McNaughton[2], Carol A. Barnes[2] and Paul F. Worley[3]

[1] *Virion Systems, Inc., 9610 Medical Center Drive, Suite 100, Rockville, Maryland 20850, USA*

[2] *Department of Psychology and Division of Neural Systems, Memory and Aging, University of Arizona, Life Sciences North, Room 384, Tucson, Arizona 85724, USA*

[3] *Departments of Neuroscience and Neurology, Johns Hopkins University School of Medicine, Johns Hopkins University, 725 N. Wolfe St., Baltimore, Maryland 21205, USA*

Correspondence should be addressed to J.F.G. (jguzowsk@welchlink.welch.jhu.edu)

We used fluorescent *in-situ* hybridization and confocal microscopy to monitor the subcellular distribution of the immediate-early gene *Arc*. *Arc* RNA appeared in discrete intranuclear foci within minutes of neuronal activation and subsequently disappeared from the nucleus and accumulated in the cytoplasm by 30 minutes. The time course of nuclear versus cytoplasmic *Arc* RNA accumulation was distinct, and could therefore be used to infer the activity history of individual neurons at two times. Following sequential exposure of rats to two different environments or to the same environment twice, the proportion of CA1 neurons with cytoplasmic, nuclear or overlapping *Arc* expression profiles matched predictions derived from ensemble neurophysiological recordings of hippocampal neuronal ensembles. *Arc* gene induction is thus specifically linked to neural encoding processes.

Since the observation that c-*fos* is dynamically regulated in the brain[1], methods that detect immediate-early gene (IEG) RNAs and proteins have gained widespread use as neuronal activity markers in behavioral studies. Despite this, the association between IEG expression and specific neural coding has remained largely unclear. This uncertainty stems from the methodologies used in most IEG studies, which have made it difficult to distinguish gene induction that is specifically linked to information processing from activity that may be due to stress, novelty, motor activity or other processes that accompany the behavioral response. This problem is common to many brain-imaging methods, and can be overcome in part using techniques that permit repeated observations of the same subjects, such as fMRI and PET. Presently, IEG expression analysis does not permit an intra-subject comparison because of limitations inherent in current histochemical procedures; instead, these procedures require separate groups of animals to be used for different behavioral or control conditions.

We describe here an experimental IEG approach, termed cellular compartment analysis of temporal activity by fluorescent in-situ hybridization (catFISH), that allows neuronal populations activated by two experiences to be discriminated and quantified. Thus, it is possible to detect not only net changes in total neuronal activation, but also the ensemble representation for each event. We used catFISH to test the specificity of the IEG response in a two-environment exploration protocol. Previous studies using parallel ensemble recording methods indicate that a fraction of CA1 neurons are specifically activated during exploration of a given environment (typically, 30–50%, depending on environment size)[2–4], and that the cell groups demonstrating strong activity in each of two distinct environments are uncorrelated. Our initial studies showed that a similar proportion of CA1 cells activated RNA expression of the IEG *Arc*[5] (also termed Arg3.1; ref. 6) following exploration of a given environment. The correspondence between the percentage of activated neurons detected by *Arc* catFISH and by single-unit recording studies in similar experimental conditions led us to hypothesize that IEG expression may be induced in CA1 neurons following periods of intense activity, such as occurs when a cell expresses a 'place field'. If true, the cells detected by *Arc* catFISH should meet predictions derived from electrophysiological studies. Specifically, rats reintroduced into the same environment should reactivate the same ensemble[7], whereas a different ensemble should be activated if the rat is placed in a novel environment[2]. In fact, because of the stochastic nature of hippocampal neuronal activation, the percentage of cells responding in one or both environments in a two-environment protocol can be predicted: if pA is the percentage of cells responding only to environment A and pB is the percentage of cells responding only to environment B, then those responding to both environments A and B are given by $p_A \times p_B$ and those responding to neither environment are given by $(1 - p_A) \times (1 - p_B)$.

The experimental data obtained using *Arc* catFISH matched the predicted frequencies and thus provide strong support for the conclusion that *Arc* gene expression in CA1 neurons is linked to neural encoding processes.

RESULTS

While developing a sensitive fluorescent *in-situ* hybridization (FISH) procedure to detect IEG mRNAs, we made an unexpected observation. Distributed neurons in the hippocampus and cortex of normal rats showed a characteristic intranuclear signal, which consisted of one or two discrete foci of intense fluorescence. The intranuclear location of the antisense IEG riboprobes was confirmed by colocalization of the IEG RNA signal with the nuclear counterstain DAPI (4′,6-diamidino-2-phenylindole) and the use of laser scanning confocal microscopy

Fig. 1. Different cell staining profiles detected using *Arc* FISH. These 0.7-micron single optical plane confocal images are from the CA3 region of control rats. (**a–c**) Cells subjected to FISH with a digoxigenin-labeled *Arc* antisense probe, which was detected with a CY3 fluorescent substrate (red; see Methods). (**a**) Characteristic *Arc* intranuclear foci. (**b**) Cytoplasmic-only labeling of *Arc* RNA. (**c**) An *Arc* intranuclear foci/cytoplasmic double-positive cell. (**d**) Double labeling with *Arc* (green) and *zif268* (red) antisense riboprobes demonstrates distinct pairs of intranuclear foci for *Arc* and *zif268*. *Arc* intranuclear foci are indicated by short arrows (**a**, **c**, **d**). The *zif268* intranuclear foci in (**d**) are indicated by the longer arrows. Nuclei are counterstained with DAPI (blue) in all panels; final magnification for all images is 320×.
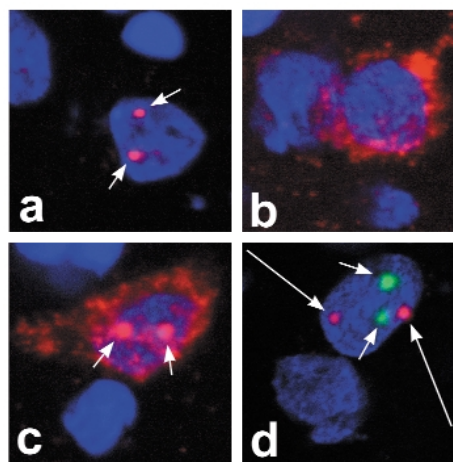
(**Fig. 1a**). In addition to cells with intranuclear foci, we observed cells with three other distinct staining profiles: cells contained only IEG RNA cytoplasmic staining (**Fig. 1b**), both intranuclear and cytoplasmic staining (**Fig. 1c**) or no IEG RNA staining. The cytoplasmic location of the IEG RNA signal was determined by colocalization with an antisense riboprobe to the β isoform of calcium and calmodulin kinase II (data not shown). The cytoplasmic and intranuclear IEG signal was detected with antisense, but not sense, IEG riboprobes, and was destroyed by pretreatment with RNase A (**Figs. 1 and 2**). Double-labeling experiments identified distinct intranuclear foci for the IEGs *Arc* and *zif268*, often in the same nucleus (**Fig. 1d** and **Fig. 2a**). Similar intranuclear/cytoplasmic RNA labeling is seen for other IEGs, including c-*fos*, *cox-2*, *Homer 1A* and *Narp*[8] (data not shown).

To examine the possibility that the intranuclear foci represented sites of IEG RNA transcription, we determined the effect of maximum electroconvulsive shock (MECS) treatment on their appearance in hippocampal CA1 neurons. IEG mRNAs are rapidly induced by MECS[9], presumably by activating transcription. MECS caused a dramatic increase in the number of neurons with intranuclear IEG foci (**Fig. 1a–f**) that appeared within two minutes and continued to increase in fluorescence intensity at five minutes. By 15 minutes, the percentage of neurons exhibiting intranuclear foci began to decrease, and IEG mRNA was first detected in the cytoplasm. By 30 minutes, the percentage of neurons exhibiting intranuclear IEG foci decreased to caged control levels, and the IEG mRNA was located predominantly within the cytoplasm. Double labeling with *Arc* and *zif268* antisense riboprobes revealed similar time courses of expression up to 30 minutes. At 60 minutes, cytoplasmic *zif268* RNA levels were still elevated above control levels, whereas cytoplasmic *Arc* RNA levels were similar to control levels. The loss of *Arc* but not *zif268* cytoplasmic signal at 60 minutes may be due to the rapid and specific transport of *Arc* mRNA to dendritic processes[5], a feature that distinguishes *Arc* from other identified IEGs.

Arc RNA expression dynamics were examined in an exploration protocol. *Arc* was selected for these studies because preliminary studies showed it to be dynamically expressed throughout the cortex and hippocampus in response to behavioral stimulation, and because its basal expression in caged control rats is low. Individual rats were placed in environment A for five minutes. To ensure uniform and complete environmental experience during this time, the animals were picked up every ten seconds and
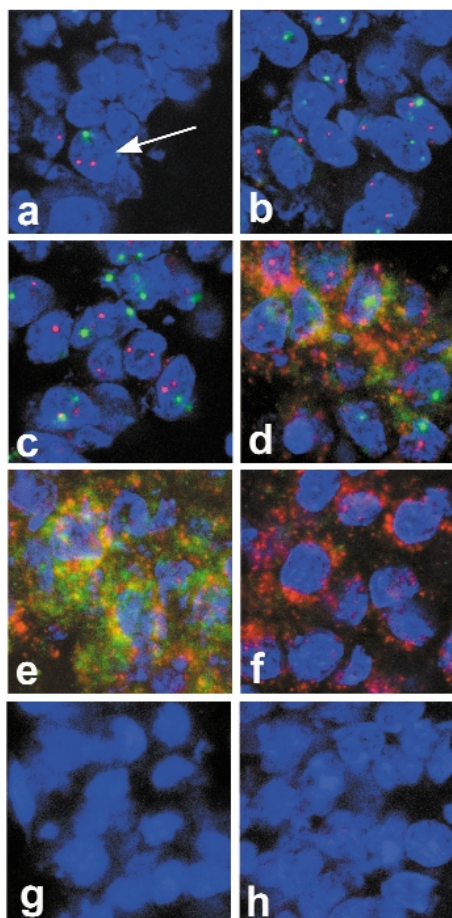

Fig. 2. Time course of *Arc* and *zif268* RNA induction in CA1 neurons after seizure (MECS). Two color FISH using *Arc* (green) and *zif268* (red) antisense riboprobes on sections from rats killed at different times following MECS: (**a**) caged control (no MECS) (**b**) 2 min (**c**) 5 min (**d**) 15 min (**e**) 30 min, and (**f**) 60 min after MECS. (**g**) Pretreatment of sections with RNase A (100 μg per ml) destroys *Arc* and *zif268* staining at all time points. This field is from a rat 5 minute after-MECS. (**h**) No staining is observed on sections from a rat brain with a digoxigenin-labeled *Arc* sense riboprobe 30 min after MECS. For all images, nuclei are stained with DAPI (blue). Final magnification, 200×. The arrow in (**a**) denotes a single neuron in which intranuclear foci for *Arc* and *zif268* are present.

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Fig. 3. Time course of *Arc* RNA induction in different brain regions following exploration of a novel environment. Representative fields from the hippocampal CA1 and CA3 principal cell layers, deep layers of the parietal cortex (PAR CX), or the dentate gyrus granule cell layer (DG). *Arc* RNA staining is indicated by CY3 fluorochrome (red), and nuclei are stained with DAPI (blue). Final magnification is 200× for all regions except the dentate gyrus, shown at 120×. The handling of the behavioral groups is described in the text. Note that *Arc* gene expression is strongly induced in neurons of CA1, CA3 and PAR CX by a limited behavioral experience, and that the time course of *Arc* RNA expression is indistinguishable among these brain regions. In contrast, the staining pattern in the DG did not obviously change over the time interval examined—in all groups, a low percentage of *Arc*-positive cells were found distributed throughout the DG.
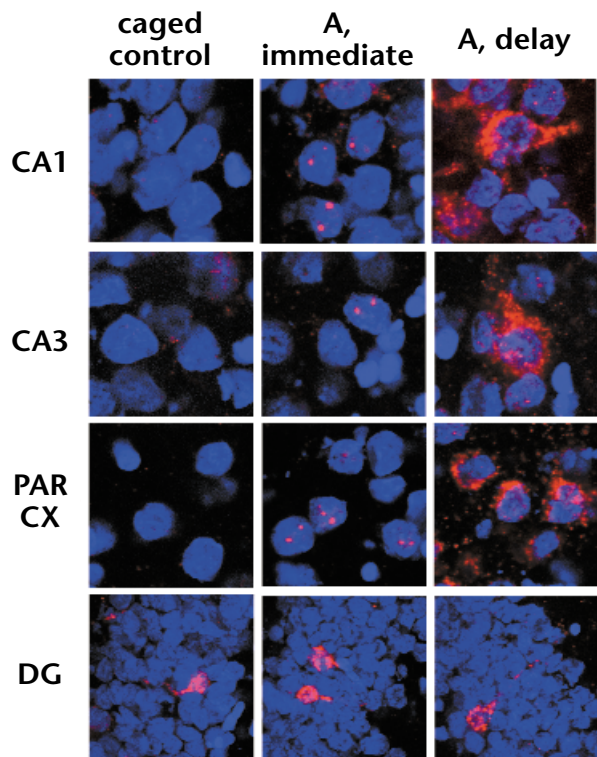

replaced in one of nine randomly selected locations. After 5 minutes, the rats were either immediately killed (A/immediate) or returned to their home cages for 25 minutes before being killed (A/delay). The expression pattern and kinetic profile of *Arc* RNA induction was similar in CA1, CA3 and cortical neurons (**Fig. 3**). In these regions, rats from the exploration groups showed a dramatic increase in cells positive for *Arc* RNA relative to caged controls. In contrast, dentate gyrus granule cells showed no obvious increase in *Arc* expression following exploration. In the A/immediate group, *Arc* signal was located predominantly in intranuclear foci (**Fig. 3**). In CA1, 45% of neurons showed intranuclear foci compared with 8% in CA1 of rats maintained in their home cages (**Fig. 4**). For comparison, 95% of CA1 neuronal nuclei contained *Arc* intranuclear foci 5 minutes after a MECS stimulus (134 of 141 neuronal nuclei); thus, the vast majority of CA1 neurons can induce *Arc* transcription given a sufficient stimulus. In the A/delay group, *Arc* RNA was prominent in the cytoplasm, and intranuclear foci decreased to control levels (**Figs. 3** and **4**). The percentage of CA1 neurons with cytoplasmic *Arc* mRNA was 44% (**Fig. 4**). In agreement with neurophysiological data, *Arc* induction in CA3 was similar to CA1, whereas a smaller proportion of cells were labeled in the dentate gyrus[11]. These observations confirm the time course and cellular compartmentalization of *Arc* RNA induction following behavioral activation.

The synchronized, time-dependent shift in the FISH signal from the nucleus to the cytoplasm (**Figs. 2** and **3**) suggested that the subcellular distribution of *Arc* RNA could be assayed to determine the activity history of neurons at two times. The presence of intranuclear IEG foci would indicate activation within the epoch of 2–20 minutes before the animal was killed, whereas cytoplasmic IEG signal would indicate activation within an epoch >30 minutes before death.

We examined *Arc* induction patterns in rats exposed sequentially to two environments. Rats were first placed in environment A for 5 minutes and returned to their home cages for 20 minutes. Rats were then returned to the same environment A (A/A group) or placed in a similar, but recognizably different environment B (A/B group) for five minutes and then immediately killed. The animals were handled using the identical protocols (see above) in the two environments, and their behavior was qualitatively indistinguishable. In the A/A group, the majority of the cells (90%) activated in the first exploration session were again activated in the second exploration session (**Fig. 4**). This single population of cells represented 40% of neurons in CA1. In contrast, the A/B group showed 3 populations of neurons (cytoplasmic only, intranuclear foci only or combined cytoplasmic and intranuclear foci) that represented 22%, 23% and 16% of

Fig. 4. *Arc* catFISH defines CA1 neuronal ensembles encoding distinct environments. Rats explored environments designated A and B as described in the text. The distinct staining profiles seen in A/A and A/B groups demonstrate that the induction of *Arc* transcription in CA1 is highly specific to the nature of the behavioral experience. The A/immediate, A/delay, and A/B/delay groups define the temporal resolution properties of *Arc* catFISH. From each rat (n = 3 rats per group), 97–146 (mean, 120) neurons were counted; the total number of neurons analyzed for this experiment was 2157. The percentage of positive cells for each staining profile was determined for each individual rat; reported values indicate the group mean. *p < 0.002 relative to caged controls, ANOVA with Scheffe *post-hoc* analysis. #p < 0.05 relative to the other 2 cell populations for that group by paired *t*-test.
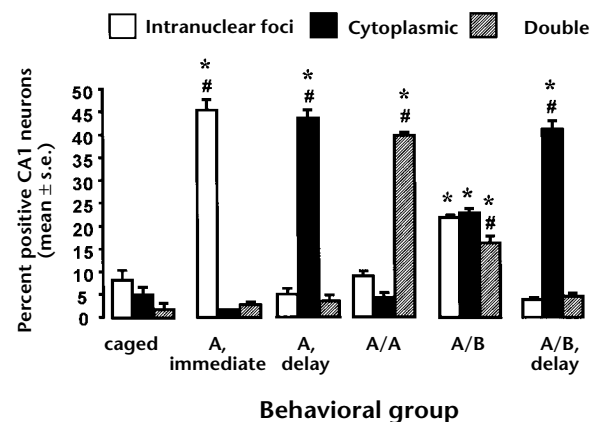
total CA1 neurons, respectively. The experimentally observed frequencies for each of the 3 populations were essentially identical to the predicted 24% (cytoplasmic), 24% (intranuclear foci) and 16% (cytoplasmic and intranuclear foci; χ^2 = 0.21, d.f. = 2; *p* = 0.90); these predicted frequencies are based on the experimentally observed values indicating that approximately 40% of CA1 neurons induce *Arc* RNA expression in response to exploration of either environments A or B. These findings support the hypothesis that *Arc* catFISH provides visualization of CA1 neuronal ensembles activated by two sequential experiences.

Rats in the A/B/delay group, who received the same handling as the A/B group and were then returned to their home cages for 25 minutes before being killed, demonstrate an important property of the *Arc* catFISH assay (**Fig. 4**). Because of the short half-lives of IEG RNAs in general[12] and the specific transport of *Arc* RNA to dendritic processes[5,6,10,13], the *Arc* signal from one event does not persist for long in the catFISH assay. In this case, the *Arc* RNA signal induced by exposure to environment A was undetectable at one hour, and only *Arc* signal from exposure to environment B was detected. These properties account for the extremely low background and tight temporal resolution possible using *Arc* catFISH.

DISCUSSION

The induction of *Arc* RNA expression in CA1 neurons confirmed two predictions derived from electrophysiological studies of hippocampal pyramidal cells in spatial foraging protocols: first, *Arc* RNA expression was induced in a single subset of the pyramidal cell population upon sequential visits to the same environment, and second, *Arc* RNA expression was induced in two overlapping pyramidal cell subsets upon sequential visits to two different environments. Although the factors that cause a given pyramidal cell to fire in a particular location are complex and only partially understood[14–18], the present findings provide strong support for the conclusion that the experience-dependent induction of *Arc* gene expression is linked to input-specific neural activity patterns and is not a generalized response to stress, novelty or motor activity that accompanies the exploratory behavior.

It is important to note that a similar percentage of CA1 neurons was engaged (as indicated by *Arc* RNA induction) by either the A or B environments in the A/B group (**Fig. 4**). The number of 'A-responsive' neurons (cytoplasmic plus double labeled) was 39% and the number of 'B-responsive' neurons (intranuclear foci plus double labeled) was 38%. Therefore, one could not have concluded that the response to the different environments was specific using a standard IEG detection method and separate groups of rats for the A and B conditions. Furthermore, methods such as PET or fMRI, which detect changes in metabolic or hemodynamic activity related to net neuronal activity, would also fail to discern that the responses to the different environments were distinct. *Arc* catFISH provides information about the content of the neuronal activity that previously could only have been obtained using electrophysiological recording techniques. Although *Arc* catFISH does not provide the real-time resolution of recording methods, information can be obtained for a large number of neurons across multiple brain structures. Thus, *Arc* catFISH may provide an important complementary approach to electrophysiological methods in identifying neural networks responsible for establishing high level internal representations of the external world.

The intranuclear foci detected in the present study likely represent newly transcribed RNA at allelic sites. This conclusion is based on the rapid appearance of the foci after neuronal activation, their paired morphology and the presence of distinct sites detected with two different IEG antisense riboprobes in double-labeling experiments (**Figs. 1** and **2**). Intranuclear sites of transcription have previously been described that are similar in morphology to the observed intranuclear IEG foci and additionally hybridize with intronic RNA sequence probes[19,20]. The ability to visualize allelic transcription *in vivo* provides a direct assay of gene activation that is temporally more precise than conventional assays of total cellular mRNA and greatly enhances the sensitivity and specificity of IEG methods for behavioral studies. Additionally, IEG catFISH may prove valuable in studying dynamics of signal-transduction pathways. For example, the rapid appearance of intranuclear IEG foci in our study indicates that signal transduction from the synapse to the nucleus must occur within seconds of a stimulus; these findings place considerable temporal constraints on the molecular pathways that could account for the observed induced gene expression.

The current findings provide evidence that the expression of specific IEGs may be induced in hippocampal pyramidal cells by the neuronal activity accompanying the development of an internal representation of a specific environmental context. Although other studies have examined the induction of another IEG, c-*fos*, in the hippocampus in response to behavioral training[21–23], the methods used in these earlier studies could not provide both the cellular and temporal resolutions necessary to show that IEG induction in hippocampal neurons is quantitatively and qualitatively similar to the activation of hippocampal 'place' cells detected using electrophysiological recording methods. Finally, because *Arc* gene expression and the stabilization of hippocampal place fields depend on the activation of NMDA receptors[5,24], *Arc* gene expression may be involved in the long-term stabilization of newly formed internal representations of experience.

METHODS

Behavioral handling procedures. Nine-month-old male F344 rats (Harlan Sprague-Dawley) were handled before the experiment to familiarize them to the experimenter and to handling in general. Environment A was circular and surrounded by 30-cm-high walls. Environment B, located in an adjacent room, was a square platform without walls, and was elevated ten cm above a supporting table. Lighting intensity was similar in the two environments, and each room contained unique distal cues. Both of the 3600-cm² environments were divided into 9 grids, each 400 cm². During each five-min exploration session, the rat was picked up, wherever it was, and released into the center of a different grid square every ten seconds on a semi-random schedule. By the end of an exploration session, the rats were placed in each of the nine grid squares three times. This procedure ensured that the rats sampled the entire environment. Intervals between exploration sessions and death for rats of the different behavioral groups are described in Results. For the seizure time course study (**Fig. 2**), MECS were induced in adult rats using a constant-current signal generator (ECT unit, Ugo Basil) as described[25].

Fluorescent *in-situ* hybridization. At the appropriate time after handling, rats were killed by decapitation using a rodent guillotine. Brains were rapidly removed and quick-frozen in a beaker of isopentane equilibrated in a dry ice/ethanol slurry and stored at –70°C until further processing. Coronal brain sections (20 μm) were prepared using a cryostat and arranged on slides (Superfrost Plus, VWR) so that all experimental groups were represented on each slide. Slides were air dried and stored frozen at –20°C until use.

A commercial transcription kit (MaxiScript; Ambion, Austin, Texas) and premixed RNA labeling nucleotide mixes containing either digoxigenin or fluorescein-labeled UTP (Roche Molecular Biochemicals) were used to generate cRNA riboprobes. Riboprobes were purified on G-50 spin columns (Pharmacia). The yield and integrity of riboprobes was confirmed by gel electrophoresis. The plasmid used to generate the *Arc* antisense and sense riboprobes contained a previously described[5], nearly full-length cDNA (~3 kbp) of the *Arc* transcript.

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The plasmid used to generate the *zif268* antisense and sense riboprobes has been described elsewhere[26].

Slide-mounted brain sections were fixed in 4% buffered paraformaldehyde, treated with 0.5% acetic anhydride/1.5% tri-ethanolamine, dehydrated through serial ethanol solutions, incubated in chloroform, rehydrated through serial ethanol solutions and equilibrated in 2× SSC. Slides were incubated in 1× prehybridization buffer (Sigma) for 30 min at room temperature. Riboprobe (100 ng) was diluted to 100 µl in a commercial hybridization buffer (Amersham), heat denatured, chilled on ice, and then added to each slide. Coverslips were added to the slides, and hybridization was carried out at 56°C for 16 hours. Slides were washed to a final stringency of 0.5× SSC at 56°C; these washes included an earlier wash step at 37°C in 2× SSC with RNase A (10 µg per ml). Endogenous peroxidase activity was quenched with 2% H_2O_2 in PBS, slides were blocked (with agent from the DirectFISH kit; NEN Life Sciences, Boston, Massachusetts), and incubated with an appropriate horseradish peroxidase (HRP)-antibody conjugate (Roche Molecular Biochemicals) overnight at 4°C. Slides were washed three times in Tris-buffered saline (with 0.05% Tween-20), and the conjugate was detected using DirectFISH. Slides were coverslipped with antifade media containing DAPI (Vectashield; Vector Labs, Burlington, California) and sealed.

For combined *Arc* and *zif268* FISH, the digoxigenin-labeled *zif268* antisense and the fluorescein-labeled *Arc* antisense riboprobes were added together during the hybridization step. The two probes were then detected sequentially. Following detection of the digoxigenin-labeled *zif268* probe with anti-digoxigenin HRP-antibody conjugate and the cyanine-3 substrate kit (CY3 DirectFISH), the slides were treated with 2% H_2O_2 in PBS to quench the residual HRP activity. The fluorescein-labeled *Arc* probe was then detected with anti-fluorescein HRP and the fluorescein substrate kit (GREEN DirectFISH). For *Arc* single labeling, digoxigenin-labeled *Arc* antisense riboprobe was detected with anti-digoxigenin HRP-antibody conjugate and CY3 DirectFISH.

Confocal microscopy and cell counting. Stained slides were analyzed using a Zeiss LSM 410 confocal microscope equipped with 4 lasers (UV laser, and 488, 543 and 633 nm lasers). Images were collected with a Zeiss 40×/1.2 water-immersion lens. PMT assignments, pinhole sizes and contrast values were kept constant across different confocal sessions. Areas of analysis were *z* sectioned in 0.7-micron optical sections. Z-section image series were collected on optical (Fujistu) or Zip (Iomega) data-storage disks. Using the LSM 410 software, each cell was characterized through several serial sections, and only cells containing whole nuclei were included in the analysis. This approach, which is essentially an optical dissector technique[27], minimizes sampling errors (due to partial cells) and stereological concerns, as minor variations in cell volumes do not influence sampling frequencies.

Cells were counted by an experimenter blind to the behavioral conditions. The DAPI stain revealed nuclei of two distinct morphologies. The majority of nuclei in the CA1 layer (~80%) were large and stained diffusely with DAPI. Only these presumptive neurons were included in the analysis. The remainder of the cells, presumably glial cells, had much smaller nuclei, stained strongly with DAPI and did not express either *Arc* or *zif268* RNAs. Assignments of cells as negative or as showing intranuclear foci, cytoplasmic staining or intranuclear/cytoplasmic staining was made only after viewing the multiple optical sections that comprised each individual cell; this method was necessary to assure accurate assignment and likely reduced counting errors due to staining artifacts. The designation 'intranuclear-foci positive' was given to cells that contained one or two of the characteristic, intense intranuclear areas of fluorescence; the designation 'cytoplasmic positive' was given to cells that contained perinuclear/cytoplasmic labeling over multiple optical sections. The total number of CA1 neurons analyzed for each rat ranged from 97–146, with an average of 120. This number of cells was obtained by counting 2 or 3 slides in the same general region of the dorsal hippocampus (anterior–posterior, ~–3.6 mm from Bregma[28]) for each animal and 2 or 3 microscope fields for each slide. The values are reported as the percent of neurons positive for a given staining profile per total counted neurons for each rat.

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