Research

Mapping neuronal activation and the influence of adrenergic signaling during contextual memory retrieval

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We recently described a critical role for adrenergic signaling in the hippocampus during contextual and spatial memory retrieval. To determine which neurons are activated by contextual memory retrieval and its sequelae in the presence and absence of adrenergic signaling, transcriptional imaging for the immediate-early gene *Arc* was used in control and mutant mice lacking norepinephrine and epinephrine. This imaging approach permits the identification of neuronal genomic activation specific to one of two behavioral epochs in the same animal. Analysis revealed several brain regions that were more greatly activated by re-exposure to a salient versus neutral context 1 d after training in control mice (e.g., hippocampal CA3 and CA1, the amygdala, the dorsolateral caudate/putamen, the primary motor cortex, and parts of the rhinal cortices). In mice lacking norepinephrine and epinephrine, many of these regions exhibited significantly reduced activation (e.g., hippocampal CA1), while other regions did not (e.g., hippocampal CA3). In consideration with previous results, the current findings suggest a hypothesis in which adrenergic signaling may be critical for the transfer of retrieved contextual information from CA3 to CA1, where it would be compared to online sensory information coming directly from the cortex.

One goal for understanding memory is to define the neurons activated during memory storage and retrieval. Electrophysiologic recordings and brain lesions have been mainstays in this pursuit. More recently, imaging techniques have been used. These include functional brain imaging studies in humans using positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), which are noninvasive and permit repeated measurements; however, they cannot resolve activity at the cellular level (Schacter and Wagner 1999; Mayes and Montaldi 2001). For this, induction of immediate-early gene (IEG) expression ("genomic activation") has been used in animals, offering high sensitivity and cellular resolution that can be analyzed throughout the brain (Clayton 2000). A disadvantage, however, is that each animal is assessed under a single condition.

A technique that overcomes this disadvantage uses fluorescence in situ hybridization (FISH) to the IEG *Arc*. Arc (Lyford et al. 1995), also termed Arg3.1 (Link et al. 1995), is an <u>a</u>ctivity-<u>r</u>egulated, <u>c</u>ytoskeleton-associated protein implicated in synaptic plasticity and memory (Steward et al. 1998; Guzowski et al. 2000). Basal expression of *Arc* is very low in most neurons. However, transcription is rapidly induced by neuronal activation, and FISH to *Arc* reveals two small, intense intranuclear foci within 2 min of activation (Guzowski et al. 1999). By ~20 min the nuclear signal has disappeared and perinuclear cytoplasmic labeling is transiently observed before disappearing due to dendritic transport of *Arc* mRNA (Steward et al. 1998). These properties allow one to determine the recent activation history of a neuron at two distinct times, referred to as <u>c</u>ellular <u>c</u>ompartment <u>a</u>nalysis of <u>t</u>emporal activity by <u>FISH</u> (catFISH) (Guzowski et al. 1999, 2001). *Arc* catFISH has been used to examine activation of hippocampal pyramidal neurons in identical and distinct contexts (Guzowski et al. 1999), generating results supported by electrophysiologic recordings (Kubie and Ranck 1983; Thompson and Best 1990; Wilson and McNaughton 1993).

One goal of this study was to determine whether *Arc* catFISH would identify neurons uniquely activated as a result of exposure to a salient versus neutral context (the former resulting in the retrieval of a contextual fear memory). If it could, activation of these neurons would likely reflect a variety of processes related to contextual fear, including retrieval of the contextual fear memory, increased arousal or fear following retrieval, or conditioned responses generated by fear, such as freezing. Our analysis included the hippocampus for several reasons. First, induction of *Arc* occurs readily in hippocampal pyramidal neurons following a change in context (Guzowski et al. 1999). Second, the hippocampus is critical for contextual memory storage and discrimination of contexts during memory retrieval (Kim and Fanselow 1992; Kim et al. 1992; Phillips and LeDoux 1992; Holt and Maren 1999). Third, antisense knock-down of *Arc* expression in the hippocampus reduces the expression of long-term potentiation and spatial memory (Guzowski et al. 2000). This is likely due to a role for *Arc* in consolidation because short-term synaptic plasticity and short-term memory remain intact. Fourth, induction of *Arc* also occurs when animals are returned to a previously experienced context. This could be due to a role for Arc in habituation, reconsolidation, or consolidation of new memory, processes that likely require memory retrieval (Nader et al. 2000; Myers and Davis 2002). While induction of *Arc* is not necessary for memory retrieval, it seems likely that it may identify neurons genomically activated as a result of retrieval.

Besides the hippocampus, we examined additional brain regions, a number of which have not been studied using *Arc* catFISH before, to determine whether this technique could identify neurons in these regions uniquely activated following expo-

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sure to the salient versus neutral context. We anticipated that regions such as the amygdala, for example, might exhibit such labeling, given its critical role in fear and conditioned responding (Fanselow and LeDoux 1999).

A second goal was to determine whether activation of neurons specific to a salient context depends on adrenergic signaling. We recently demonstrated a critical and specific role for norepinephrine (NE) in contextual and spatial memory retrieval that is not due to a role in arousal, fear, or performance (Murchison et al. 2004). Those findings were initiated by the study of mice genetically altered to lack the endogenous ligands for the adrenergic receptors, NE and epinephrine (NE/E), via targeted disruption of the dopamine β-hydroxylase gene (*Dbh*) (Thomas et al. 1995, 1998). Intracerebral infusions indicated that β_1-adrenergic receptor signaling in the hippocampus is necessary and sufficient for contextual memory retrieval mediated by NE. As a result, we anticipated that a subset of neurons identified by *Arc* catFISH as being activated specifically during exposure to the salient context might not be activated in the absence of NE/E, and that at least some of those neurons would reside in the hippocampus, because of the role of NE in that structure for contextual memory retrieval. We also anticipated that additional brain regions, such as the amygdala, might exhibit reduced salient context-specific activation in the absence of NE/E because of differences in fear and conditioned responding.

Results

Arc catFISH in mice

We first examined the time course of *Arc* expression in our mice because previous studies were performed in rats. We used re-exposure to the training apparatus 1 d after fear conditioning as the stimulus for induction of *Arc* to match our main experimental design (see below). Mice were sacrificed 5–35 min after re-exposure. Figure 1 identifies the brain regions examined using *Arc* catFISH. At 5 min there were significant increases in nucleus-specific labeling that returned to baseline by 20 min in all brain regions examined except the dentate gyrus (DG, not shown) (Fig. 2). In rat, *Arc* transcription initiated by a single stimulus persists for hours in the DG, as compared to minutes in other excitatory cell populations of hippocampus and neocortex (Vazdarjanova et al. 2002), in agreement with the current findings in mice. In all other regions, cytoplasm-specific labeling peaked at ~30 min, and the percentages of labeled cells were equivalent to those for nucleus-specific labeling at 5 min, suggesting that essentially all nucleus-positive neurons become nucleus-negative, cytoplasm-positive neurons between 5 and 30 min. These observations are consistent with the time course of *Arc* induction in rats (Guzowski et al. 1999), and indicate that *Arc* catFISH can be used in mice to analyze neuronal activation in many brain regions following context exposure.
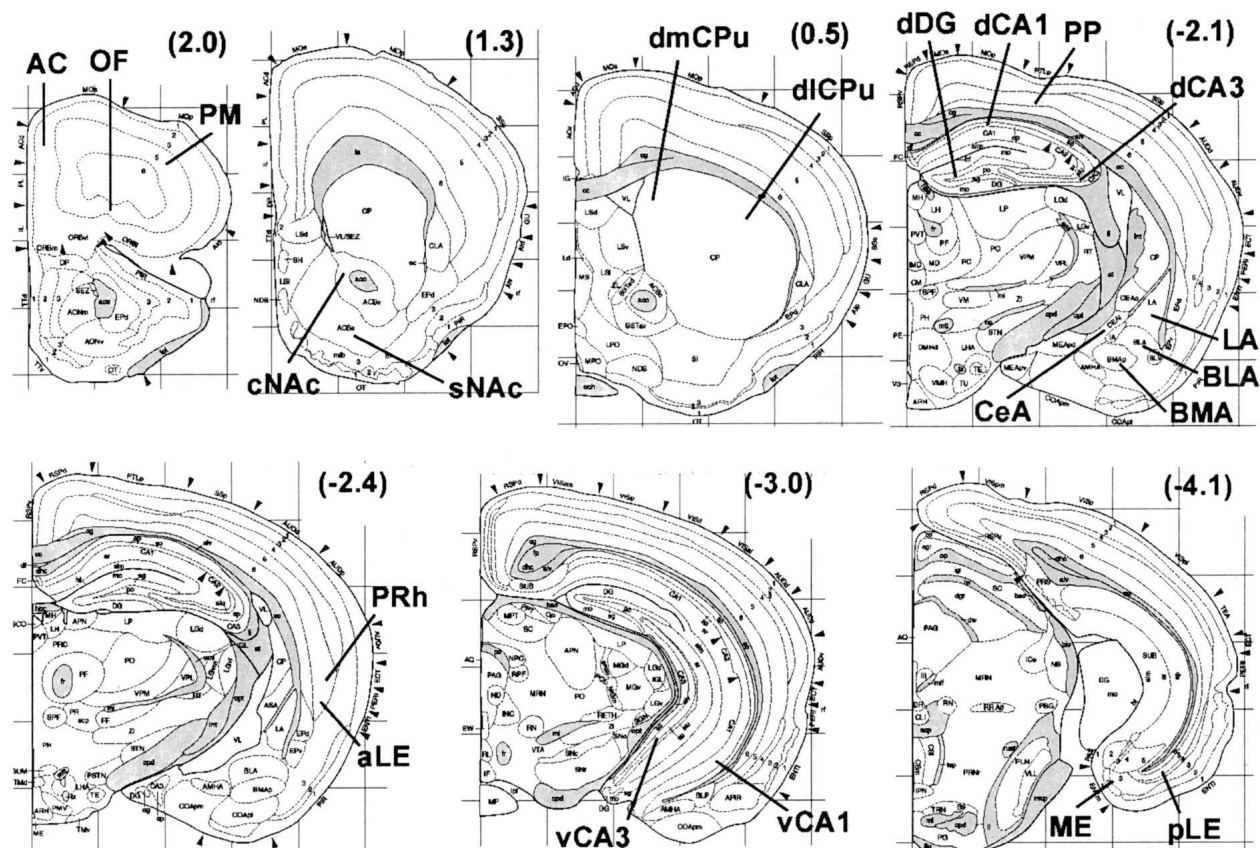
Figure 1. Coronal mouse brain diagrams with regions of analysis indicated. Numbers in parentheses are anterior–posterior distances from Bregma in millimeters. Abbreviations of regions–for hippocampus: dCA3 and dCA1 are dorsal CA3 and CA1; vCA3 and vCA1 are ventral CA3 and CA1; for amygdala: LA, BLA, BMA, and CeA are lateral, basolateral, basomedial, and central nuclei; for striatum: dlCPu and dmCPu are dorsolateral and dorsomedial caudate/putamen; sNAc and cNAc are the shell and core regions of the nucleus accumbens; for cortex: ME, pLE, and aLE are medial, posterior lateral and anterior lateral entorhinal; PRh is perirhinal; PP is posterior parietal; OF is orbitofrontal; AC is anterior cingulate; PM is primary motor. Diagrams are reprinted with permission from Elsevier Science © 2000 (Hof et al. 2000).
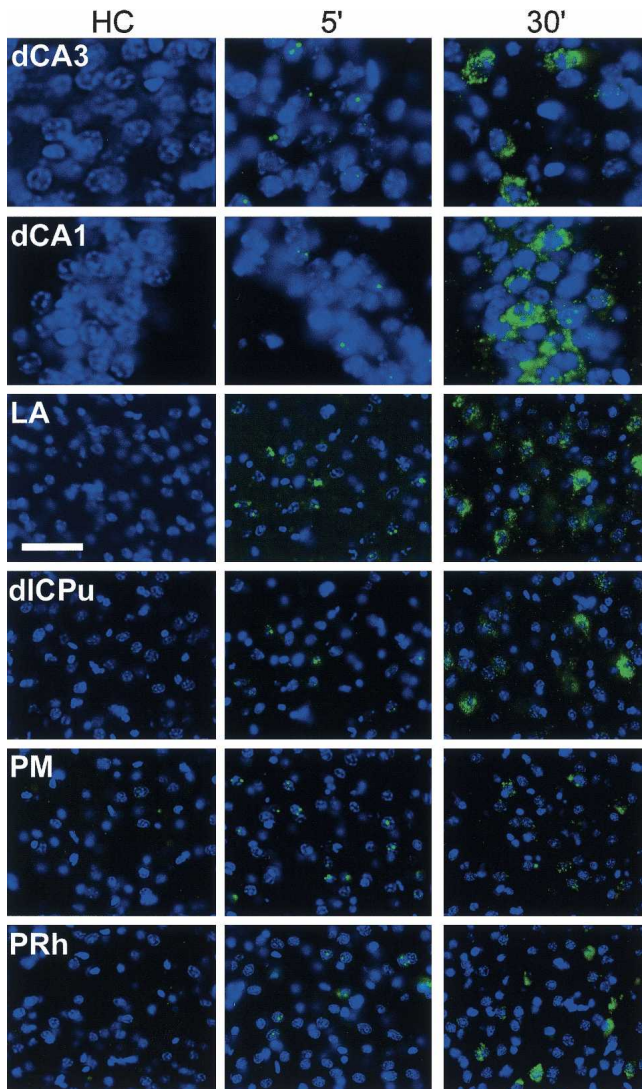
Figure 2. *Arc* expression before (HC) and after (5 min and 30 min) re-exposure to the training context. Sections are from mice that were sacrificed 1 d after training with shock. For 5 min and 30 min, mice were returned to their home cage after exposure to context S on Day 2 for 5 min and 30 min before sacrifice, respectively. Nuclei are blue and *Arc* RNA is green. Almost no labeling is present in HC. At 5 min, small intranuclear foci of labeled *Arc* RNA are present in some neurons in each brain region. At 30 min, perinuclear cytoplasmic labeling of *Arc* mRNA is present and intranuclear labeling is similar to that for HC. Regions are as per Figure 1. The scale bar is 40 μm for all regions except dCA3 and dCA1, where it is 20 μm.

Design of *Arc* catFISH

To identify neurons activated during and following retrieval of contextual fear, paradigms using two contexts were used. Context S (shock or salient) was the training apparatus and context N (non-shock or neutral) was a distinct context not associated with shock. For the main two experimental groups, neuronal activation in the salient versus neutral context was assessed. Mice were exposed to both contexts on the training day, receiving shock in context S (Fig. 3A). These mice were then re-exposed to each context on the testing day 1 d later. One of these groups was exposed to context S then N (group S1 = context S first), the other to context N then S (group S2 = context S second) to control for order effects. For a third group of mice, neuronal activation in two neutral contexts was assessed. Mice were exposed to context

N then S during training without receiving shock, and the next day were exposed to context S then N (group NS = no shock during training). A fourth group of mice was used to assess basal levels of *Arc* expression following fear conditioning. These mice were exposed to both contexts on the training day, receiving shock in context S. They were then sacrificed from their home cage the next day (group HC = home cage). Using these paradigms, fear conditioning (assessed by freezing) was apparent in groups S1 and S2 but not NS, as expected (Fig. 3B). The $Dbh^{-/-}$ mice froze significantly less than controls, consistent with an impairment in contextual memory retrieval (Murchison et al. 2004). Groups S1 and S2 were combined to create group SX because significant differences within genotype were absent.

Total *Arc* expression: Training history and adrenergic signaling

To examine how *Arc* expression varies between regions, treatment groups, and genotypes, total *Arc* labeling was analyzed. Importantly, total percentages of *Arc* expression (nuclear plus cytoplasmic plus double-labeled/total cells analyzed) did not differ by genotype in any brain region except for the primary motor cortex, where *Arc* was reduced in the $Dbh^{-/-}$ mice because of a large difference in salient context-specific *Arc* expression (see below). These data indicate that there is not a general impairment in induction of *Arc* in the absence of NE/E. Two common patterns of *Arc* induction were observed between treatment groups. One pattern identified brain regions that were primarily context-sensitive. In these regions there were increases in total labeling in the non-shock group compared to the home-cage group, without further significant increases in the shock group compared to the non-shock group (Fig. 4). This pattern was observed in the hippocampus, core of the nucleus accumbens, dorsomedial caudate/putamen, and the perirhinal, posterior parietal, and orbitofrontal cortices. The other pattern identified regions that were sensitive to training condition. It was similar to the first pattern except that there were significant increases in total labeling in the shock group compared to the non-shock group. This pattern was observed in the amygdala, shell of the nucleus accumbens, dorsolateral caudate/putamen, and the entorhinal, anterior cingulate, and primary motor cortices. Unlike all other regions, in the DG
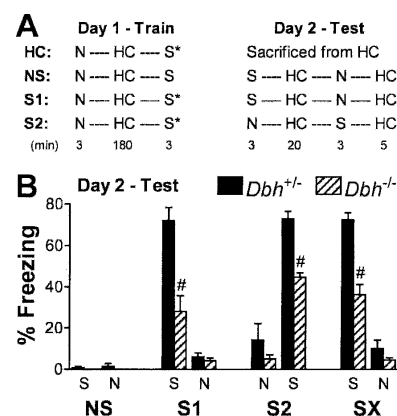
Figure 3. Protocol for *Arc* catFISH and freezing behavior of mice. (*A*) The order and duration of context exposures are shown. All groups were trained with exposure to context N followed by a 3-h epoch in their home cage (HC) and then exposure to context S. All mice received shock (*) on Day 1 in context S except group NS. Mice were tested the next day as shown without any shock. NS, S1, and S2 groups were sacrificed after the second HC epoch on Day 2. (*B*) Freezing during testing on Day 2. Group SX represents the combined data from groups S1 and S2 (thus *n* = 8 per genotype). For all figures, levels of significance are indicated as follows: (*) *P* < 0.05; (∧) *P* < 0.01; (#) *P* < 0.001.
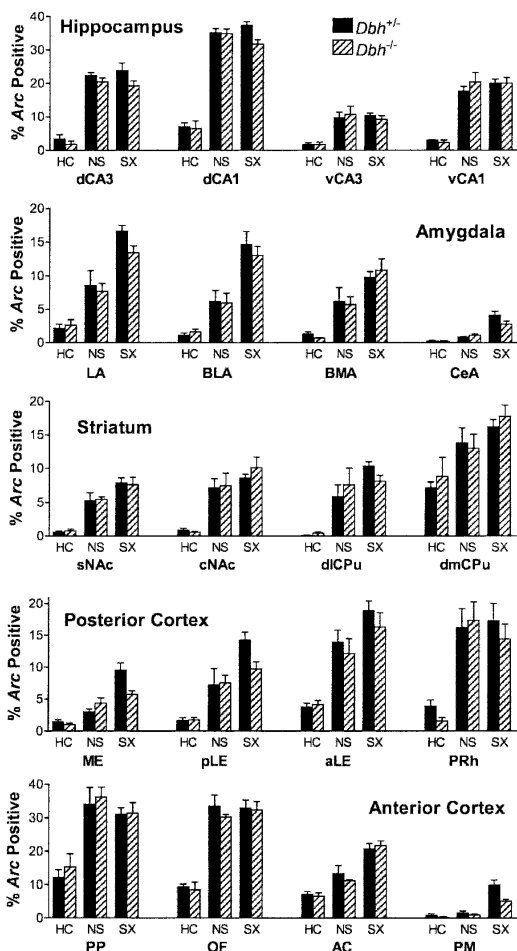
Figure 4. Total *Arc* labeling by brain region, treatment group, and genotype. Shown are the combined percentages of cells labeled for *Arc* either in the nucleus, the cytoplasm, or both. Regions are as per Figure 1 and groups are as per Figure 3. The main effect of treatment was significant for all regions ($P < 0.001$). There were no significant main effects of genotype except for PM cortex ($P = 0.03$) and no significant interactions between treatment and genotype. When collapsed by genotype, labeling in NS was significantly higher ($P < 0.01$) than HC for all regions except PM. In addition, SX was significantly higher ($P < 0.05$) than NS for all regions in the amygdala and entorhinal cortex, as well as the AC and PM cortex.

there was an increase only in double-positive labeling with context exposure (Fig. 5A). This was anticipated in part from the extended time course of nuclear labeling in the DG. These same overall labeling patterns described above were also apparent when labeling was parceled into the three categories that indicate when each neuron was activated: cytoplasmic (Cy, specific for the first epoch), nuclear (Nu, specific for the second epoch), and double-labeled (Do, common to both epochs) (Fig. 6; data not shown for regions outside the hippocampus). Labeling was parceled in this manner so that neurons activated during each behavioral epoch could be quantified and compared (see below).

## Identification of salient context-specific labeling using *Arc* catFISH

To identify brain regions that contained neurons activated during and following contextual memory retrieval, we examined whether the shock groups S1 and S2 would exhibit significantly more uniquely activated neurons in context S versus N. There-

fore, labeling resulting from context N was subtracted from that for context S for each mouse (S − N = residual labeling). Because double labeling (Do) is common to both contexts, S − N is represented by the difference between cytoplasm- and nucleus-specific labeling (Cy − Nu for group S1; Nu − Cy for group S2). We then tested whether the residual labeling was significantly different from zero. For the non-shock group, values were expected to be not significantly different from zero (i.e., Cy = Nu) in order to satisfy the basic premise of catFISH. Importantly, this was confirmed for all brain regions examined in both genotypes except the DG (Figs. 5–7). The different outcome for the DG was expected based on the extended time course of *Arc* labeling in the granule cells. For the shock groups, we asked whether residual *Arc* labeling might be significantly greater than zero in any brain region. If it were, this would indicate that *Arc* catFISH could be used to identify regions containing neurons uniquely activated as a result of contextual fear memory retrieval or its sequelae, such as increased arousal, fear, or conditioned fear responses like freezing. To control for possible order effects on induction or detection of *Arc*, groups S1 and S2 were combined to create group SX.
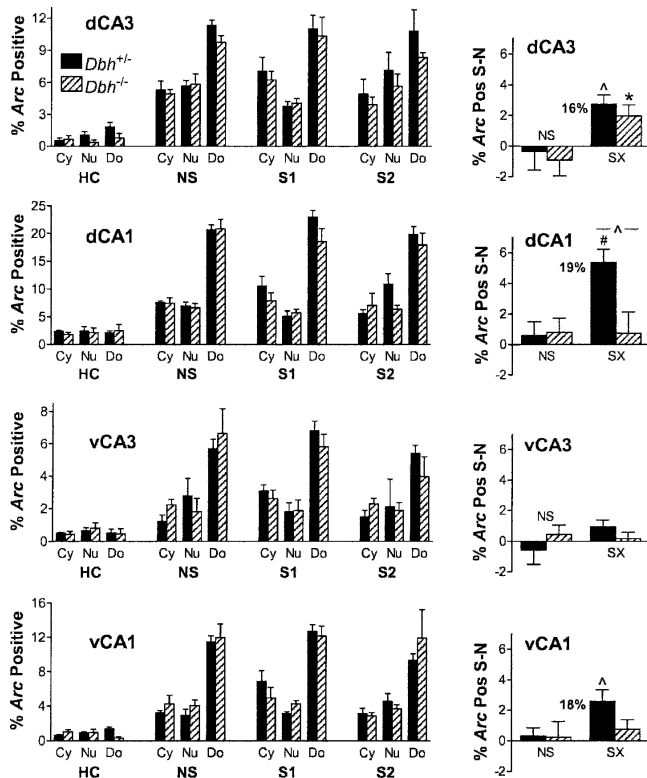
Salient context-specific labeling in the hippocampus

We first examined the hippocampus for significant salient context-specific labeling for *Arc* in control mice because lesion and reversible inactivation studies have demonstrated an important role for the hippocampus in contextual memory storage and retrieval (Kim and Fanselow 1992; Kim et al. 1992; Phillips and LeDoux 1992; Holt and Maren 1999). We also examined the hippocampus because adrenergic signaling in this region is necessary for contextual fear memory retrieval 1 d after training (Murchison et al. 2004). However, it is not known in which subfield(s) of the hippocampus activation might be affected during memory retrieval in the absence of NE/E.

In the dorsal hippocampus, we found significant salient context-specific labeling in dCA3 and dCA1 in control mice, which was apparent when inspecting percentages of cytoplasm- and nucleus-specific labeling in the shock groups (S1 and S2; left part of Fig. 6), and was shown quantitatively by the residual
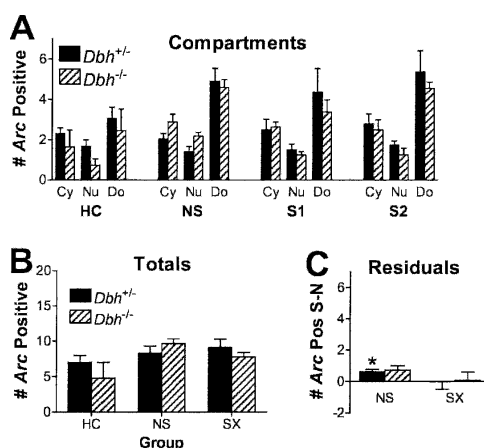
Figure 5. Compartment analysis of *Arc* labeling in the dentate gyrus. Data are presented as per Figures 4 and 6, except that instead of reporting the percentage of cells labeled, the number of labeled cells per section is given owing to the sparse labeling in the DG. (*A*) The main effect of compartment labeling was significant for all four groups. (*B*) The main effect of treatment was significant. (*C*) For control mice, there was significant bias in the NS group that reflected the greater amount of cytoplasmic to nuclear labeling in the DG. No significant salient context-specific labeling was observed.

Figure 6. Compartment analysis of *Arc* labeling and identification of salient context-specific labeling in the hippocampus. On the *left* are the percentages of cells labeled either in the cytoplasm (Cy, unique labeling for the first epoch), the nucleus (Nu, unique labeling for the second epoch), or both (Do, double, common to both epochs) for each treatment. Only the main effect of *Arc* labeling by compartment was significant, and this was true for groups NS, S1 and S2 (but not HC) for each region (*P* < 0.01). On the *right* are the mean residual labeling percentages after subtracting the values for context N from context S for each mouse, as a percentage of all cells analyzed, including unlabeled neurons. Significant residual (salient context-specific) labeling was observed in dCA3, dCA1, and vCA1 for the control mice and dCA3 for the *Dbh*$^{-/-}$ mice. There was a significant difference in residual labeling between genotypes for dCA1. Numbers next to *Dbh*$^{+/-}$ SX bars indicate the percent increase in *Arc* labeling in context S relative to context N when significant residual labeling is present.

labeling percentages (right part of Fig. 6). Note that the percentages are absolute, that is, they reflect the status of all neurons examined, including the majority that was not labeled for *Arc*. To aid interpretation, the percent increase in *Arc*-positive neurons is also given next to the bars for the control mice. Interestingly, there was significant salient context-specific labeling in dCA3 but not dCA1 in the *Dbh*$^{-/-}$ mice, and there was a significant difference between genotypes in dCA1 but not dCA3. In the dDG there was no salient context-specific labeling in either genotype using the standard analysis (Fig. 5) or a second analysis that took into account the extended duration of nuclear labeling.

The ventral hippocampus was also examined because studies suggest that some of its functional roles may differ from the dorsal hippocampus (Kjelstrup et al. 2002; Maren and Holt 2004). Labeling was about half as frequent as that observed in the dorsal hippocampus (Fig. 6). Owing to very sparse labeling and lack of salient context-specific effects in the dDG, the vDG was not quantified. In vCA3 there was no significant salient context-specific labeling. However, in vCA1 there was significant salient context-specific labeling in control but not *Dbh*$^{-/-}$ mice, similar to dCA1.

Salient context-specific labeling in other brain regions

Because of the success in using *Arc* catFISH to identify salient context-specific labeling in the hippocampus, and because of the attractive within-subject design of *Arc* catFISH, we asked whether salient context-specific activation could be observed in additional brain regions. These regions were either predicted to exhibit salient context-specific activation based on prior functional studies, known to be areas of considerable input or output with respect to the hippocampus, and/or were observed to exhibit obvious salient context-specific activation during the course of the study.

Further analysis began with the amygdala because it is critical for acquisition and expression of conditioned fear (Kim and Fanselow 1992; Helmstetter and Bellgowan 1994; Muller et al. 1997). In the amygdala, there was significant salient context-specific labeling in all four regions examined in control mice: the lateral, central, basolateral, and basomedial nuclei (Fig. 7). In contrast, only in the lateral nucleus was there significant salient context-specific labeling (40% of control) in *Dbh*$^{-/-}$ mice. Furthermore, in the lateral, basomedial, and central nuclei, salient context-specific *Arc* induction in *Dbh*$^{-/-}$ mice was significantly impaired relative to controls.

In the course of these studies we noted striking salient context-specific labeling throughout the dorsolateral half of the caudate/putamen (dlCPu). For comparison, labeling was also quantified in the dorsomedial CPu (dmCPu). Significant salient context-specific labeling was present in control and *Dbh*$^{-/-}$ mice in the dlCPu. However, salient context-specific labeling in the mutants was only 30% of that for controls, a significant reduction. No salient context-specific labeling was observed in the dmCPu. As for other structures in the striatum, studies suggest that the nucleus accumbens (NAc) plays an important role in contextual fear conditioning (Riedel et al. 1997; Haralambous and Westbrook 1999; Levita et al. 2002). Using *Arc* catFISH, we found significant salient context-specific labeling in the shell of the
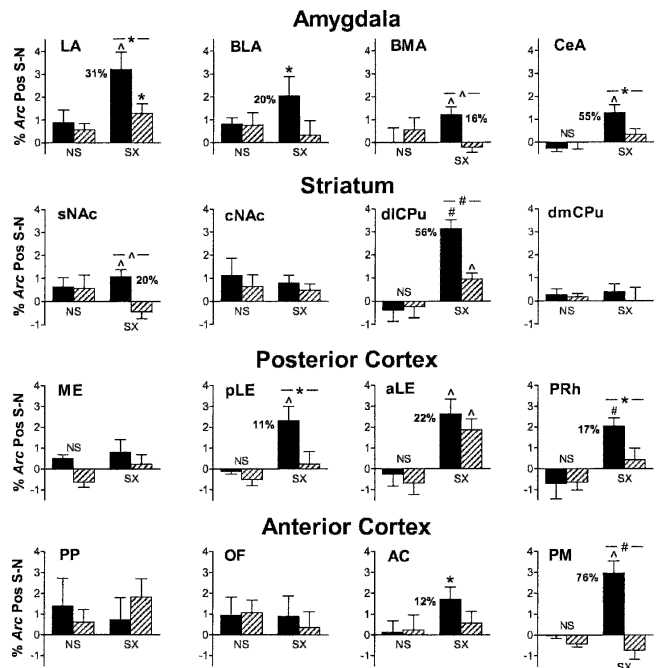
Figure 7. Salient context-specific labeling in additional brain regions. Data are presented as in Figure 6 except that percentage labeling by compartment is not shown. Salient context-specific labeling is observed in several brain regions in controls and in some cases in *Dbh*$^{-/-}$ mice, although only in the aLE is the labeling equivalent to that in controls.

NAc in control but not $Dbh^{-/-}$ mice, and a significant difference between genotypes (Fig. 7). In contrast, no salient context-specific labeling was observed in the core of the NAc.

Labeling in the cortex was also examined. Several parahippocampal cortical regions were studied because of their extensive connections with the hippocampus and their role in mnemonic processes (Eichenbaum 2000; Witter et al. 2000; Brown and Aggleton 2001; Egorov et al. 2002). No salient context-specific labeling was present in the medial entorhinal cortex; however, the posterior lateral entorhinal cortex exhibited significant salient context-specific labeling that was present only in control mice (Fig. 7). More rostrally, there was significant salient context-specific labeling in both genotypes in the anterior lateral entorhinal cortex, while in the adjacent perirhinal cortex there was significant salient context-specific labeling that was present only in control mice.

In the frontal cortex, we noted striking salient context-specific labeling in the primary motor cortex. When this was quantified and compared to other regions of the frontal cortex, we found salient context-specific labeling in the primary motor cortex and to a lesser degree in the anterior cingulate cortex that was present only in control mice (Fig. 7). In contrast, there was no salient context-specific activation of the orbitofrontal cortex. Finally, when we examined the posterior parietal sensory association area, no salient context-specific labeling was observed.

Discussion

Applicability of *Arc* catFISH

This study highlights the general applicability of *Arc* catFISH for studying neuronal activation. The results are consistent with studies using *Arc* catFISH in the rat (Guzowski et al. 1999) and extend those observations to the mouse and to many additional brain regions. For interpreting results from *Arc* catFISH, it is important to stress that induction of *Arc* is not meant to indicate whether a neuron fired action potentials during a behavioral epoch. In general, the relationship between neural activity and IEG activation is complex (Clayton 2000; Fields et al. 2001). Rather, induction of *Arc* should be regarded as a marker of "genomic activation" that likely represents a threshold or specific pattern of postsynaptic activity.

For the purpose of studying memory retrieval and its sequelae, we were able to identify brain regions using *Arc* catFISH that had significantly greater labeling unique to the salient versus the neutral context. *Arc* was chosen as an IEG marker of neuronal activity because of its previously described utility for within-subjects analysis using catFISH (Guzowski et al. 1999, 2001). Because Arc is implicated in synaptic plasticity and memory formation (Guzowski et al. 2000), it was not clear whether *Arc* catFISH would identify neurons uniquely activated during memory retrieval. The fact that it did could reflect a role for Arc in reconsolidation following memory retrieval. Our results in control mice are consistent with results from previous imaging studies examining the IEGs Fos or *zif268* following retrieval tests for contextual fear conditioning that used brief training protocols (Milanovic et al. 1998; Hall et al. 2001a; Thomas et al. 2002; Frankland et al. 2004).

We observed some double labeling for *Arc* in all brain regions. This was expected because there were many similarities as well as some important differences between exposure to a neutral and a salient context. For example, in both situations animals in the same room were aroused, picked up by the tail, and placed into a relatively novel context. Significant overlap in neuronal activation could be present because of similar sensorimotor experiences and arousal in both situations. Indeed, it was imperative that this be identified and subtracted in order to detect labeling due to specific differences between the two situations (e.g., retrieval, fear, freezing).

Critical to the analyses performed in this study on the role of adrenergic signaling, basal levels of *Arc*, as well as the levels induced by a neutral context, were unaffected in the $Dbh^{-/-}$ mice. This is perhaps surprising because lesions of the locus coeruleus, which supplies much of the adrenergic innervation to the forebrain, cause reductions in Arc labeling (Cirelli and Tononi 2000). However, those experiments measured IEG expression following prolonged wakefulness with repeated novel object exposure, whereas our experiments examined short-term induction of *Arc* in response to a change in context. Another difference of potential importance is that lesions incapacitate adrenergic terminals, whereas the adrenergic terminals are intact (except for the absence of NE/E) in the $Dbh^{-/-}$ mice (Jin et al. 2004).

Memory retrieval, the hippocampus, and adrenergic signaling

Using *Arc* catFISH, we examined how NE might act to promote contextual memory retrieval in the hippocampus. We focused on neuronal activation in the hippocampus during retrieval because infusion of β_1-adrenergic receptor antagonists into the dorsal hippocampus selectively impairs contextual fear memory retrieval, as does systemic NE/E deficiency in $Dbh^{-/-}$ mice (Murchison et al. 2004). In this and the previous study, freezing to the training context is reduced but not eliminated in the mutant mice and in control mice treated with a β_1 antagonist. There are several potential explanations for this. For example, it could be that retrieval is impaired but not eliminated because factors in addition to NE promote some retrieval in the hippocampus. Residual freezing could also be present in response to elements contained within the context (cued fear), which is normal in the mutant mice (Murchison et al. 2004).

Importantly, neither blocking β_1 receptors in the dorsal hippocampus nor systemic deficiency of NE/E alters fear in general as assessed by freezing in response to the training tone 1 d after conditioning or in response to the training context 1 wk after conditioning (Murchison et al. 2004). It was our hypothesis then that in the absence of NE/E, one or more subfields of the hippocampus would exhibit impaired activation during contextual memory retrieval. Our results indicate that CA3 is activated normally in the absence of NE/E. In contrast, CA1 exhibits salient context-specific activation that is significantly and considerably reduced in the absence of NE/E.

One consideration is whether the increased labeling for *Arc* in CA1 following exposure to the salient context is due to arousal, fear, or freezing, for example, rather than memory retrieval. Importantly, results from several studies demonstrate minimal or no change in Fos or *zif268* levels in CA1 when testing cued fear conditioning after habituation to the context, or when testing contextual fear conditioning ~1 mo after training (Hall et al. 2001a,b; Frankland et al. 2004). Under those conditions subjects exhibited high levels of freezing (fear) to the context or cue without induction of Fos or *zif268* in CA1, dissociating IEG expression and arousal, fear, and freezing behavior.

One could argue that induction of *Arc* reflects processes that are distinct from those reflected by induction of Fos or *zif268*. For example, even though adrenergic signaling in the hippocampus mediates contextual memory retrieval, salient context-specific labeling for *Arc* (but not Fos or *zif268*) in the hippocampus could reflect increased arousal, fear, or freezing that results from fear memory retrieval. However, it is interesting to note that results examining induction of Fos in the hippocampus following contextual fear memory retrieval in control and $Dbh^{-/-}$ mice (Tho-

mas et al. 2004) mirror those for the induction of *Arc* presented here. Furthermore, a general deficit in arousal in the *Dbh*$^{-/-}$ mice that would impinge upon induction of *Arc* might have been manifest when mice were placed in the neutral context (arousing because it is relatively novel). A large majority of the *Arc* induction observed in the salient context was also observed in the neutral context. However, such induction was entirely normal in the *Dbh*$^{-/-}$ mice. Of course, it is possible that the magnitude or pattern of activation induced by arousal in a relatively novel, but neutral, context differs from that for arousal in a salient context.

The patterns of salient context-specific Arc induction that we have observed in the hippocampus are interesting to consider in light of models suggesting CA3 is important for contextual memory storage and retrieval, while CA1 is important for comparing memory of context coming from CA3 to the current context coming from the perforant path directly to CA1 (Treves and Rolls 1994; Lisman 1999). Based on these models, we hypothesize that perforant path activity to the hippocampus is normal in the absence of NE, allowing normal contextual memory storage in CA3 and normal online contextual information processing in CA1. This hypothesis is consistent with the normal activation of CA1 in a neutral context and normal activation of CA3 in the neutral and salient contexts in the *Dbh*$^{-/-}$ mice. The model and results suggest that activation of CA3 during contextual memory retrieval fails to be properly transmitted to CA1. This could be for several reasons. β_1-adrenergic receptors, which are critical for the actions of NE in promoting memory retrieval, are expressed primarily by CA1 pyramidal neurons and dentate granule cells (Nicholas et al. 1993). Thus, it is possible that β_1-adrenergic receptors expressed by CA1 pyramidal neurons play a key role in gating the strength of the Schäffer collateral input from CA3. Alternatively or in addition, β_1-adrenergic receptors expressed by DG cells could indirectly gate the firing of CA3 pyramidal neurons via the mossy fiber pathway. The latter seems less likely given the normal induction of *Arc* in dCA3 in the *Dbh*$^{-/-}$ mice during retrieval. However, because the relationship between induction of IEGs and neuronal firing is complex (Clayton 2000; Fields et al. 2001), it is possible that the synaptic activation that leads to normal *Arc* induction does not lead to appropriate firing in CA3.

Salient context-specific *Arc* labeling in other brain regions

We predicted that when retrieval was impaired, activation in other brain regions would also be altered. This is because impaired retrieval leads to reduced fear and freezing. In the amygdala, salient context-specific labeling was present in all four nuclei examined in control mice, consistent with a requirement for each of these in fear conditioning (Goosens and Maren 2001). In contrast, in *Dbh*$^{-/-}$ mice salient context-specific labeling was present only in the lateral nucleus, where there was significantly less labeling than in controls.

In the striatum, salient context-specific labeling in the dlCPu was striking, both because it represented ~30% of total *Arc* labeling in this region, and because it was anatomically extensive (dorsal to ventral and rostral to caudal). Interestingly, the dlCPu receives cortical input largely from the somatosensory and motor cortices, whereas the medial CPu receives input from the visual, auditory, and olfactory cortices (McGeorge and Faull 1989). Because salient context-specific labeling was significantly reduced in the *Dbh*$^{-/-}$ mice in this region, it seems reasonable to hypothesize that the dlCPu is important for mediating behavioral output, for example, freezing. This is consistent with there being salient context-specific labeling throughout the dlCPu because freezing entails fixation of posture (motor inhibition) across the

somatic axis. The primary motor region of the prefrontal cortex exhibited a similar pattern of labeling to that for the dlCPu, suggesting a potential role in mediating freezing as well.

The parahippocampal cortices (perirhinal, postrhinal, and entorhinal) are multimodal association areas that process and relay sensory information between the hippocampus and other cortical regions (Insausti et al. 1997; Burwell and Amaral 1998; Dolorfo and Amaral 1998; Naber and Witter 1998; Witter et al. 2000). Lesion studies of the entorhinal or perirhinal cortex suggest that these regions may contribute to contextual fear conditioning (Corodimas and LeDoux 1995; Phillips and LeDoux 1995; Maren and Fanselow 1997). We observed varying degrees of salient context-specific activation that depended on the area examined. Interestingly, the anterior lateral entorhinal cortex exhibited salient context-specific activation, but it was not dependent on NE/E, similar to what was observed in CA3. This pattern of activity suggests that this region may process information either en route to the hippocampus (e.g., upstream of DG/CA3) or independently of the hippocampus. Two of the regions (posterior lateral entorhinal and anterior perirhinal) exhibited salient context-specific activation that was not present in the absence of NE/E. These regions may subserve contextual information processing downstream of the hippocampus because the labeling was dependent on NE/E and was present in the deep layers of these regions, areas that receive much of the cortical hippocampal output via CA1 and the subiculum.

Conclusions

This study demonstrates that *Arc* catFISH can be used in mice to map activity in many regions of the brain during distinct behavioral epochs. Within the hippocampus, NE is required for normal activation of CA1 but not CA3 during retrieval of a salient contextual memory. Because normal behavior is restored in *Dbh*$^{-/-}$ mice when testing contextual fear following infusion of a β-adrenergic receptor agonist into the dorsal hippocampus (Murchison et al. 2004), we hypothesize that the lack of NE-dependent retrieval in the hippocampus in untreated *Dbh*$^{-/-}$ mice results in reduced salient context-specific activation of multiple "downstream" brain regions (e.g., the amygdala, dlCPu, and some cortical areas) important for mediating appropriate, context-dependent behavior. This could be investigated in future studies by examining IEG induction following context exposure after infusing a β_1-adrenergic receptor antagonist into the dorsal hippocampus.

In addition, it would be interesting to map contextual memory retrieval in even greater detail in future studies. For example, one could compare salient context-specific activation in proximal versus distal regions (along the transverse axis) of CA1 and the subiculum because anatomic studies indicate distinct differences in connectivity within these regions and in their extrahippocampal projections (Naber et al. 2000; Witter et al. 2000). A clearer picture of activation within the parahippocampal cortices could be obtained by analyzing many more areas and their various layers. Extensive analyses such as these using catFISH would be greatly aided by automated methods for quantifying labeled neurons that are now under development (Lin et al. 2003).

Materials and Methods

Animals and behavior

Dbh$^{-/-}$ mice were rescued prenatally and housed as described (Ouyang et al. 2004). Studies were in accordance with NIH guidelines and had the approval of IACUC at the University of Pennsylvania. Sex-matched littermate *Dbh*$^{+/-}$ mice (4–5 mo old) were used as controls because they have normal tissue levels of NE/E

Table 1. Approximate mean total number of cells analyzed per mouse by brain region

Hippocampus				Amygdala				Striatum				Cortex							
dCA3	dCA1	vCA3	vCA1	LA	BLA	BMA	CeA	sNAc	cNAc	dlCPu	dmCPu	ME	pLE	aLE	PRh	PP	OF	AC	PM
240	320	340	380	440	400	380	560	640	720	480	720	520	360	300	380	520	540	480	320

See Figure 1 for anatomic abbreviations and locations.

and are phenotypically indistinguishable from $Dbh^{+/+}$ mice (Thomas et al. 1998). Four mice of each genotype were used for each treatment group.

Fear conditioning was performed as described in context S (ENV-010MC; Med Associates), which was a clear Plexiglas box with a metal bar floor 20 × 16 cm and 21 cm tall (Murchison et al. 2004). On day 1, following 2 d of handling, mice were placed for 3 min in context N, which was a Plexiglas cylinder with a green wire grid floor, 21 cm diameter, 24 cm tall, having vertical white and green stripes around two-thirds of the wall and olfactory cues distinct from context S. About 3 h later they were placed in context S (same room) for 3 min (see Fig. 3A). At 2 min, a tone was activated for 30 sec and a 2-sec, 1-mA footshock coterminated with the tone for mice in all groups except the no shock (NS) group. For basal *Arc* expression, mice trained on day 1 were removed from the home cage (group HC) on day 2. For analysis of the time course of *Arc* expression, mice were sacrificed at 5, 15, 20, 30, or 35 min after exposure to context S for 3 min on day 2. For analysis of memory retrieval, exposure to contexts S and N was performed on day 2 for 3 min each without shock. There was a 20-min interval in the home cage between contexts S and N. Percent freezing was estimated by scoring the presence or absence of nonrespiratory movement every 5 sec. Only mice that were resting before context testing or death (group HC) were used.

catFISH

Mice were sacrificed on day 2 after being removed from their home cage or 5 min following the second context exposure. Their brains were rapidly dissected and stored ($-80°C$). Six brains (at least one from each group) were mounted together, and coronal sections (20 µm) were cut by cryostat (HM505E; Microm). Sections were thaw-mounted onto slides and stored ($-80°C$). Digoxigenin-labeled antisense and sense *Arc* riboprobes (~3 kb) were prepared from linearized mouse cDNA (P. Worley, Johns Hopkins University, Baltimore, MD) using a kit (Roche). FISH was performed as described (Guzowski et al. 1999) using antidigoxigenin-horseradish peroxidase (Roche) and TSA-Direct fluorescence amplification (PerkinElmer). Slides were coverslipped with anti-fade medium containing DAPI (Vectashield; Vector Laboratories) and sealed. Sections were viewed using epifluorescence (Eclipse E600W; Nikon). To detect FITC, 494-nm excitation and 520-nm emission wavelengths were used. To detect DAPI, 360-nm excitation and 456-nm emission wavelengths were used. Images (1280 × 1024 pixels) were acquired using a monochrome CCD camera (ORCA-100; Hamamatsu Photonics). RGB TIFF images were recorded using Image-Pro Plus (Media Cybernetics). For compartment analysis, the entire field was analyzed (80× magnification, 106 × 85 µm, 83 nm/pixel for CA3 and CA1; 40× magnification, 212 × 170 µm, 83 nm/pixel for other regions). In all, 20 images (1 µm) were recorded along the z-axis of each section, and Autodeblur software (AutoQuant Imaging) was used for sharpening. Small, bright, uniformly DAPI-stained nuclei (from putative glial cells) were not analyzed. All other whole nuclei were analyzed from top to bottom. Nucleus-positive (Nu) neurons had two small, intense intranuclear fluorescent foci; cytoplasm-positive (Cy) neurons contained perinuclear/cytoplasmic labeling in multiple optical sections. Four adjacent sections were analyzed bilaterally for each brain region from each mouse. Approximate mean total numbers of cells analyzed per mouse for each brain region are given in Table 1. There were a total of 32 mice used for catFISH (4 mice × 2 genotypes × 4 conditions). The total number of cells analyzed for each of the 20 regions besides the DG ranged from ~8000 to ~23,000. For the DG, there were estimated to be >300 cells/field; thus, the number of positive cells/field is reported because negative cells were not scored owing to sparse labeling under all conditions.

Statistical analysis

Data were analyzed with *Statistica* 6.0 (StatSoft) using factorial two-way ANOVA of genotype and condition. Depending on the experiment, condition was either "context" for behavioral data, "treatment group" for total *Arc* labeling, or "type of labeling" for *Arc* compartment analysis. Post hoc comparisons were made using Duncan's range test. For analysis of context-specific labeling, each animal served as its own control for catFISH because of the within-subject design. Therefore, the difference for labeling between contexts (neutral − neutral or salient − neutral) was calculated for each animal. These were averaged to obtain summary data for each group. The null hypothesis was that the mean residual values would not be significantly different from zero. This was our planned comparison and was tested using a one-sample *t*-test. Our one additional planned comparison was between genotypes within the same group, which was made using a Student's unpaired *t*-test. Because these two planned comparisons were on related rather than independent data, no adjustment was made for α < 0.05. Levels of significance are indicated as: *, $P < 0.05$; ∧, $P < 0.01$; #, $P < 0.001$.

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