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Research report

Complementary activation of hippocampal–cortical subregions and immature neurons following chronic training in single and multiple context versions of the water maze

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ABSTRACT

Neurobiological studies of memory typically involve single learning sessions that last minutes or days. In natural settings, however, animals are constantly learning. Here we investigated how several weeks of spatial water maze training influences subsequent activation of neocortical and hippocampal subregions, including adult-born neurons. Mice were either trained in a single context or in a variant of the task in which the spatial cues and platform location changed every 3 days, requiring constant new learning. On the final day, half of the mice in each training group were tested in a novel context and the other half were tested in their previous, familiar context. Two hours later mice were perfused to measure subregion-specific expression of the immediate-early gene zif268, a marker of neuronal activation. None of the training paradigms affected the magnitude of adult neurogenesis. However, different neuronal populations were activated depending on prior training history, final context novelty, or a combination of these 2 factors. The anterior cingulate cortex was more activated by novel context exposure, regardless of the type of prior training. The suprapyramidal blade of the dentate gyrus and region CA3 showed greater activation in mice trained in multiple contexts, primarily after exposure to a familiar context. In immature granule neurons, multiple context training enhanced activation regardless of final context novelty. CA1 showed no significant changes in zif268 expression across any training condition. In naïve control mice, training on the final day increased zif268 expression in CA3, CA1 and the anterior cingulate cortex, but not the dentate gyrus, relative to mice that remained in their cages (transport controls). Unexpectedly, immature granule cells showed a decrease in zif268 expression in naïve learners relative to transport controls. These findings suggest novel and complementary roles for hippocampal, neocortical, and immature neuronal populations in learning and memory.

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1. Introduction

Spatially guided behavior is associated with distinct patterns of activation across hippocampal and neocortical subregions that can be observed through electrophysiological recordings and immediate-early gene (IEG) expression [1–7]. Within the dentate gyrus (DG) spatial exploration and spatial learning also induce expression of IEGs in adult-generated neurons [8–13]. In most of these studies, neuronal activation has been measured following acute experiences in a single environment, but it is known that prior experience can shape activity. For example, hippocampal Arc expression is increased when the escape platform is moved to a novel location in the water maze [14], anterior cingulate Fos expression is dependent on the age of a water maze memory [15], and the number of Fos+ hippocampal neurons increases when familiar spatial cues are rearranged in a radial maze task [16]. Additionally, mature adult-born neurons are more likely to be active during water maze retention tests if they were sufficiently integrated at the time of original training [10], and new neurons are particularly activated by water maze re-training at remote time-points after a previous training session [9]. While it is known that neuronal activity is modulated by both previous and current spatial experiences, it remains unclear how neuronal populations are recruited during spatial learning situations in animals that are highly familiar with a context or highly experienced at learning new spatial contexts. Such regular learning is common in natural populations.

To investigate the effects of extended prior experience on neuronal activation we trained mice for several weeks in a standard spatial water maze or a variant where the spatial context changed every 3 days. We chose the water maze because it is highly hippocampal-dependent [17–19], it induces IEG expression in the hippocampus and neocortex [10,11,14,15], and behavioral performance is readily quantifiable, in contrast to exploration paradigms where learning cannot be measured. On the final day of training

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we trained mice in a novel or familiar environment and measured expression of the activity-dependent IEG zif268 [20], which has been used as a reliable marker of activation of adult-born neurons [8,9,12,21] and of hippocampal and cortical brain regions [6,7,22,23]. Neuronal activation induced by water maze training was examined in adult-born granule neurons, the granule cell population as a whole, and CA3 and CA1 pyramidal cells. In addition, we examined zif268 expression in the anterior cingulate cortex (ACC), because this region has been shown to be an important site for long-term storage of spatial memory in other tasks [6,7,15,24]. Contrary to our expectations, we found no effects of long-term water maze training on new neuron survival but did find enhanced activity in selective neuronal populations that differed according to prior training condition (single vs. multiple contexts), final context exposure (familiar vs. novel), or a combination of these two factors. Collectively, these results point to new roles for adult-born neurons and spatial memory networks that have not been addressed with acute behavioral testing procedures.

2. Methods

2.1. Animals and general procedures

A total of 40 male C57Bl/6 (National Cancer Institute Animal Production Area, Frederick, MD) mice were used in this study. Mice arrived at 8 weeks of age and were allowed 1 week to acclimate to the vivarium prior to any experimentation. Mice were housed 4/cage with *ad libitum* availability of food and water and 12:12 h light:dark schedule with lights on at 6:00 a.m. Each mouse was given a single injection of BrdU (200 mg/kg, I.P. in 0.9% NaCl with 0.007 N NaOH) 31 days prior to sacrifice.

2.2. Behavioral procedures

All mice (including controls) were handled during the week prior to testing to familiarize them with the experimenter and minimize stress associated with water maze training. Mice were trained for several weeks in a spatial water maze. On training days, all mice (including controls) were brought to the testing area 30 min prior to testing. The tails of all mice (including controls) were numbered with a marker to aid animal identification during training. On the day prior to spatial training, mice were given 2 water maze trials with the curtains drawn (i.e., minimal cues) and the platform in the center of the pool, cued by an object hanging overhead. For spatial training, mice were placed in the N, S, E, or W points of the water maze in a systematically random and counterbalanced fashion. Mice were given 90 s to find a 10 cm platform submerged 1 cm below the surface of the water, which was made opaque by the addition of white nontoxic paint. If they did not find the platform during this time, they were guided there by the experimenter. Mice were trained with 4 trials each day with 2–4 min inter-trial intervals. Training occurred either in a single context throughout the entire experiment or in contexts that changed every 3 days ($n = 16$ for each; see Fig. 1a for complete breakdown of groups tested). In the standard context, used for single context training, there were prominent cues on 3 walls and a curtain behind which the experimenter remained during the trial. For multiple context training, distinct contexts were created using a variety of curtains and cues (flags, 3 dimensional objects such as buckets and tables) positioned in various arrangements. Inevitably, some cues (primarily static features of the room) were used in multiple contexts, but efforts were made to make the contexts as distinct as possible. The platform location remained constant within each context but moved when contexts changed (Fig. 2h). The single and multiple context-trained mice were further subdivided into groups that were only trained after BrdU injection and groups that were trained both before and after BrdU injection. For the post-BrdU trained groups, mice were trained for 24 days beginning 7 days after BrdU injection. For the pre/post-BrdU trained groups, mice were similarly trained for 24 days but also received 9 additional days of training ending 2 days prior to BrdU injection. Naïve cage control mice received BrdU but underwent no water maze training on days 0–41.

On the final day of the experiment (day 42), mice in each group were split into subgroups and either tested in the previous, familiar (F) context or a novel (N) context to examine the role of context novelty on zif268 expression. Naïve control mice either remained in their cage (cageC, i.e., transport controls) or were tested in a single block of spatial water maze trials for the first time (cageN). Mice in all other groups were tested with a single block of spatial water maze trials in either the same familiar context as on the prior day (singleF and multiF) or in a completely novel context (singleN and multiN). The final testing context was the same for all mice except the singleN group. Thus, most mice experienced the same context on the final day, but the degree of novelty associated with it varied across groups. The block of 4 trials lasted approximately 20 min, after which mice remained undisturbed in their home cage in the testing area until 2 h after the 1st trial, at which time mice were rapidly euthanized.

2.3. Histological methods

Mice were anesthetized with isoflurane and perfused with 4% paraformaldehyde. Brains were post-fixed for 24 h in 4% paraformaldehyde, transferred to a 20% sucrose solution for at least 24 h, and then sectioned coronally at 40 μm on a freezing sliding microtome. Fluorescent immunostaining was performed as previously described [25], on sections containing dorsal hippocampus. To measure experience-dependent activity in hippocampal and cortical subregions, zif268 immunostaining with chromogenic detection was performed. Sections were incubated for 3 days, free floating, in PBS containing 0.5% tween20, 3% goat serum and rabbit anti-zif268 antibody (1:1000; Santa Cruz Biotechnology, sc-189). Sections were then washed and incubated with a biotinylated goat anti-rabbit secondary antibody (1:250; Jackson Immunolabs) for 60 min, detected using Vector SG peroxidase substrate (Vector labs), cleared, and coverslipped under Permount. To assess activity in young neurons, combined fluorescent immunostaining against zif268 and doublecortin (DCX) was performed as previously described [25]. Briefly, free-floating sections were incubated simultaneously in rabbit anti-zif268 (1:1000; Santa Cruz Biotechnology, sc-110), and goat anti-DCX (1:200; Santa Cruz Biotechnology, sc-8066), followed by visualization with Alexa conjugated secondary antibodies (Invitrogen). For combined BrdU and DCX immunostaining, free floating sections were treated with 2 N HCl and then incubated simultaneously with rat anti-BrdU (1:2500; Accurate Antibodies, OBT0030) and anti-DCX antibodies (as above). For BrdU+ cell counting, immunostaining with BrdU visualization was performed as previously described [25]. Briefly, slide-mounted sections were pre-treated with heat, trypsin, and HCl to denature DNA, incubated with mouse anti-BrdU (1:100), and visualized with avidin–biotin–HRP (Vector Labs) followed by DAB.

2.3.1. Histological data analysis

Analysis of immunoperoxidase-stained zif268 expression in the DG, CA3 and CA1 was performed on 2 sections spaced 240 μm apart containing dorsal hippocampus (between −1.8 and −2.4 mm relative to Bregma). Zif268 expression in the anterior cingulate cortex (Cg1 and Cg2, according to Ref. [26]) was examined in 4 sections, each spaced 120 μm apart, between 0 mm and 1.0 mm relative to Bregma. Images of the hippocampus and cortex were acquired with a 4× objective, using Stereoinvestigator 9.0 software (Microbrightfield). To ensure consistent levels of brightness and quality, all sections were imaged during the same session and with the same exposure and gain settings. Images were analyzed using ImageJ software (http://rsbweb.nih.gov/ij/). Boundaries were traced and the percentage of area within each brain subregion that exhibited above-threshold zif268 staining intensity was calculated, as an estimate of the proportion of cells in the subregion showing activation. The same thresholds were applied across all sections, but due to the greater cell packing in CA1 and greater staining intensity in the anterior cingulate cortex, the threshold for these regions was 22% lower than that used for the DG and CA3, in order to avoid saturation/ceiling effects that could obscure differences between groups.

To assess the behavioral activation of adult-born neurons, DCX+ neurons were exhaustively examined for co-labeling with zif268 in one hemisphere of a single dorsal hippocampal section. The mean number of DCX+ cells examined per mouse was 374, spanning both the infrapyramidal and suprapyramidal blades. DCX+ cells were initially inspected under epifluorescence using a 60× oil-immersion objective, and putative double-labeling was confirmed via confocal microscopy as previously described [25]. Specifically, cells with a zif268 intensity of 1.4× background were considered positive. Zif268 expression was also observed in BrdU+ cells but due to the small number of BrdU-labeled cells these cells were not examined further. Additionally, the total number of DCX+ cells was divided by the sampled volume of the granule cell layer to obtain a density measure for this marker of immature neurons. To determine the proportion of BrdU+ cells expressing DCX, all BrdU+ cells in both hemispheres of a single dorsal hippocampal section (mean = 34 per mouse) were examined for co-expression of DCX via confocal microscopy. For the survival analysis, DAB-labeled BrdU+ cells were counted in a 1:12 series through the entire DG, and the total count was multiplied by 12 to provide a stereological estimate of the total number of BrdU+ cells in the DG.

2.4. Statistical analysis

Comparisons between groups were performed by ANOVA with Bonferroni post hoc tests as required.

3. Results

3.1. Behavioral performance in single- and multiple context variants of the water maze

Comparison of Pre/Post-BrdU and Post-BrdU trained mice in the single context condition by 2-way repeated measures ANOVA showed a significant main effect of training day ($F_{23,322} = 8.5$, $P < 0.0001$) and a significant interaction ($F_{23,322} = 5.2$, $P < 0.0001$) but no main effect of prior training ($F_{1,322} = 1.1$, $P > 0.3$). Post hoc tests

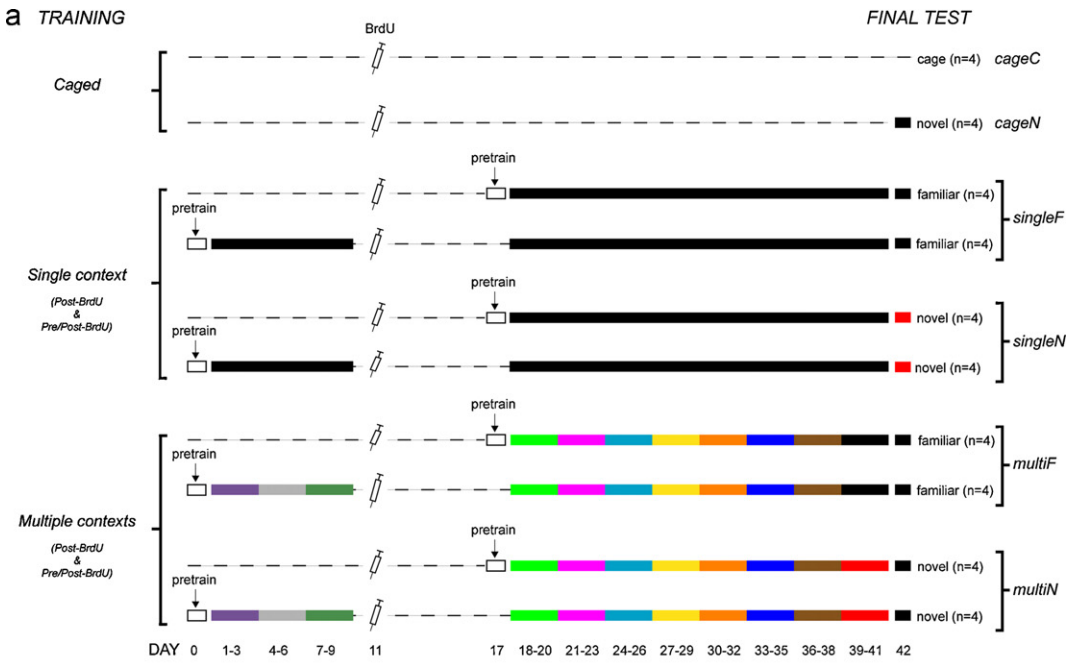

Fig. 1. Experimental design. (a) Timeline. All mice received BrdU injections on day 11 and were perfused on day 42. Cage control mice were brought to the testing room with the rest of the mice but received no other treatment through day 41. Mice in other groups were trained in a spatial water maze task, either in a single spatial context, shown in black, or in multiple contexts, represented by other colors. Some mice received water maze training only after BrdU administration (Post-BrdU), while others (Pre/Post-BrdU) received water maze training both before and after BrdU administration. On the final day of the experiment (day 42), mice were subjected to a single block of 4 water maze trials in either a familiar or novel context (or no training for some control mice) and were perfused 2 h after the initial training trial. (b) Panoramic images of 4 contexts. The context shown on top is the standard context, used to train mice in the single context condition and to test most of the groups on the final day. The other 3 contexts are the 2nd, 5th, and 8th contexts used for Pre/Post-BrdU training of mice in the multiple context condition. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

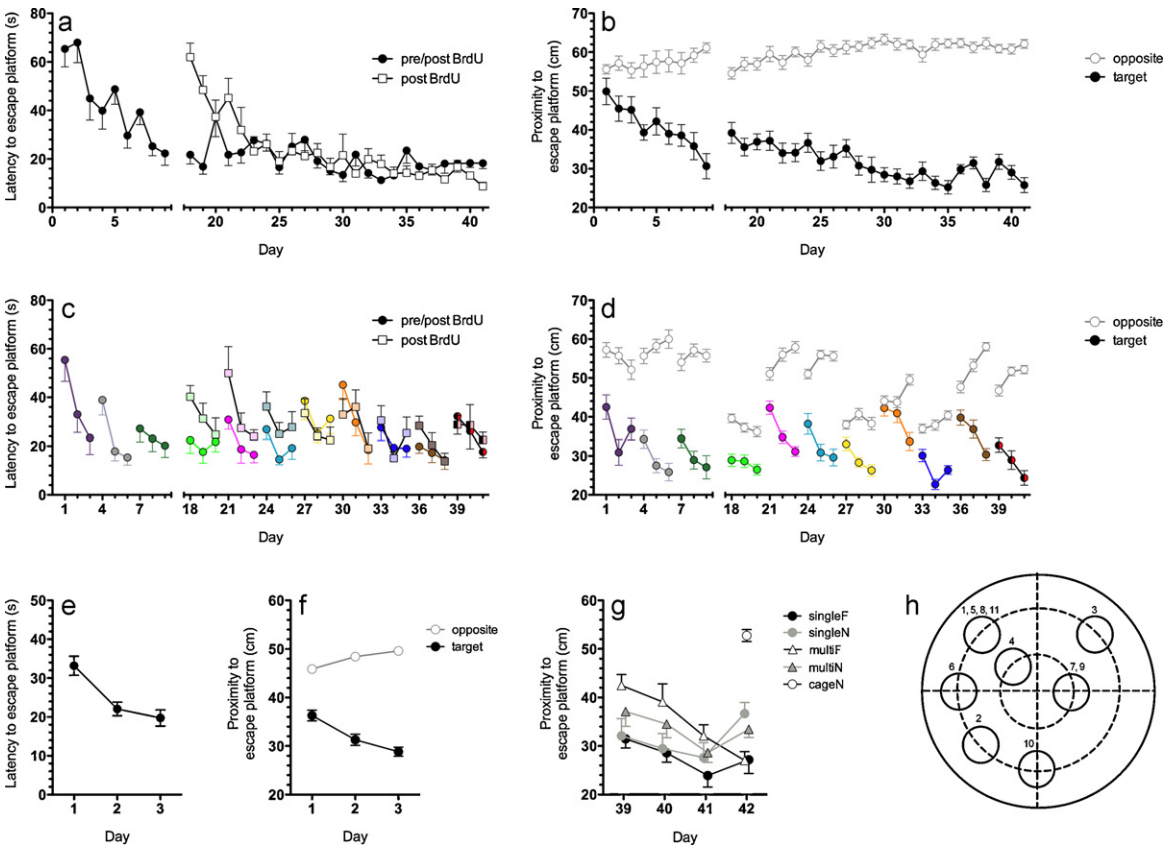

Fig. 2. Behavioral performance in single- and multiple-context water mazes. (a) Latency to find the hidden platform decreased across in the single-context training condition. (b) Proximity, i.e., mean within-trial distance, to the platform location decreased over days of training for mice in the single-context training condition. Here, and in (d) Post-BrdU and Pre/Post-BrdU groups are pooled for clarity. Therefore, days 1–9 reflect behavioral performances for only the Pre/Post-BrdU group and days 18–41 reflect behavioral performances for both groups. (c) Latency to find the hidden platform for the multiple-context groups. (d) Proximity scores for the multiple-context groups. (e) Averaged latencies for the 3 days of training in each context for mice in the multiple-context condition. (f) Averaged proximity scores for the 3 days of training in each context for mice in the multiple-context condition. (g) Proximity scores for the final 4 days of the experiment (days 39–42). Days 39–41 show performance at the end of training and day 42 shows performance as mice are tested further in either the previous context (singleF, multiF) or a novel context (singleN, multiN) to induce zif268 expression. One group of mice did not receive training but was only tested on day 42 (cageN). (h) Platform locations for the multiple-context enriched mice. Numbers indicate the contexts (sequentially ordered) that used a given platform location.

showed that Pre/Post-BrdU trained mice were significantly faster to locate the hidden platform on days 18, 19 and 21, as expected due to prior training on days 1–9 (post hoc all $P < 0.01$). To confirm that improved escape latencies reflect spatial learning we also analyzed the average proximity to the escape platform over days [27]. As with the latency measure, proximity to the platform increased significantly over days (i.e., average within-trial distance to the platform decreased; Fig. 2b, ANOVAs for both groups both $P < 0.001$). Over days 18–41, there was no main effect of prior training on performance; however, there was a prior training × day interaction such that mice in the Pre/Post-BrdU group outperformed mice in the Post-BrdU group on day 19 (2-way repeated measures ANOVA, effect of training day $F_{23,322} = 8.5$, $P < 0.0001$; effect of prior training $F_{1,322} = 0.00003$, $P > 0.9$; interaction $F_{23,322} = 3.3$, $P < 0.0001$). Since there was no main effect of prior training, the Pre/Post-BrdU and Post-BrdU groups are pooled in Fig. 2b.

Mice in the multiple-context condition showed improved escape latencies and spatial proximity to the platform across days within each spatial context, i.e., performance typically improved over 3 days and then was impaired as mice were challenged to learn a new spatial context (Fig. 2c and d). To measure performance, latency and proximity scores across contexts were averaged for each mouse for each day. Two-way repeated measures ANOVAs showed no differences between mice in the Post-BrdU and Pre/Post-BrdU multiple context training groups ($F_{1,28} \leq 1.0$, $P > 0.3$) therefore the 2 groups were pooled. Mice trained in multiple contexts showed reduced latency over days 1–3 ($F_{2,30} = 29.7$, $P < 0.0001$; Fig. 2e), and post hoc comparisons indicated significant differences in latency between days 1 and 2 and between days 1 and 3 ($P < 0.001$ for both). Proximity to the platform location changed over days ($F_{2,30} = 82.6$, $P < 0.0001$; Fig. 2f), with post hoc tests revealing significant reductions in the proximity score each day (all comparisons $P < 0.001$). To investigate the possibility that mice formed an allocentric representation of the room that carried over across contexts, we compared the latencies to 20 cm annuli surrounding the previous versus current platform locations on the first trial in a novel context. On average, 42% ± 12% (SEM) swam to the current platform location first, which was not different from chance (one sample t-test, $T_6 = 0.7$, $P = 0.5$), suggesting poor performance was due to the altered context and not simply because the platform had moved to a new location as in spatial match to place testing [28].

On the final day (day 42), mice in each group were either exposed to the same context seen over the last 3 or more days or to a novel context (see Fig. 1 schematic). Proximity scores for the final 4 days are shown in Fig. 2g. Collectively, performance of mice in novel contexts was impaired relative to those in familiar contexts on the final day, as expected (2-way ANOVA; main effect of final context novelty, $F_{1,28} = 13$, $P = 0.001$; effect of prior training condition, $F_{1,28} = 0.6$, $P = 0.5$; interaction, $F_{1,28} = 0.5$, $P = 0.5$). To investigate how performance changed from day 41 to day 42 when the final novel/familiar context was introduced, a 2-way (group × day) repeated measures ANOVA was performed. There

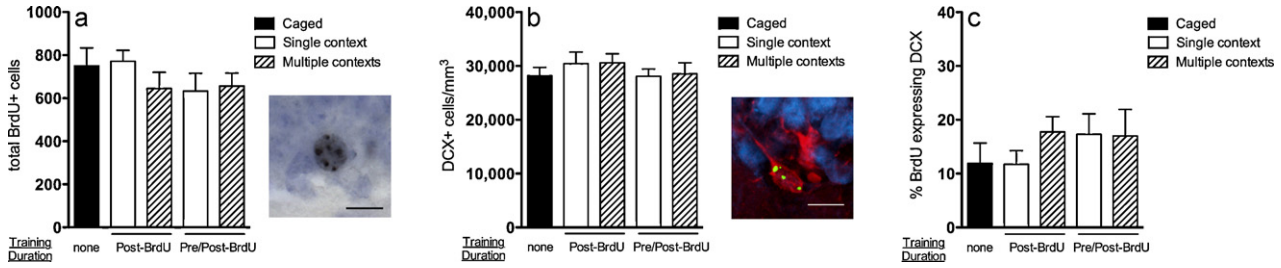

Fig. 3. Adult neurogenesis following spatial water maze training. (a) Stereological counts of 31-day-old BrdU+ cells showed no significant training effects. Inset shows a representative BrdU+ cell in the DG. (b) The density of DCX+ cells in the dorsal dentate gyrus was not different between training groups. Inset shows confocal image of a representative BrdU+/DCX+ cell. (c) The proportion of BrdU+ cells expressing DCX was not different between groups. Bars represent mean ± SEM, $n = 7–8$ in each group. Scale bars 10 μm.

was a significant group × day interaction ($F_{3,28} = 6$, $P < 0.01$), and post hoc tests revealed that mice tested in a novel context on day 42 performed significantly worse than on day 41 (singleN $P < 0.01$, multiN $P = 0.05$). In contrast, mice in the multiple context condition that were trained in a familiar context on day 42 (multiF) continued to improve their performance relative to day 41 ($P < 0.05$). Mice trained in the same context through the entire experiment (singleF) reached asymptotic performance many days earlier, and were not different on day 41 compared to day 42 ($P = 0.2$).

3.2. Water maze training did not affect neuronal survival or the rate of expression of the immature marker doublecortin

Long-term exposure to enriched housing conditions and running wheels enhance survival of young granule neurons in adult mice [25,29]. We hypothesized that long-term exposure to spatial water maze training, a strongly hippocampal-dependent behavior, would also enhance new neuron survival in these mice. The total number of BrdU+ cells was estimated for each training condition and subjected to a 1-way ANOVA. Mice exposed to novel and familiar contexts on the final day were grouped, since this exposure was after the normal time period of cell death [30–32] and in all likelihood too close to the time of perfusion to have an effect. No significant group differences were found in the number of surviving BrdU+ cells (Fig. 3a; $F_{4,31} = 0.8$, $P = 0.5$) or in the density of DCX+ cells (Fig. 3b; 1-way ANOVA, $F_{4,35} = 0.5$, $P = 0.7$). Additionally, the proportion of BrdU+ cells expressing DCX across groups was not different (Fig. 3c; $F_{4,34} = 0.7$, $P = 0.6$).

3.3. Distinct subregion-specific patterns of activation following spatial water maze training and exposure to familiar versus novel contexts

Because behavior was not different in the mice trained post-BrdU and pre/post-BrdU, these groups were pooled for analyses of zif268 expression on the final day. Within each hippocampal or cortical subregion, zif268 expression was analyzed using 2-way ANOVAs with training (single or multiple context) and final testing context (familiar or novel) as factors (Fig. 4). In both blades of the DG, more cells expressed zif268 in multiple context-trained mice than in single context-trained mice (for both blades, $F_{1,24} > 5$, $P < 0.05$). In the suprapyramidal blade there was a trend for more zif268 in multiple context-trained mice exposed to the familiar context on day 42 (interaction $F_{1,25} = 2.7$, $P = 0.11$; post hoc vs. multiN $P = 0.08$, post hoc vs. singleF $P < 0.01$). A similar pattern of activation was also observed in CA3, where multiple context-trained mice had more zif268 expression than single-trained mice (main effect of training: $F_{1,25} = 7$, $P < 0.05$), and there was a significant training × final test context interaction ($F_{1,25} = 5.5$, $P < 0.05$) with the greatest zif268 expression in mice in the multiF condition (post hoc, $P < 0.05$ vs. multiN and $P < 0.01$ vs. singleF). In contrast to the DG and CA3, zif268 expression in CA1 showed no significant differences across trained groups. Lastly, the ACC showed significantly enhanced activation in response to novel context exposure, regardless of prior training status (main effect of final test context, $F_{1,05} = 5.3$, $P < 0.05$).

Zif268 expression was also examined in control groups, which received no prior training and were exposed to the spatial water maze for the first time on day 42 (cageN) or remained in their cages (cageC) just prior to perfusion (Fig. 4c). As expected based on studies of naïve rodents learning a spatial water maze [14,33], cageN mice showed enhanced zif268 expression relative to cageC mice in regions CA3, CA1 and ACC (main effect of brain region, main effect of experience and interaction all $P < 0.01$; post hocs – CA3 $P < 0.05$, CA1 $P < 0.001$, ACC $P = 0.05$; Fig. 4c). In contrast, zif268 levels did not differ significantly between these two groups in the DG (post hoc both blades $P > 0.05$).

3.4. Multiple context training enhances activation of adult-born neurons

Zif268 expression was quantified in adult-born granule cells in the DG identified via fluorescent immunostaining for DCX (Fig. 5). Activation was measured as the proportion of DCX+ neurons co-expressing zif268. In the infrapyramidal blade, DCX+/zif268+ expression showed no main effect of training or of final test context and no interaction between these two factors (all $P > 0.1$). In the suprapyramidal blade, however, there was a significant effect of training, with a greater proportion of DCX+ neurons expressing zif268 in mice subjected to multiple context training compared to the single context training condition ($F_{1,22} = 10.0$, $P < 0.01$; Fig. 5c). There was no effect of final context on DCX+/zif268+ expression ($F_{1,22} < 0.1$, $P = 0.9$).

Zif268 expression in DCX+ cells was assessed in control mice, which remained in their home cage until the final day. Using 2-way ANOVA with blade (supra- or infrapyramidal) and training (novel training or no training) as factors, significant main effects of blade and training and a significant interaction (all $F > 5$, $P < 0.05$) were found (Fig. 5d). Zif268 expression in DCX+ neurons was greater in the suprapyramidal blade than in the infrapyramidal blade, as seen in the overall granule cell population (Fig. 4c). But in contrast to what was seen in mature neurons in all examined subregions, DCX+ cells in the suprapyramidal blade of the cageC group, had significantly greater zif268 expression compared to those in the cageN group ($P < 0.01$). DCX+/zif268+ expression was also significantly greater in the suprapyramidal blade than in the infrapyramidal blade of cageC mice ($P < 0.01$).

To further examine activation of immature neurons as a function of prior training experience, we compared all groups that were trained in a novel context on the final day. One-way ANOVA showed that mice in the multiN condition had greater suprapyramidal DCX+/zif268+ expression than both singleN cageN mice, which

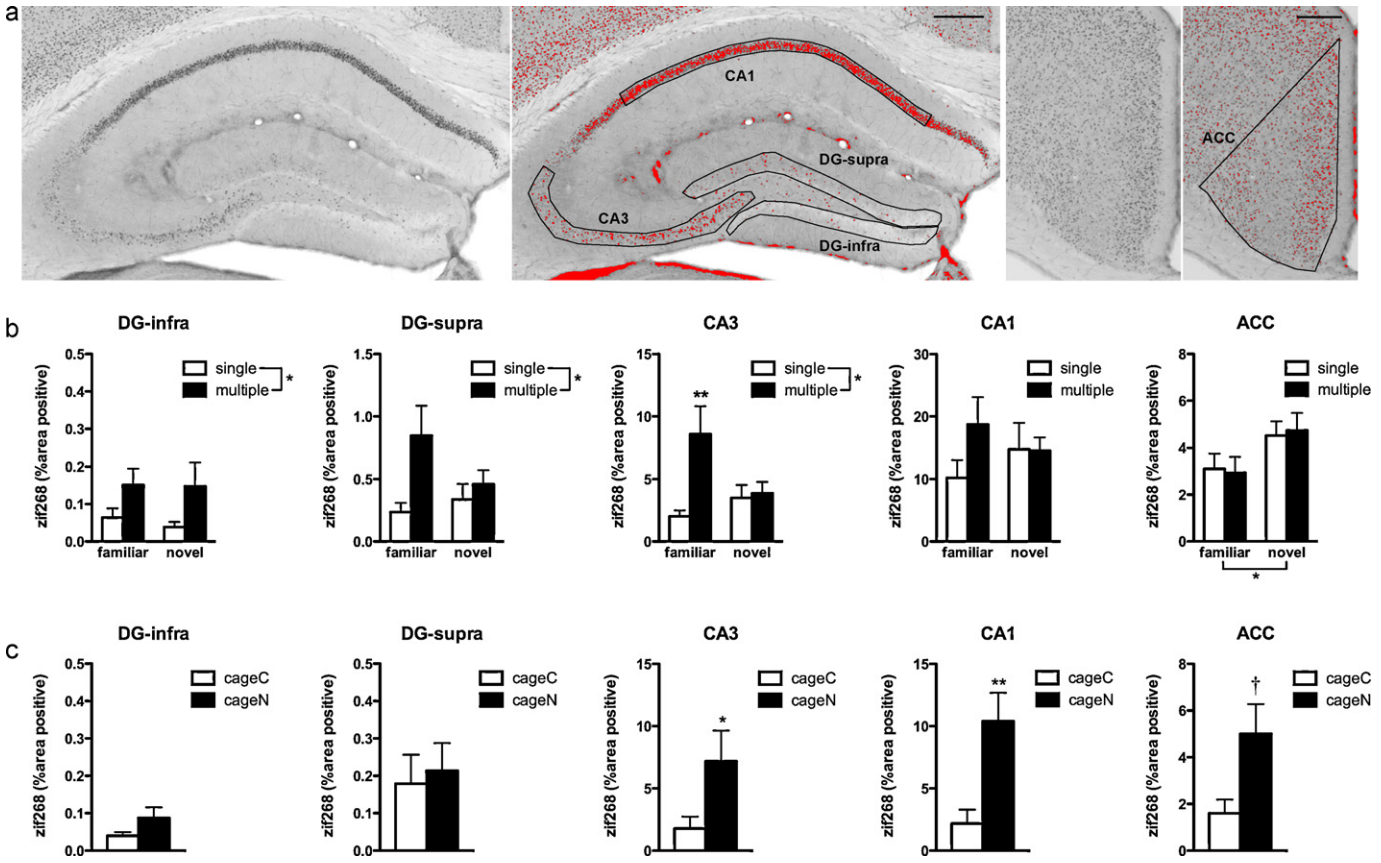

Fig. 4. Distinct activation patterns across hippocampal–cortical subregions. (a) Images of hippocampus and anterior cingulate cortex illustrating subregions analyzed. Red regions in the right panels indicate cells expressing supra-threshold levels of zif268 (i.e., positive cells); thresholded hippocampal image is adjusted to illustrate subregion-specific differences in threshold in the same image. (b) Both blades of the dentate gyrus DG, as well as CA3, both showed significantly greater activation in mice receiving multiple context training. There was also a significant interaction such that CA3 activation was greater in multiple context-trained mice after exposure to a familiar context and a trend for such an interaction in the suprapyramidal blade of the DG. Activation in CA1 was not significantly different across groups. Activation in the ACC was greatest following exposure to a novel environment, regardless of prior training. (c) In regions CA3, CA1 and ACC, naïve control mice showed increased zif268 expression following training in the water maze relative to caged mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\dagger}0.05 < P < 0.10$; Scale bars 250 μm.

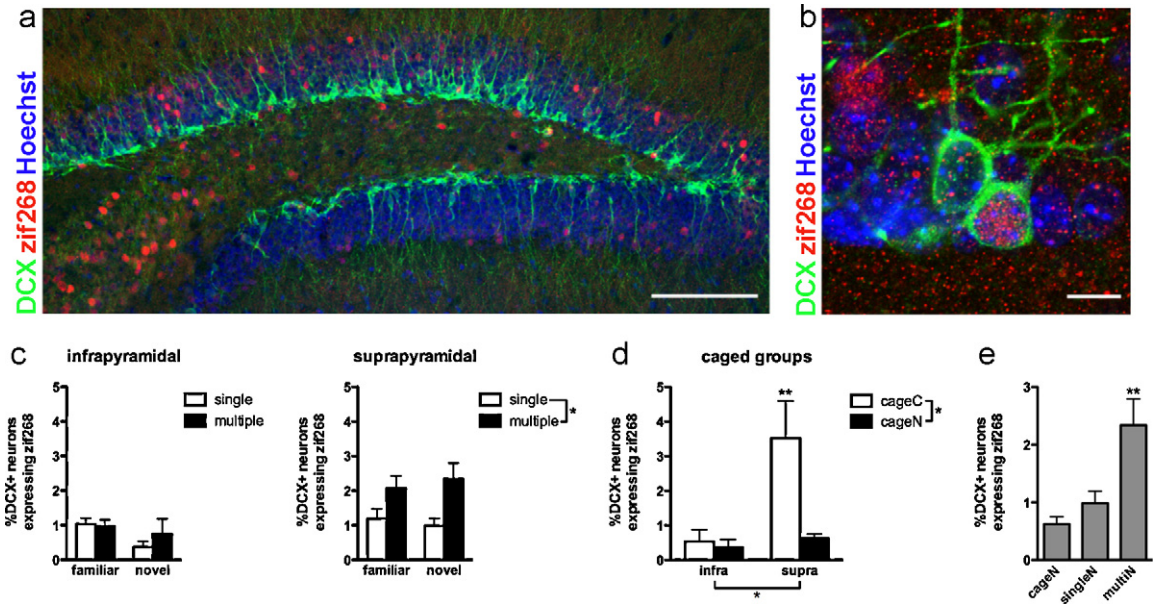

Fig. 5. Activation patterns in adult-born neurons. (a) Confocal photograph of DCX+ immature neurons and zif268 expression throughout the DG following water maze exposure. (b) Example of a DCX+/zif268+ young neuron. (c) Immature neurons in the suprapyramidal blade showed significantly greater activation in multiple context-trained mice, regardless of whether activation was induced by exposure to a novel or familiar context. (d) In the suprapyramidal blade, DCX+ neurons showed significantly greater zif268 expression in caged controls (cageC) compared to previously naïve mice trained in the water maze for the first time (cageN). (e) Activation of immature neurons in mice that were trained in a novel context on the final day. Mice that were previously trained in multiple contexts had greater activation of DCX+ neurons than naïve mice and mice trained in a single context. $^{*}P < 0.05$; Scale bars 100 μm (a) and 10 μm (b).

were not significantly different from each other ($F_{2,16} = 6.1$, $P < 0.01$; post hoc multiN vs. singleN and cageN both $P < 0.05$, post hoc singleN vs. cageN $P > 0.2$; Fig. 5e).

4. Discussion

In the current study we used chronic standard water maze training or a novel variant where contextual cues regularly changed to examine (1) how prolonged spatial learning affects survival of adult-born neurons and (2) how prior spatial learning experiences modulate activity during exploration of novel and familiar spatial contexts. We found no effect of chronic spatial learning on new neuron survival. However, after chronic training in static or varying versions of the spatial water maze, distinct patterns of activation were revealed across neuronal populations. Specifically, the ACC showed enhanced activation following exposure to a novel context on the final test day (day 42), regardless of prior training (single vs. multiple). In contrast, the DG (supra) and CA3 were preferentially activated in multiple context-trained mice but only when tested in a familiar context. CA1 appeared to have a similar pattern for the means across groups, but zif268 expression showed no statistically significant differences. Lastly, immature granule neurons in the suprapyramidal blade showed enhanced activation in mice that were repeatedly trained in different spatial contexts, with no preferential activation in novel versus familiar contexts on the final day. In addition to chronically trained mice, control groups were transported to the testing room in their home cages throughout the training period and either remained in their home cages on the final test day or were trained in the water maze for the first time. Training increased zif268 expression in CA3, CA1 and the ACC but not in the DG. We unexpectedly found that young granule cells were actually inhibited during initial exposure to the water maze relative to their very high rate of activation in the cage control condition. Collectively, these results suggest novel complementary roles for hippocampal and neocortical regions in spatial learning and memory.

4.1. Survival of adult-born hippocampal neurons

Experience-dependent effects on neuronal survival have been demonstrated with environmental enrichment [29] and spatial water maze learning [34], with the direction of change varying depending on factors such as task difficulty and cell age [35,36]. It is somewhat surprising, therefore, that the current study found no change in young granule cell survival or DCX+ cell density, since our experimental design contained features of both of these paradigms and involved training mice during a prolonged period when new neurons die and could be potentially rescued [30–32]. However, although several studies have found changes in new neuron survival in rats [34,37] (but see Ref. [39]), the water maze may have less of an effect on new neuron survival in mice [38,40]. Another possibility is that survival was increased when the labeled granule cells were very young and was subsequently decreased as they matured, as previously described in rats [41,42]. The sum of these two changes could result in a lack of net effect on survival with prolonged learning. A previous study has suggested that the stress of spatial water maze training has a negative effect on young neuron survival, since decreased survival was eliminated with pretraining [38], but a similar effect of pretraining was not observed in the current study. Enhanced survival of new neurons is consistently seen in mice with environmental enrichment [8,29,43]. There are several possible reasons why long-term water maze training did not produce the survival effect seen with enrichment, including the length of daily exposure (brief versus continuous), sex differences (males versus females, which have been used in virtually all mouse enrichment studies), or differences in stress levels produced by water maze learning and environmental enrichment.

4.2. Activation in naïve mice

Previous studies have shown that training naïve animals in the water maze increases IEG expression relative to cage controls in CA1 and CA3 [14,33] and that non-naïve behaving animals typically have greater IEG expression in the hippocampus and neocortex relative to cage controls [1,15,23,44,45]. Our data showing increased zif268 expression in CA3, CA1 and the ACC relative to cage controls are consistent with these findings. The DG, however, did not show increased activation following water maze exposure in naïve mice relative to cage controls, in apparent contrast with several studies reporting IEG increases in behaving animals relative to cage controls [1,3,10] and two studies showing increased Fos and Arc in naïve water maze-trained rats relative to cage controls [11,14]. It is unlikely that lack of expression reflects a problem with immunostaining or with the behavioral manipulations because zif268 levels were increased other brain regions (CA3, CA1) in the same sections. Currently, little is known about the function of the DG and the factors that activate populations of DG neurons, but stress can decrease IEG expression relative to cage controls [46], and DG IEG expression can be reduced by novelty [47]. Species differences and/or distinct functions of zif268 [48] versus Fos and Arc may also explain differential expression of these IEGs in the current study as compared to previous studies. Furthermore, electrophysiological and IEG data indicate that the same DG neurons are often reactivated by different experiences, including sleep [1,2,5]. Thus, the DG of naïve trained mice, relative to cage controls, was either (1) not significantly activated, (2) did not undergo zif268-dependent forms of plasticity with 2 h post-training, or (3) did not activate additional populations of neurons during water maze acquisition.

Unlike the other neurons examined, young adult-born granule cells in the current study showed reduced zif268 expression in naïve water maze-trained naïve mice relative to the cage control condition. Other studies have reported a lack of increase in zif268 protein in young neurons following behavioral stimulation [8,49], but this is the first study to our knowledge to find a significant reduction in IEG expression in new neurons with novel experience. This decrease could reflect inhibition of young granule cells in stressful conditions, since mice that were familiar with the water maze (single/multiple context-trained mice) had numbers of DCX+/zif268+ neurons that were intermediate between those in the highly stressful first exposure and (presumably stress-free) cage controls. Alternatively, it is possible that relatively high levels of zif268 in young neurons in cage controls reflect sleep-related consolidation activity [1,50], since undisturbed mice spend much of their time sleeping during the light phase, and adult-born neurons are required for consolidation and long-term spatial memory [39,51,52]. A previous study [53] has found increased zif268 activation following REM sleep in the DG of rats exposed to a novel environment; cage control mice in the current study were briefly handled upon transport to the testing room and then slept for much of the subsequent ~3 h prior to perfusion. These experiences and/or social interactions that occurred as a result of transport and handling could have led to increased zif268 expression in new neurons during subsequent sleep bouts. It will be useful to determine specific conditions under which resting animals show neuronal activation and expression of various IEGs. Although stress and/or sleep effects could potentially explain the inhibition of zif268 in young neurons induced by water maze training in naïve mice, neither of these factors seems likely to play a role in the changes observed within the groups of chronically trained mice, which were all equally awake and probably all mildly stressed during the training period.

4.3. Response to novel conditions in the ACC of chronically trained mice

Chronically trained mice were highly familiar with the task demands, unlike naïve mice, which had experiences (swimming in a spatial water maze in a novel room or remaining in the home cage) that differed in spatial layout and procedural aspects as well as novelty. Thus, differences in activation patterns across chronically trained mice can be more easily linked to training history or final context novelty than those in the naïve mice.

Only neurons in the ACC showed a selective response to the novel context condition; pyramidal neurons in CA3 and CA1 and both the overall population of DG neurons as well as the immature subset showed no increase in response to the novel spatial context. The ACC response to novelty is unexpected in light of studies showing increased ACC activation following retrieval of remote memory relative to recent memory [6,7,15]. Although we did not have test groups that were directly comparable to previous studies, one might have expected that mice in the singleF group, which experienced a context that was well-trained and presumably well-consolidated, would have shown the highest levels of activation, but this was not the case. Our finding that mice in the singleF group had less ACC activation than mice in the singleN group and comparable activation to mice in the cageN group, despite retrieving a memory that was as old (24/41 days) as that in typical rodent studies of remote memory, suggests that a key factor required for activation of the ACC during remote memory retrieval is an extended training–testing interval during which the animals are not exposed to the testing environment.

In contrast to the ACC response, the DG showed identical responses in the novel and familiar spatial conditions. This was unexpected since lesion data specifically points to a role for the DG in detecting spatial novelty [54,55]. This disconnect between IEG activation and lesion-induced deficits may reflect different requirements for detecting spatial rearrangement of objects within a context, as in the earlier studies, and learning an entirely new context, as in the current study.

4.4. Effects of prior training on hippocampal activation in chronically trained mice

Unlike ACC activation, hippocampal activation was related to prior training, and in some cases there was an interaction between the training type and novelty. For example, mice in the multiF group had elevated zif268 expression in CA3, and there was a trend toward this same effect in the suprapyramidal blade of the DG (and perhaps CA1). This activation pattern could reflect differential learning during the final training episode, since these were the only mice that showed improvement from the prior day's performance levels. The other groups, which showed lower levels of zif268 expression, were at different stages of behavioral performance. Mice trained in a novel context on the final day (multiN, singleN) had just begun to form a memory of the spatial context and platform location, as evidenced by impaired performance compared to the prior day. At the other extreme, mice trained in the same context throughout the entire experiment (singleF) performed as well as mice in the multiF group on the final day, but their performance had plateaued approximately 2 weeks earlier and they showed no evidence of continued learning. Increased zif268 in the multiF mice on the final day may therefore reflect activation related to mastery of the current context and/or early stages of memory consolidation. This interpretation fits with previous data showing mossy fiber sprouting and, presumably, increased connectivity between the DG and CA3 after 4–5 days of spatial water maze training [56]. An unresolved question is whether increased activation on the 4th day of training in a given context is dependent on extensive prior training.

The parallels between zif268 activity and behavior on the final day do not hold as well for infrapyramidal DG granule cells and immature granule neurons in the suprapyramidal blade. Neuronal activation in these populations was determined by prior experience rather than current stimuli; these neurons were more active in mice trained in multiple contexts than mice trained in a single context and were completely unaffected by the novelty/familiarity of the final context. It is interesting to note that the mature granule cells of the suprapyramidal blade showed an activity pattern that resembled that of CA3 and CA1 pyramidal cells, while the pattern in the infrapyramidal blade was very different, resembling only the immature granule cells. Previous studies have found that experiences such as undirected exploration or spatial water maze training rapidly increase expression of immediate-early genes c-Fos and Arc in mature granule cells within the suprapyramidal but not infrapyramidal blade [1,3,11,57]. This difference may be driven by differences in GABAergic inhibition to the two blades [58]. The current study found that although activation is always lower in the infrapyramidal blade than in the suprapyramidal blade, mature granule cells in the infrapyramidal blade can be rapidly activated by spatial water maze experience if the animals have undergone prior training. The increased activation in the multiple exposure animals, which are still learning on the final day of training, along with previous findings that the infrapyramidal blade is activated 6–8 h after exposure to a novel environment [1] seems consistent with the possibility that the infrapyramidal blade is involved in memory consolidation.

An interesting question is how prior experience affects immature neurons that are not even born when training begins. Granule cells in mice can be activated shortly after they are 2 weeks old, and some retain doublecortin until they are greater than 4 weeks of age [12]. Thus, it is possible that zif268 expression in doublecortin-positive neurons reflects participation in memory formation only during the final 1–2 weeks of training and subsequent retrieval of those memories on the final day. A second possibility is that prior memory of the training affects subsequent perception, strategy, or other aspects of behavior and it is this upstream difference, rather than a change in the young neurons themselves, that affects recruitment of immature neurons.

There are several possible consequences of increased activation of young granule neurons. Previous studies have found that a small proportion of granule neurons, often the same population, are active in different contexts [2,3,5,22]. Growth in the active population seen here could reflect the recruitment of additional populations of neurons in order to better pattern-separate related contexts, perhaps partly via enhanced integration of immature neurons by water maze training [59,60]. Alternatively, it has been proposed that a developmental window of increased activity may allow immature neurons to bind together temporally related experiences [61]. A twist on this idea is the possibility that increased activation of (infrapyramidal and) immature neurons during multiple context learning serves to relate similar experiences, perhaps by recruiting additional neurons that were recently activated by similar contexts. Repeated context learning may therefore promote the formation of a schematic understanding of the related spatial water maze contexts, akin to that which is formed during the systems consolidation of memory [62,63]. Such a role would be consistent with IEG imaging studies and neurogenesis ablation studies that have implicated young neurons [9,39,51] and the infrapyramidal blade [1] in memory consolidation and the processing of remote memory. In any case, the activity and/or plasticity in these granule neurons is unusual in its dependence on the prior experience and could be thought of as an instance of metaplasticity at a cellular level.

Taken together, we have shown differential responses of hippocampal and neocortical neuronal populations following learning in novel and familiar environments, as a function of prior training. These data suggest that young granule cells, mature granule cells, hippocampal pyramidal cells, and ACC neurons act in complementary ways to integrate new information with previous experience.

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