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Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus

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Throughout adulthood, new neurons are continuously added to the dentate gyrus, a hippocampal subregion that is important in spatial learning. Whether these adult-generated granule cells become functionally integrated into memory networks is not known. We used immunohistochemical approaches to visualize the recruitment of new neurons into circuits supporting water maze memory in intact mice. We show that as new granule cells mature, they are increasingly likely to be incorporated into circuits supporting spatial memory. By the time the cells are 4 or more weeks of age, they are more likely than existing granule cells to be recruited into circuits supporting spatial memory. This preferential recruitment supports the idea that new neurons make a unique contribution to memory processing in the dentate gyrus.

New neurons are continuously generated in the subgranular zone of the hippocampus throughout adulthood[1–7]. The majority of these newborn neurons become integrated into existing dentate gyrus circuitry within 3 weeks, extending axons along the mossy fiber tract to CA3 and receiving excitatory synaptic input from perforant path afferents[8–13]. Whether these new neurons are subsequently integrated into memory networks is unknown. Previous studies have used irradiation or antimitotic agents such as methylazoxymethanol acetate (MAM) to suppress adult neurogenesis in rodents. Such manipulations impair learning in trace eye-blink[14] and fear[15] protocols, but not in several other hippocampus-dependent tasks[15–17]. Because suppression of adult neurogenesis after these manipulations is typically incomplete[18,19], learning may be supported by either residual adult-generated neurons or even existing granule cells[1].

To circumvent this issue, we used immunohistochemical approaches to visualize the recruitment of new neurons into circuits supporting water maze memory in intact mice. We labeled adult-generated granule cells with the proliferation marker 5-bromo-2′-deoxyuridine (BrdU)[20,21]. To identify dentate gyrus neurons processing spatial information, we quantified expression of the immediate-early genes *c-fos* (also known as *Fos*) and *Arc* (encoding the activity-regulated cytoskeletal-associated protein), after behavioral testing[22]. A major advantage of the water maze is that training in the hidden version of this task produces stable, long-lasting spatial memories in mice[23]. In addition, unlike with some forms of hippocampal learning such as contextual fear[24] or trace eye-blink[25] conditioning, the expression of this memory is continuously dependent on the hippocampus, including the dentate gyrus[23,26–28]. Our experiments reveal that adult-generated neurons are recruited into dentate gyrus circuits supporting spatial memory in an age-dependent manner. Furthermore, we show that by the time new neurons are 4–8 weeks of age, they are preferentially recruited into circuits supporting spatial memory compared with existing granule cells.

RESULTS

Recruitment of new neurons into spatial memory networks

Changes in the expression of immediate-early genes such as *c-fos* are correlated with neuronal firing, and therefore they can be used as neuronal activity markers in the brain[22]. In preliminary studies, we first established that Fos expression is limited to neurons (**Supplementary Results** and **Supplementary Fig. 1** online) and is selectively upregulated in the dentate gyrus after both water maze training and testing (**Supplementary Results** and **Supplementary Fig. 2** online).

We next examined whether adult-generated granule cells are incorporated into spatial memory circuits in the dentate gyrus. Mice were initially treated with BrdU and different groups of mice were then trained in the water maze either 1, 2, 4, 6 or 8 weeks later. Spatial memory was tested in all groups of mice 10 weeks after BrdU treatment (**Fig. 1a**). Mice were killed 90 min after this test, and Fos and BrdU expression were quantified using immunohistochemical approaches. We predicted that if BrdU-labeled neurons are incorporated into dentate gyrus circuits supporting spatial memory at the time of training, then there should be Fos expression in $BrdU^+$ cells following spatial memory testing.

Over the course of water maze training, mice required progressively less time to escape to the platform, and escape latencies did not differ between groups (data not shown). This training produced stable and long-lasting spatial memory in all groups: mice showed a strong spatial bias for the area of the pool where the platform was located during training in each of the three probe tests (**Fig. 1b**; **Supplementary Results** and **Supplementary Fig. 3** online). After the probe test, we identified many Fos^+ and $BrdU^+$ cells in the dentate gyrus (**Fig. 1c,d**).

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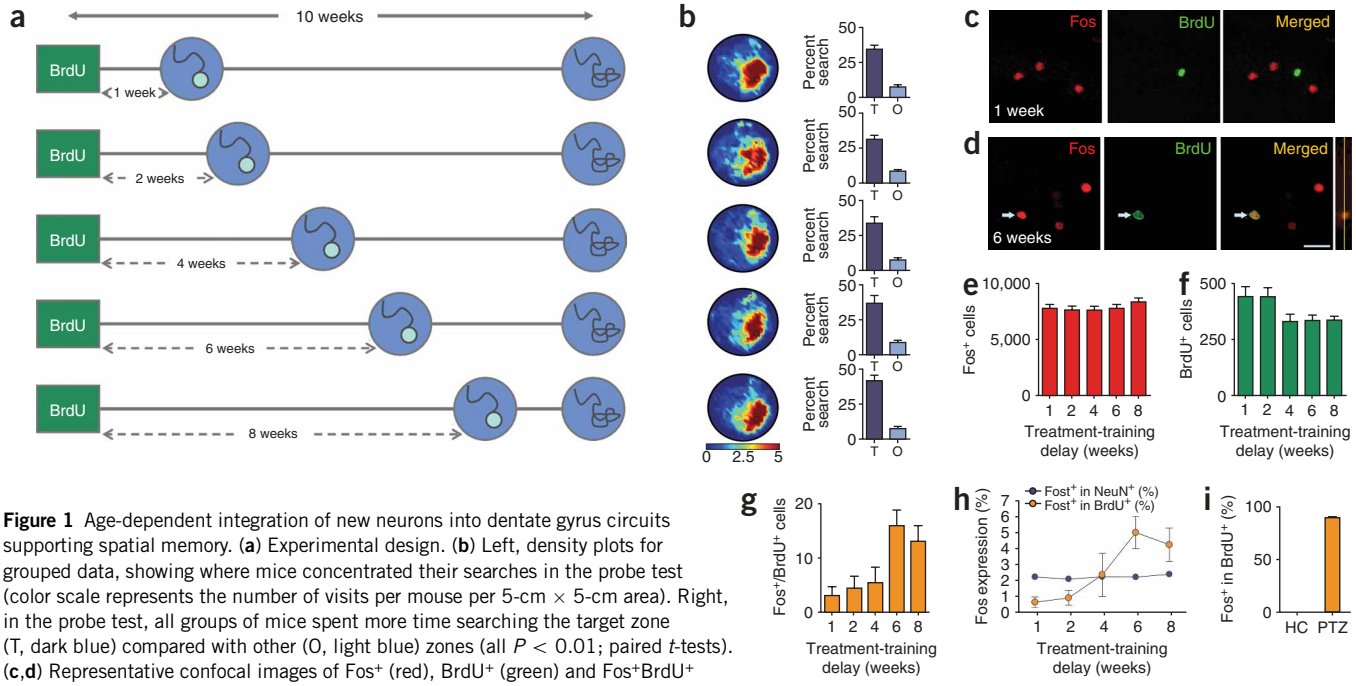

Figure 1 Age-dependent integration of new neurons into dentate gyrus circuits supporting spatial memory. (**a**) Experimental design. (**b**) Left, density plots for grouped data, showing where mice concentrated their searches in the probe test (color scale represents the number of visits per mouse per 5-cm × 5-cm area). Right, in the probe test, all groups of mice spent more time searching the target zone (T, dark blue) compared with other (O, light blue) zones (all $P < 0.01$; paired *t*-tests). (**c**,**d**) Representative confocal images of Fos$^+$ (red), BrdU$^+$ (green) and Fos$^+$BrdU$^+$ (arrow) cells in the dentate gyrus following probe testing in the 1-week (**c**) and 6-week (**d**) groups (scale bar, 20 μm). (**e**) After probe testing, similar numbers of Fos$^+$ cells were identified in the dentate gyrus in all groups ($P > 0.05$; ANOVA). (**f**) Numbers of BrdU$^+$ cells in the dentate gyrus were higher in the 1-week and 2-week groups ($P < 0.05$; ANOVA). (**g**) The number of Fos$^+$BrdU$^+$ cells increased as the treatment-training interval lengthened ($P < 0.01$; ANOVA). (**h**) Fos expression was elevated in BrdU$^+$ (orange) cells relative to NeuN$^+$ (blue) cells in groups of mice with longer delays between BrdU treatment and training ($P < 0.01$; ANOVA, significant delay × cell type interaction). The probability of Fos expression was higher in BrdU$^+$ cells than in NeuN$^+$ cells only in the 6- and 8-week groups (all $P < 0.05$; Fishers LSD test). (**i**) Ten weeks after BrdU treatment, Fos expression in BrdU$^+$ cells was much greater in PTZ-treated mice compared with home cage (HC) control mice ($P < 0.001$; ANOVA). Error bars represent ± s.e.m.

Fos expression was similar in all groups (**Fig. 1e**), consistent with the idea that activity in these neurons is always required for the expression of water maze memory[23,26,27]. We also identified BrdU$^+$ cells in the dentate gyrus in all groups of mice. These numbers of BrdU$^+$ cells were higher in the mice trained 1 or 2 weeks after BrdU treatment (**Fig. 1f**), indicating that exercise, learning or enrichment associated with water maze training promotes survival of 1–2-week-old neurons, as previously reported[29–31]. Most notably, the overlap between the Fos$^+$ and BrdU$^+$ populations depended on the time of training. As the delay between BrdU treatment and water maze training lengthened, the number of Fos$^+$BrdU$^+$ cells increased, suggesting that as adult-generated granule cells mature, they are increasingly likely to be incorporated into dentate gyrus circuits supporting spatial memory (**Fig. 1g**). Because Fos is only expressed in neurons after behavioral testing (**Supplementary Results** and **Supplementary Fig. 1**), all double-labeled cells identified in the dentate gyrus are neuronal.

The design used here rules out several alternative interpretations of these data. First, mice were the same age at the time of BrdU treatment, and so age-dependent differences in the proliferation and differentiation of adult-generated granule cells cannot account for differences in Fos expression in these new neurons[32]. Second, the fixed time between BrdU treatment and mouse death ensured that all BrdU$^+$ cells were the same age at the time of death, and so developmental stage cannot account for differences in Fos expression in these new neurons. Third, mice in each group underwent exactly the same experience before death, and therefore group differences in numbers of Fos$^+$BrdU$^+$ cells are specifically related to spatial memory processing and not related to nonspecific aspects of the probe tests (for example, swimming or physical exercise, stress or arousal).

New neurons are preferentially recruited

To determine the age at which these adult-generated granule cells are incorporated into spatial memory networks, we next estimated the total number of neurons in the granule cell layer by counting the number of cells expressing the mature neuronal marker neuronal-specific nuclear protein, NeuN. This analysis indicated that the probability of existing granule cells expressing Fos after a probe test ranged between 2.1% and 2.4% across groups (**Fig. 1h**). In mice in the 1- and 2-week groups, the probability of Fos expression in BrdU$^+$ cells was lower than in the mature neuron population, suggesting suboptimal recruitment of these new neurons at this age. In the 4-week group, the amount of Fos expression in the BrdU$^+$ population was similar to that of mature neurons, indicating that by 4 weeks of age, new neurons are recruited into spatial memory networks in the dentate gyrus. Perhaps most notably, by the time new neurons are 6–8 weeks of age, they are as much as twice as likely as mature neurons to be recruited into spatial memory networks. This preferential recruitment was most pronounced in the innermost portion of the dentate gyrus (**Supplementary Results** and **Supplementary Fig. 4** online), where the vast majority of BrdU$^+$ cells were located[33]. This raises the possibility that there are regional differences in the propensity of new granule cells to be recruited into spatial memory circuits. Throughout the granule cell layer, Fos expression in BrdU$^+$ cells after probe tests was higher than in home-cage control mice and much lower than in mice treated with the chemical convulsant pentylenetetrazol (PTZ) (**Fig. 1i**).

These data show that as new neurons mature, they become preferentially recruited into spatial memory networks. In all groups, Fos expression was quantified 10 weeks after BrdU treatment. At this time, approximately 90% of BrdU$^+$ cells in the granule cell layer are neuronal

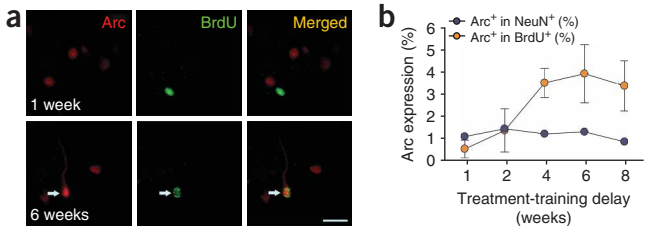

Figure 2 Induction of Arc in new neurons. (**a**) In Experiment 1, the overlap between Arc$^+$ and BrdU$^+$ populations depended on the time of training. Representative confocal images of Arc$^+$ (red), BrdU$^+$ (green) and Arc$^+$BrdU$^+$ (arrow) cells in the dentate gyrus following probe testing in either the 1-week or 6-week groups are shown (scale bar, 20 μm). (**b**) Comparison of Arc expression in mature (NeuN$^+$, blue) versus adult-generated (BrdU$^+$, orange) granule cells. Arc expression was elevated in BrdU$^+$ cells relative to NeuN$^+$ cells in groups of mice with longer delays between BrdU treatment and training ($P < 0.05$; ANOVA, significant delay × cell type interaction). The probability of Arc expression was higher in adult-generated (BrdU$^+$) cells compared to mature (NeuN$^+$) cells only in the 4-week, 6-week and 8-week groups (all $P < 0.05$; Fishers LSD test). Error bars represent ± s.e.m.

(**Supplementary Results** and **Supplementary Fig. 5** online). As Fos expression is limited to neurons, our analyses likely underestimate the recruitment of new neurons into spatial memory networks in the dentate gyrus. Nevertheless, this preferential recruitment suggests that these neurons have a competitive advantage compared with existing granule cells, and, consistent with this, immature neurons have a lower threshold for long-term potentiation and are more excitable than mature granule cells[5,34,35].

Arc induction in new neurons

To evaluate whether this temporally specific pattern would apply to another marker of neuronal activation, we examined *Arc* expression in the same mice. Activity that is associated with both memory formation and memory retrieval induces robust Arc expression in neurons[36–38] (**Supplementary Results** and **Supplementary Fig. 1**). Like Fos expression, Arc expression likely mediates the stabilization of new and reactivated memory circuits[22,36–38]. Similar to our results with Fos, we found that the overlap between the Arc$^+$ and BrdU$^+$ populations depended on the time of training (**Fig. 2a**). The percentage of BrdU$^+$ cells expressing Arc increased as the delay between BrdU treatment and training increased (**Fig. 2b**). In the 2-week group, Arc expression was similar in BrdU$^+$ and existing cells, suggesting that recruitment of new neurons into spatial memory networks in the dentate gyrus may occur as early as 2 weeks. However, in mice in the 4–8-week groups, there was a threefold increase in Arc expression in BrdU$^+$ cells relative to the existing granule cell population, indicating that maximal recruitment does not occur until later on.

We confirmed this preferential recruitment in an additional experiment where the delay between BrdU treatment and training was varied, but the delay between training and testing was kept constant (**Supplementary Results** and **Supplementary Fig. 6** online). These data support the conclusion that as adult-generated granule cells mature, they are increasingly likely to be incorporated into dentate gyrus circuits supporting spatial memory. The data confirm that as new neurons mature, they are preferentially recruited. Finally, these analyses indicate that our effects apply to another immediate-early gene and are thus not specific to *c-fos*.

Recruitment is specific to the dentate gyrus

The subgranular zone is one of two major neurogenic regions in the brain. Olfactory precursor cells divide in the subventricular zone and new neurons migrate via the rostral migratory stream to the granule olfactory bulb[4,39]. Because neurons in the olfactory bulb would not be expected to participate in spatial memory, we next examined the pattern of Fos and BrdU expression in the olfactory bulb in the same mice.

After the probe test, we found many Fos$^+$, BrdU$^+$ and Fos$^+$BrdU$^+$ cells in the granule cell layer of olfactory bulb (**Fig. 3a–c**). However, the probability of Fos expression in BrdU$^+$ cells did not differ between groups (**Fig. 3d**), indicating that the age-dependent recruitment of new neurons into spatial memory networks was specific to the dentate gyrus.

One-week-old neurons are not transiently recruited

The low number of double-labeled neurons in the 1-week group suggests that new neurons are not recruited into spatial memory networks at this young age. An alternative possibility, however, is

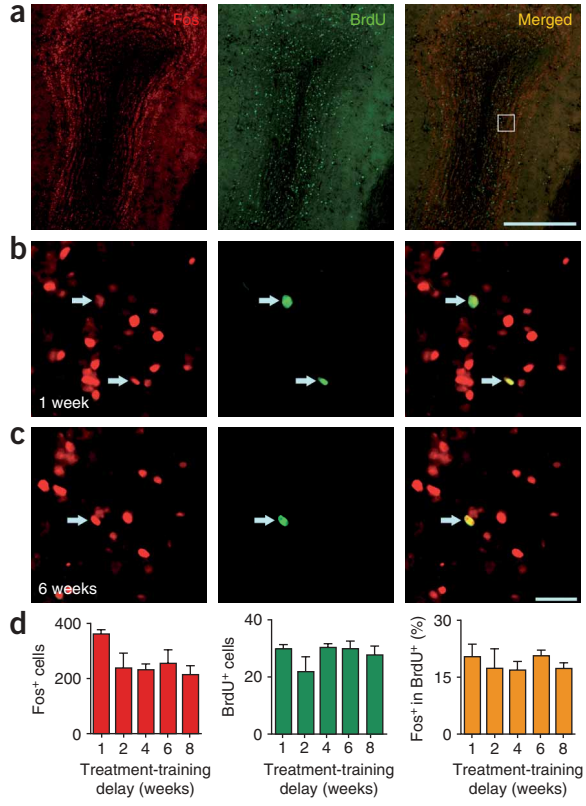

Figure 3 Age-dependent integration of new neurons into circuits supporting spatial memory does not occur in the olfactory bulb. (**a**) Low-magnification examples of Fos immunofluorescence, BrdU immunofluorescence and merged images of the olfactory bulb (scale bar, 1 mm). (**b**,**c**) High-magnification confocal images of the olfactory bulb showing Fos$^+$, BrdU$^+$ and Fos$^+$BrdU$^+$ cells following probe tests in either the 1-week (**b**) or 6-week (**c**) groups (scale bar, 20 μm). (**d**) Quantification revealed similar numbers of Fos$^+$ and BrdU$^+$ cells in both groups (all $P > 0.05$, ANOVA). Notably, the probability of BrdU$^+$ cells expressing Fos did not vary as a function of treatment-training interval ($P > 0.05$, ANOVA), indicating that age-dependent recruitment of new neurons into spatial memory networks is specific to the dentate gyrus. Cells counts are per 100-μm × 100-μm area. Error bars represent ± s.e.m.

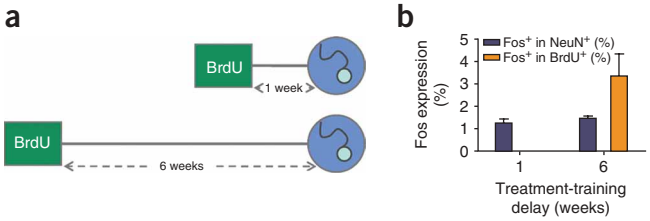

Figure 4 Are 1-week-old neurons transiently incorporated into dentate gyrus circuits supporting spatial memory? (**a**) Mice were trained either 1 week or 6 weeks following BrdU treatment, and Fos and BrdU expression was quantified after the third day of training. (**b**) The probability of Fos expression in the mature neuronal population (NeuN$^+$) was similar in both groups. However, Fos expression in BrdU$^+$ cells depended on the delay between BrdU treatment and training ($P < 0.01$; ANOVA, significant delay × cell type interaction). In the 6-week group, Fos expression in the BrdU$^+$ cells was significantly greater than in the NeuN$^+$ cells ($P < 0.05$, Fishers LSD test), indicating that these cells are preferentially activated following water maze training. Error bars represent ± s.e.m.

that these new neurons are transiently incorporated into spatial networks, but do not subsequently survive and therefore cannot be detected at later time points[2,29]. To evaluate this possibility, we trained mice either 1 week or 6 weeks after BrdU treatment and examined Fos expression induced by training alone (**Fig. 4a**). Following training, many Fos$^+$BrdU$^+$ cells were identified in the 6-week group. In contrast, no Fos$^+$BrdU$^+$ cells were identified in the 1-week group (**Fig. 4b**), suggesting that 1-week-old neurons are not transiently recruited into spatial memory networks in the dentate gyrus. Notably, Fos expression in the mature neuron population in the dentate gyrus was similar in both groups of mice. Furthermore, in the 6-week group, Fos expression in the BrdU$^+$ cell population exceeded that in the mature neuron population, indicating that 6-week-old neurons are preferentially activated during water maze training, consistent with our first experiment.

The lack of recruitment of 1-week-old neurons was confirmed in an additional experiment where spatial memory was tested 1 d after the completion of training. Under these conditions, we found a complete absence of Fos$^+$BrdU$^+$ cells following the probe test (**Supplementary Results** and **Supplementary Fig. 7** online). Together, these results indicate that 1-week-old neurons do not become functionally integrated into spatial memory circuits in the dentate gyrus, not even transiently.

Recruitment depends on the BrdU treatment-training delay

Our results suggest that as adult-generated neurons mature, they become increasingly likely to be incorporated into dentate gyrus networks supporting spatial memory. In the first series of experiments, however, a fixed 10-week survival period was used, and therefore retention delays varied across groups. To control for this potential confound, we trained three additional groups of mice and then tested retention 4 weeks later. These mice were treated with BrdU either 6 weeks before, 1 week before or 1 week after the start of water maze training (**Fig. 5a**). During the retention test, all mice searched selectively in the region of the pool that formerly contained the escape platform (**Fig. 5b**). Although Fos$^+$ and BrdU$^+$ cells were identified in the dentate gyrus in all groups, significant overlap between these two populations was only observed in the mice trained 6 weeks after BrdU treatment. In contrast, there were significantly fewer Fos$^+$BrdU$^+$ cells in groups of mice that were treated with BrdU either 1 week before or 1 week after training (**Fig. 5c**). These results confirm that the degree of overlap between the Fos$^+$ and BrdU$^+$ populations is dependent on the delay between BrdU treatment and training, and not on the delay between training and testing. They further indicate that once recruited, adult-generated neurons are stably maintained in spatial memory networks for at least 4 weeks.

In addition, the mice which were treated with BrdU post-training provide an ideal control for Fos expression in BrdU$^+$ cells associated with non-mnemonic aspects of the probe test (such as swimming or physical exercise, stress or arousal). In these mice there were very few Fos$^+$BrdU$^+$ cells, indicating that these nonspecific aspects of the testing procedure do not significantly contribute to gene expression in these new neurons in the dentate gyrus, and furthermore, that these new neurons are not simply more likely to be activated during memory retrieval. There were similarly few Fos$^+$BrdU$^+$ cells in mice trained 1 week after BrdU treatment. The fact that the numbers of Fos$^+$BrdU$^+$ cells in groups of mice treated with BrdU either 1 week before or 1 week after training were similar suggests that there must be very little recruitment of 1-week-old neurons into spatial memory networks in the dentate gyrus.

Fos expression correlates with memory strength

To more closely examine the relationship between memory strength and Fos expression, we next trained mice that have deficits in spatial learning. To do this, we used mice carrying a point mutation of the αCaMKII gene (T286A), which abolishes this enzyme's ability to autophosphorylate[40]. In this experiment, CaMKII^{T286A} homozygous mice and their wild-type littermate controls were treated with BrdU and trained 6 weeks later (**Fig. 6a**). CaMKII^{T286A} homozygous mice were impaired in a probe test 1 d after training (**Fig. 6b**), which is consistent with the idea that autophosphorylation of CaMKII at the Thr286 residue is important for spatial learning[40]. The learning impairments in CaMKII^{T286A} homozygous mice are likely to be due to deficits in synaptic plasticity throughout the hippocampus (and possibly other forebrain regions). In contrast, similar numbers of BrdU$^+$ cells were identified in wild-type and CaMKII^{T286A} homozygous mice (**Fig. 6c**),

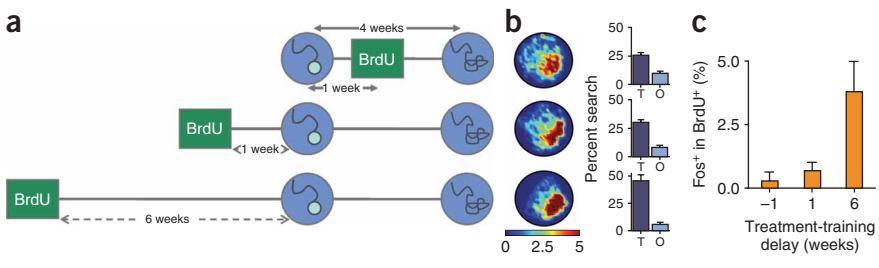

Figure 5 Overlap between Fos$^+$ and BrdU$^+$ populations depends on the delay between BrdU treatment and training. (**a**) In this experiment, we varied the delay between BrdU treatment and training, although the retention delay was fixed at 4 weeks. (**b**) Performance in probe tests was similar in all groups. Left, density plots for grouped data, showing where mice concentrated their searches in the probe test. Right, in the probe test, all groups of mice spent more time searching the target zone (T, dark blue) compared with other (O, light blue) zones (all $P < 0.001$; paired t-tests). (**c**) Following the probe test, the percentage of BrdU$^+$ cells expressing Fos was greatest in the mice trained 6 weeks after BrdU treatment ($P < 0.01$; ANOVA). Error bars represent ± s.e.m.

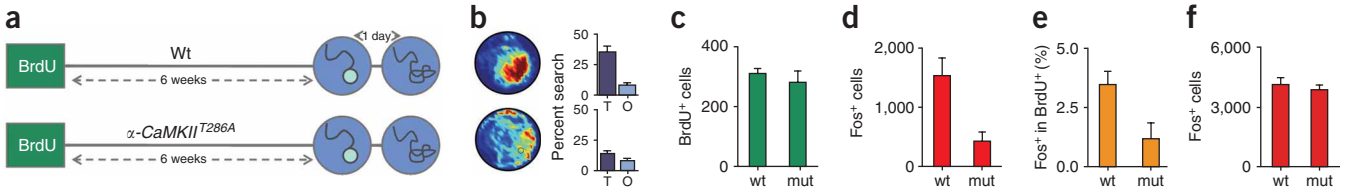

Figure 6 Fos expression in the dentate gyrus is correlated with memory strength. (**a**) To explore the relationship between Fos expression and memory strength, α-*CaMKII*^{T286A} mice and their wild-type (wt) littermate controls were trained in the water maze 6 weeks after BrdU treatment. Spatial memory was tested 1 d later. (**b**) In the probe test, wild-type mice searched more selectively than α-*CaMKII*^{T286A} mice (density plot, left). Although both groups of mice spent more time searching the target zone (T, dark blue) compared with other (O, light blue) zones, wild-type mice spent significantly more time in the target zone compared to α-CaMKII^{T286A} mice ($P < 0.05$; ANOVA, significant genotype × zone interaction). (**c**) The number of BrdU$^+$ cells in the dentate gyrus was similar in wild type and α-CaMKII^{T286A} mutant (mut) mice ($P > 0.05$; ANOVA). (**d**) Following the probe test, the number of Fos$^+$ cells was reduced in α-CaMKII^{T286} mutants (mut) relative to wild-type controls ($P < 0.05$; ANOVA). (**e**) Similarly, the percentage of BrdU$^+$ cells expressing Fos was reduced in α-CaMKII^{T286} mutants (mut) relative to wild-type controls ($P < 0.05$; ANOVA). (**f**) Fos expression in the dentate gyrus was similar in wild type and α-CaMKII^{T286A} mutant (mut) mice following restraint stress ($P > 0.05$; ANOVA). Error bars represent ± s.e.m.

suggesting that adult neurogenesis was normal in the CaMKII^{T286A} homozygous mice (although it is still possible that maturation of new neurons is affected). Consistent with the learning deficits, the numbers of both Fos$^+$ (**Fig. 6d**) and Fos$^+$BrdU$^+$ (**Fig. 6e**) cells in the dentate gyrus were reduced by approximately two-thirds in the CaMKII^{T286A} homozygous mice. This reduction in numbers of Fos$^+$ and Fos$^+$BrdU$^+$ cells was not due to an overall disruption of Fos regulation in CaMKII^{T286A} homozygous mice: induction of Fos after restraint stress was normal in these mutants (**Fig. 6f**).

In this experiment, wild-type and CaMKII^{T286A} homozygous mice were subjected to the same behavioral procedures and showed similar levels of adult neurogenesis and similar regulation of Fos expression following restraint stress. Therefore, the finding that the probe test fails to induce Fos in BrdU$^+$ cells in CaMKII^{T286A} homozygous mice strengthens the support for a relationship between activation of new neurons and spatial memory processing. In addition, it provides further evidence that nonspecific aspects of the test procedure (such as swimming or physical exercise, stress or arousal) do not contribute to Fos expression in both new and existing neurons in the dentate gyrus.

DISCUSSION

The continuous generation and integration of new neurons in the dentate gyrus has led to the idea that these adult-generated neurons have a function in memory. Using immunohistochemical approaches to visualize new neurons in intact animals, we showed that these new neurons are incorporated into dentate gyrus circuits supporting spatial memory in an age-dependent manner. Significant recruitment of new neurons did not occur until they were at least 2 weeks old, and beyond 4 weeks new neurons were more likely than mature neurons to be recruited into dentate gyrus circuits supporting spatial memory. This preferential recruitment is consistent with a decreased threshold for plasticity in new neurons[5,34,35] and supports the idea that new neurons make a unique contribution to memory processing in the dentate gyrus[1]. Previous studies showed that spatial learning enhances the survival of 1–2-week-old neurons, perhaps because it promotes integration of these adult-generated neurons into dentate gyrus circuits[29]. However, our results indicate that maximal integration of new neurons into spatial memory–related circuits does not occur until much later, suggesting that integration into existing dentate gyrus circuitry and subsequent integration into memory-related circuits are distinct processes.

To identify BrdU-labeled cells processing spatial memory, we quantified the expression of two immediate-early genes, *c-fos* and *Arc*. The expression profiles for Fos and Arc were remarkably similar following water maze testing. First, the expression of both Fos and Arc was limited to neurons. Second, consistent with the idea that spatial information is sparsely encoded in the dentate gyrus[41], we found that both Fos and Arc were expressed in a relatively small proportion of mature neurons following water maze testing[36]. Previous studies used similar immunohistochemical approaches to show that adult-generated granule cells can be activated by a variety of stimulation, including high-frequency electrical perforant path stimulation[42], kainate-induced seizure activity[43], water maze training[43] and spatial exploration[44]. Although these latter two behavioral studies establish that adult-generated granule cells respond to environmental stimulation, they cannot distinguish between gene expression related to memory encoding and gene expression associated with nonspecific aspects of the task (for example, swimming or physical exercise, stress or arousal). Notably, these studies do not address whether new neurons are stably incorporated into memory circuits.

We addressed this question by examining the activation of BrdU-labeled cells following recall of a spatial memory. Six key observations establish a role for these new neurons in spatial memory. First, activation of new neurons depended on behavioral expression of the spatial memory. Second, this activation was reduced in mutant mice that were unable to form a spatial memory. Third, activation of new neurons was not associated with nonspecific aspects of the probe test, such as swimming or physical exercise, stress or arousal. Fourth, activation depended on the age of new neurons at the time of training (that is, the delay between BrdU treatment and training, rather than the delay between training and testing) and did not simply reflect a lower threshold for activation at the time of retrieval. Fifth, this pattern of results was observed only in the dentate gyrus and did not extend to other brain regions that do not have a role in spatial memory. Finally, we found that new neurons were stably recruited into spatial memory circuits in the dentate gyrus, consistent with an enduring role for the hippocampus in the expression of water maze memories[23,26,27,45].

These experiments reveal that as adult-generated granule cells mature, they are increasingly likely to be recruited into spatial memory circuits in the dentate gyrus. This age-dependent recruitment tracks the morphological and physiological maturation of new neurons[3–6,11,12,33]. After migrating into the granule cell layer, adult-generated neurons initially extend axons into CA3 and dendritic processes into the molecular layer[8–10]. At this stage, immature neurons may receive GABA-mediated excitatory synaptic inputs[11,12,46]. However, major glutamatergic synaptic activation from perforant path afferents does not occur until new neurons are 2 (ref. 12) or more[11] weeks old, when spines begin to form[10]. Consistent with this, our data indicate that significant recruitment does not occur until new neurons reach 2 weeks

of age, and by the time new neurons are 4–8 weeks of age they are more likely than existing granule cells to be recruited into spatial memory circuits. This preferential recruitment was evident during acquisition, as well as during recall, and in experiments using either *c-fos* or *Arc* as markers of neuronal activation. Consistent with the idea that these new neurons are competitively advantaged compared to existing granule cells, by the time new neurons are 3 weeks or older, they have a lower threshold of activation and a lower threshold for inducing long-term potentiation relative to existing granule cells[34,35]. These distinct physiological characteristics likely promote integration into spatial memory circuits, and a recent study suggests that new neurons may maintain this competitive advantage for several months[44].

Previous studies have used irradiation or antimitotic agents such as MAM to suppress adult neurogenesis in rodents. Such manipulations have impaired learning in trace eye-blink[14] and fear[15] paradigms. Similar treatments, however, do not prevent learning in other hippocampus-dependent tasks[16]. For example, suppression of adult neurogenesis does not prevent acquisition in the Morris water maze[15,17] (although long-term retention may be affected[17]). Several issues have complicated the interpretation of these ablation-based approaches. First, these treatments have typically targeted relatively immature neurons (for example, less than 2 weeks of age[14,15]), an age at which new neurons are not yet integrated into dentate gyrus circuitry[10]. The current experiments suggest maximal recruitment does not occur until at least 4 weeks of age. Second, suppression of adult neurogenesis following irradiation or MAM treatment is typically incomplete[18,19], and therefore it is possible for learning to be supported by residual adult-generated neurons. Third, even complete suppression of adult-generated granule cells may not prevent learning, because learning may be supported by existing granule cells[1]. Rather, memories formed without the contribution of new neurons may contain degraded temporal information[1], be more prone to interference[47,48] or be less durable[17]. Here we circumvented these issues by using immunohistochemical approaches to visualize the recruitment of new neurons into spatial memory networks in intact mice. We identified a window in which new neurons are preferentially recruited into spatial memory circuits. The preferential recruitment of adult-generated granule cells suggests that they make a unique, but as yet unspecified, contribution to memory processing in the dentate gyrus[1].

METHODS

Mice. Male offspring from a cross between C57Bl/6NTacfBr (C57B6) and 129Svev (129) mice (Taconic) were used in the majority of these experiments. In one experiment, we used α-CaMKII^{T286A} knock-in mice, generated previously[40]. The α-CaMKII^{T286A} mutation has been maintained in a C57B6 background (ten or more generations). To generate experimental mice, α-CaMKII^{T286A} heterozygous mice in the C57B6 background were first crossed with 129 mice to obtain F1 hybrid mice. F1 heterozygous mice were then crossed again to obtain F2 hybrid mice. Genotypes were determined by PCR analysis of tail DNA samples, and only male wild-type and α-CaMKII^{T286A} homozygous mice were used in the experiments. All mice were bred in our colony at The Hospital for Sick Children, and mice were maintained on a 12 h light/12 h dark cycle with free access to food and water. Mice were at least 8 weeks of age at the start of experiments, and behavioral procedures were conducted during the light phase of the cycle. Experiments were conducted blind to the treatment condition of the mouse and according to protocols approved by the Animal Care Committee at The Hospital for Sick Children.

BrdU administration. BrdU was dissolved in 0.1 M phosphate-buffered saline, pH 7.4 (PBS), and heated to 50–60 °C. BrdU was injected intraperitoneally for 5 d according to the specific experimental protocols described below.

Water maze and general training procedures. The apparatus and behavioral procedures have been previously described[23]. Behavioral testing was conducted in a circular water maze tank (120 cm in diameter, 50 cm deep), located in a dimly lit room. The pool was filled to a depth of 40 cm with water made opaque by adding white, nontoxic paint. The water temperature was maintained at 28 ± 1 °C by a heating pad located beneath the pool. A circular escape platform (10 cm in diameter) was submerged 0.5 cm below the water surface, in a fixed position in one of the quadrants. The pool was surrounded by curtains; hung at least 1 m from the perimeter of the pool. The curtains were white and had distinct cues painted on them.

Before the commencement of training, mice were individually handled for 2 min per d over 7 consecutive days. In the majority of experiments, mice were trained over 5 d. On each training day, mice received six training trials (presented in two blocks of three trials; interblock interval was ~1 h, intertrial interval was ~15 s). On each trial they were placed into the pool, facing the wall, in one of four start locations. The order of these start locations was pseudorandomly varied throughout training. The trial was complete once the mouse found the platform or 60 s had elapsed. If the mouse failed to find the platform on a given trial, the experimenter guided the mouse onto the platform. Following the completion of training, spatial memory was assessed in a series of probe tests with an intertest interval of approximately 3 min. In this test, the platform was removed from the pool, and the mouse was allowed 60 s to search for it. In Experiment 2, Fos and BrdU expression were examined following training. Procedures were as described above, except that mice were trained for 3 d. On the third day of training, mice received only the first block of three trials.

Behavioral data from the training and the probe tests were acquired and analyzed using an automated tracking system (Actimetrics). Using this software, we were able to record a number of parameters during training, including escape latency and swim speed. In probe tests, we quantified performance in two ways. First, we measured the amount of time mice searched the target zone (20 cm in radius, centered on the location of the platform during training) versus the average of the time spent searching three other equivalent zones in other areas of the pool[23]. These zones each represent approximately 11% of the total pool surface. Second, we represented probe test performance as a color map (or density plot), with hot colors corresponding to areas of the pool that were more frequently visited[23].

Experimental procedures. *Experiment 1.* In the first experiment, we determined the age at which adult-generated granule cells are incorporated into spatial memory networks. To label the dividing cell population, mice were initially injected with BrdU for 5 consecutive days (50 mg per kg of body weight; two injections per d). Separate groups of mice were then trained in the water maze for 5 d, either 1 week ($n = 9$), 2 weeks ($n = 7$), 4 weeks ($n = 7$), 6 weeks ($n = 8$) or 8 weeks ($n = 9$) later. Spatial memory was assessed in all mice 10 weeks after BrdU treatment. In this test, mice were given a series of three probe tests. In addition, Fos expression in BrdU$^+$ cells was examined in home cage control mice ($n = 5$) and mice treated with the chemical convulsant, PTZ (50 mg per kg, $n = 2$), 10 weeks after BrdU treatment (50 mg per kg, two injections per d).

Experiment 2. In this experiment we evaluated whether new granule cells are only transiently incorporated into spatial networks in the dentate gyrus. Two groups of mice were trained in the water maze 1 week (n = 8) or 6 weeks (n = 8) after BrdU treatment (100 mg per kg; 5 d, two injections per d). These mice were trained for 3 d. On the third day of training, mice received only the first block of three trials. We chose this abbreviated training schedule because, in our previous experiments, we found that escape latencies reached asymptote by the fourth day of training[23]. Therefore, at this time point significant learning is still occurring and we avoid the high levels of stress typically associated with the first day of training.

Experiment 3. In this experiment, we varied the delay between BrdU treatment and training, although the retention delay between training and testing was fixed at 4 weeks. Mice were treated with BrdU (100 mg per kg; 5 d, two injections per d) either 6 weeks before ($n = 7$), 1 week before ($n = 6$) or 1 week after ($n = 6$) training. On the test day, all mice were presented with a series of three probe tests.

Experiment 4. In this experiment, we examined the relationship between memory strength and Fos expression. Six weeks after BrdU treatment (100 mg per kg; 5 d, two injections per d), α-CaMKII^{T286A} homozygous mice ($n = 8$)

and their wild-type littermates ($n = 8$) were trained in the water maze. One day following the completion of training, spatial memory was tested in a series of three probe tests. Additionally, Fos expression was examined following restraint stress in wild-type ($n = 3$) and α-CaMKII^{T286A} homozygous ($n = 3$) mice. Mice were placed in a well-ventilated Plexiglas cylinder for 60 min (volume, 60 cm^3) and perfused 60 min later.

Tissue preparation. Ninety minutes following the completion of behavioral testing, mice were deeply anesthetized and perfused transcardially with PBS and then 4% paraformaldehyde (PFA). Brains were removed, fixed overnight in PFA and then transferred to 30% sucrose solution and stored at 4 °C. Coronal sections of 50 μm were cut and divided into four sets. Each of these sets included sections at 200-μm intervals, and covered the full anterior-posterior extent on the dentate gyrus. Free-floating sections were prepared for immunohistochemistry, and additional sets were stored in a PBS solution containing 0.02% sodium azide for later processing.

BrdU, Fos and NeuN immunohistochemistry. For BrdU immunohistochemistry, the BrdU antigen was exposed by incubating the sections in 1 N HCl at 45 °C for 30 min. Immunohistochemistry was performed using primary antibodies to Fos (rabbit anti-Fos polyclonal antibody; 1:1,000, Calbiochem), BrdU (rat anti-BrdU monoclonal antibody; 1:500, Accurate Chemicals), Arc (rabbit anti-arc polyclonal antibody;1:500, generously donated by P. Worley) and NeuN (mouse anti-NeuN Alexa Fluor 488 conjugated; 1:1,000, Chemicon). We used Alexa-488 goat anti-rat, Alexa-568 goat anti-mouse and Alexa-568 goat anti-rabbit (1:500, Molecular Probes) as secondary antibodies. Sections were incubated in primary antibodies overnight at room temperature (20–25 °C) for Fos and BrdU labeling or at 4 °C for Arc and BrdU labeling. Sections were incubated with secondary antibodies and anti-NeuN primary antibody for 2 h at room temperature. Antibodies were diluted in blocking solution containing 2% goat serum, 1% bovine serum albumin and 0.2% Triton X-100 dissolved in PBS. Sections were mounted on slides with Permafluor anti-fade medium (Lipshaw Immunon).

Quantification. We quantified Fos$^+$, BrdU$^+$ and Fos$^+$BrdU$^+$ cells throughout the anterior-posterior extent of the granule cell layer. The average number of Fos$^+$, BrdU$^+$ and Fos$^+$BrdU$^+$ cells per section was then normalized for the entire dentate gyrus by multiplying this average by the number of 50-μm sections corresponding to the entire dentate gyrus[2,19,29,30,43].

Quantification of Fos$^+$cells. The density of Fos$^+$ cells was relatively low (around 2.1–2.4% of mature granule cells expressed Fos following behavioral testing) and therefore it was possible to quantify the number of Fos$^+$ cells from two-dimensional images of the entire dentate gyrus. These were acquired using a 10× objective on a Nikon Eclipse 80i epifluorescence microscope (Nikon Instruments) and Fos$^+$ cells were counted using ImageJ software (National Institute of Health). In a subset of animals we performed equivalent stereological counts of Fos$^+$ cells using a confocal microscope (Olympus IX81 DSU). These yielded similar estimates, verifying the accuracy of our method.

Quantification of BrdU$^+$and Fos$^+$BrdU$^+$cells. Numbers of BrdU$^+$ and Fos$^+$BrdU$^+$ cells were first determined using the epifluorescence microscope with a 40× objective. A subset of BrdU$^+$ and Fos$^+$BrdU$^+$ cells were confirmed with the confocal microscope. To verify that Fos$^+$BrdU$^+$ cells were double-labeled, we collected 20 focal planes, and the resulting three-dimensional image was examined to ensure that the fluorochromes originated from the same cell. Partially labeled or sectioned cells were not counted.

Quantification of NeuN$^+$cells in the dentate gyrus. To estimate the total number of mature neurons in the dentate gyrus, sections were stained for the mature neuronal marker, NeuN. Using a method adapted from a previous report[36], two *z*-stacks per dentate gyrus (one each from the upper and lower blades) were collected from four mice at 40× magnification using the confocal microscope. The total number of NeuN$^+$ cells in each 50-μm stack was counted manually and the total area of the middle plane was measured. From this we calculated the density of NeuN$^+$ cells in the dentate gyrus. From our analysis of Fos$^+$ cells, we then determined the proportion of mature neurons expressing Fos following behavioral testing.

Quantification of Fos$^+$, BrdU$^+$and Fos$^+$BrdU$^+$cells in the olfactory bulb. Three 50-μm sections containing the granule cell layer of the olfactory bulb were selected from each mouse[49]. A confocal microscope was used to quantify Fos$^+$, BrdU$^+$ and Fos$^+$BrdU$^+$ cells using the optical dissector, stereological method[50]. Cells were counted within a single *z*-stack (area, 165 × 225 μm). Cells in the uppermost 5-μm focal plane were not included in counts.

Imaging. Representative images were acquired using either a Nikon Eclipse 80i epifluorescence microscope or an Olympus IX81 DSU confocal microscope. The signal for Alexa-488 labeling was detected using a Nikon HQ FITC filter, and the Alexa-568 labeling was detected using a Nikon HQ R filter. Images were digitized with a Nikon DXM 1200F CCD camera or with a Photometrics CoolSnap CCD camera, respectively.

Statistical analyses. To evaluate whether mice searched selectively in probe tests, paired *t*-tests were performed on the zone (target versus other) data. The effects of experimental manipulations on numbers of Fos$^+$, BrdU$^+$ and Fos$^+$BrdU$^+$ cells were evaluated with ANOVAs. Significant main effects or interactions were followed up with *post hocs* (Fishers LSD test), where appropriate.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

P.W.F., N.K. and C.M.T. conceived the experiments. C.M.T., P.W.F. and A.H.W. conducted the water maze studies, N.K. and C.M.T. conducted the immunohistochemistry and quantification and C.M.T. conducted the statistical analyses. PW.F. supervised the project and wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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