

A ROLE FOR ADULT NEUROGENESIS IN SPATIAL LONG-TERM MEMORY

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Abstract—Adult hippocampal neurogenesis has been linked to learning but details of the relationship between neuronal production and memory formation remain unknown. Using low dose irradiation to inhibit adult hippocampal neurogenesis we show that new neurons aged 4–28 days old at the time of training are required for long-term memory in a spatial version of the water maze. This effect of irradiation was specific since long-term memory for a visibly cued platform remained intact. Furthermore, irradiation just before or after water maze training had no effect on learning or long-term memory. Relationships between learning and new neuron survival, as well as proliferation, were investigated but found non-significant. These results suggest a new role for adult neurogenesis in the formation and/or consolidation of long-term, hippocampus-dependent, spatial memories. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

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The dentate gyrus of the hippocampus is unique in that it is one of two regions in the adult mammalian brain that continues to produce new neurons in adulthood (Altman and Das, 1965; Dayer et al., 2003). This process is modulated by a variety of factors including hippocampal dependent learning and enriched environments (Kempermann et al., 1997; Gould et al., 1999; Ambrogini et al., 2000, 2004; Dobrossy et al., 2003). This is perhaps not surprising as the dentate gyrus is also a critical component of the medial temporal lobe, a memory system responsible for the formation of spatial and episodic memories (Eichenbaum et al., 1999). Lesions to the dentate gyrus are known to produce spatial learning deficits in rats (McNaughton et al., 1989; Gilbert et al., 2001; Xavier et al., 1999; Ferbin-teanu et al., 1999), similar to those seen following hippocampal lesions (Morris et al., 1982; Debiec et al., 2002; Mumby et al., 1999). Furthermore, tetanization of perforant path–granule cells synapses inhibits spatial learning (Moser et al., 1998) and erases existing spatial memories

(Brun et al., 2001), presumably by preventing and disrupting, respectively, the synaptic weight changes required to store memories. Existing studies suggest that adult-born neurons contribute to some forms of hippocampal-dependent learning (Shors et al., 2001, 2002; Madsen et al., 2003; Raber et al., 2004a,b) and are important for the behavioral effects of antidepressants (Santarelli et al., 2003). Behavioral roles for these new neurons might be subserved by synaptic connections which have the plasticity purported to support memory formation (Wang et al., 2000; Snyder et al., 2001; van Praag et al., 2002; Overstreet et al., 2004; Schmidt-Heiber et al., 2004).

Precisely how young neuronal plasticity might be relevant to hippocampal-dependent learning remains speculative. Technical barriers make it difficult to monitor young neuronal synaptic plasticity in behaving animals and even if such information could be obtained it would be open to many interpretations. A more tractable way to relate young neuronal plasticity to learning might be to relate cognitive performance to neuronal proliferation and survival. In rats roughly half of the adult-born granule cell population dies within 1 month (Dayer et al., 2003) and it has been reported that the survival of these neurons can be modulated by hippocampal-dependent forms of learning (Gould et al., 1999; Ambrogini et al., 2000, 2004). Spatial learning has also been found to exert bidirectional affects on the proliferation of granule neurons (Dobrossy et al., 2003). If new neurons are substrates for learned information, their addition or removal, in response to learning, may reveal insight into potential functional roles. Of course, memories that initially depend on the hippocampus may later be consolidated in cortical structures independent of, or in addition to, the hippocampus (McClelland et al., 1995; Rosenbaum et al., 2001; Debiec et al., 2002; Frankland et al., 2004; Maviel et al., 2004). Thus, relationships between neuron addition and the medial temporal lobe memory formation process need to be understood. For example, do young neurons contribute preferentially to either the acquisition or (re)consolidation of memories? How does activity-regulated neuronal survival correlate with the lifespan of a memory or its dependence on the hippocampus? By answering these questions we can determine the importance and role of adult hippocampal neurogenesis in normal, affected, and clinical situations.

Here, we examined the contribution of adult neurogenesis to learning the hippocampal-dependent Morris water maze (Morris et al., 1982) and examined the effect of maze learning on the survival and proliferation of dentate granule neurons. The following groups of male Long Evans rats were used: learners, irradiated learners (IRR-learners),

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Abbreviations: BrdU, 5'-bromo-2'-deoxyuridine; CaBP, calbindin; fEPSP, field excitatory post-synaptic potential; GCL, granule cell layer; IRR-learners, irradiated learners; LTP, long-term potentiation; MWM, Morris water maze; SGZ, subgranular zone.

and cage controls. IRR-learners were treated with 10 Gy whole brain irradiation at 40–41 days of age to eliminate adult neurogenesis. This allowed us to assay the contribution of adult-born neurons to short- and long-term memory in the water maze (learners vs. IRR-learners). All groups were injected with 5'-bromo-2'-deoxyuridine (BrdU) 1 week prior to maze training in order to assess the effect of irradiation on neurogenesis (IRR-learners vs. learners/cage controls) and learning on neurogenesis (learners vs. cage controls).

EXPERIMENTAL PROCEDURES

Animals and timeline

Six to eight male Long Evans rats (Charles River, Saint-Constant, QC, Canada) comprised each group of IRR-learners, learners, or cage controls, except the 1-week learner group in which 13 rats were used. Rats were housed on a 12 h light/dark cycle with lights on from 7:00 am to 7:00 pm. Beginning at 40 days of age rats were irradiated (IRR-learners) or sham-irradiated (learners and cage controls) to eliminate adult neurogenesis in the dentate gyrus (Monje et al., 2002). Four weeks later, when the number of neurons aged 4 weeks and younger will be virtually eliminated, rats began training in the hippocampal-dependent Morris water maze (MWM; Morris et al., 1982). Previous experiments, which have blocked neurogenesis to reveal changes in hippocampal function, have done so for 2–4 weeks prior to investigation (Shors et al., 2001, 2002; Snyder et al., 2001; Madsen et al., 2003; Santarelli et al., 2003). Either 1, 2, or 4 weeks after maze training separate groups of rats returned to the water maze for a probe trial to test retention of the previously learned information. The day following probe testing animals were killed for immunohistochemical and electrophysiological analysis. In additional experiments rats were irradiated at 64–65 days of age or 74–75 days of age (i.e. just before or after water maze training). Cage controls remained in their cages throughout the behavioral training but were otherwise age and time matched in their treatments (sham irradiation, BrdU, time of kill; Fig. 1A). All experiments were approved by the University of Toronto animal care committee and conformed to the National Institutes of Health standards for the humane treatment of animals. All efforts were made to minimize the number of animals used and their suffering.

In order to perform both immunohistochemistry and electrophysiology on some rats a perfusion–fixation technique was used which would allow one hippocampus to be sectioned into live slices. Animals were anesthetized with halothane and intra-cardially perfused with approximately 250 ml oxygenated, ice cold artificial cerebrospinal fluid. The brain was removed and the left hippocampus prepared for electrophysiology (see below). The right hippocampus was then extracted, cut into four pieces to aid fixation, and then placed in 8% paraformaldehyde for 1 week at 4 °C. The hippocampus was then stored in 1 M phosphate-buffered saline (pH 7.2) containing 0.1% sodium azide until immunohistochemical processing.

Irradiation

At 40 days of age IRR-learner rats received 10 Gy γ irradiation to the head, under sodium pentobarbital anesthesia, on 2 consecutive days. The 10 Gy was delivered over approximately 10 min. Irradiation was performed at the University of Guelph veterinary hospital using a Theratron 780-C (AECL Medical, now MDS Nordion; ^{60}Co source). In a dose-response experiment preceding the main study 40 day old rats were irradiated at several doses and proliferation was assessed with BrdU at 68 days of age (200 mg/kg, killed 2 h after injection), the same age at which behavioral testing would begin in the main experiments. Two doses of 10 Gy

was chosen based on its ability to reduce proliferation of subgranular zone precursor (SGZ) cells (418 ± 59 vs. controls: 1904 ± 334) relative to 2×7 Gy (797 ± 92) and 2×5 Gy (953 ± 164) doses (mean \pm S.E.; $n=3$ per group, except controls $n=6$). Shams (learners and cage controls) were transported to the irradiation facility and anesthetized but did not receive irradiation.

BrdU injection paradigm

The thymidine analog BrdU (Sigma) was injected 1 week prior to maze training (3 weeks after irradiation date) in order to label the DNA of cells in S-phase of mitosis. Each rat received two injections (200 mg/kg each), 12 h apart. This dosage ensures that the majority of cells actively dividing in the (SGZ) on that day will be labeled with BrdU (Cameron and McKay, 2001). By injecting at this time point relative to maze training, we aimed to label those cells responsive to hippocampal dependent learning (Gould et al., 1999).

Water maze

Hippocampal dependent spatial learning ability was tested using the MWM (Morris et al., 1982). Rats were placed in a circular pool (184 cm in diameter, 60 cm high, filled to 36 cm) containing a submerged platform in its southeast quadrant. The water was made opaque with non-toxic white paint. The room was 1075 cm long \times 655 cm wide and posters, a computer, and the rack of rat cages served as spatial cues with which the rats could learn the location of the hidden platform. Rats were allowed to remain on the platform for 10 s once they found it or, if after 60 s, a rat failed to find the platform it was placed on the platform by the experimenter. A computer assisted tracking system (VP118; HVS Image) was used for data collection. Rats were trained for 6 days, with eight trials per day and an inter-trial interval of 5 min. With equal frequency, rats were randomly placed at each of the four start positions (north, south, east, west) initially facing the pool wall. At either 1, 2, or 4 weeks after training rats were returned to the pool for a probe trial. The hidden platform was removed and rats were allowed to swim freely for 30 s. The amount of time spent in the quadrant where the platform was previously located (target) relative to the other three quadrants was used as an index of the rats' long term memory capacity.

Another group of rats was trained in a non-spatial, but cued, version of the water maze (Packard and Teather, 1997). For cued training rats received eight trials/day but the location of the hidden platform varied with each trial. The platform location was therefore not predictable based on spatial cues, but by the presence of a cue hanging directly above the platform, 1 foot above the water surface. The cue was a rod, roughly 1 foot long, painted with alternating black and white stripes. The platform was placed in each of the quadrants twice and rats were released from the four start points in a pseudorandom manner such that the number of releases close, and far, from the platform was balanced on each day. Unlike the spatial training, rats were only trained for 5 days. At 3 weeks post-training rats were subjected to a 90 s probe trial where the platform was not present but the cue hung in one of the quadrants.

Immunohistochemistry and quantification

Stereological quantification (West et al., 1991) of surviving neurons was performed by immunofluorescent co-labeling of BrdU and calbindin (CaBP) D-28k, a calcium binding protein found in mature granule neurons (Rami et al., 1987; Kempermann et al., 1997; Gould et al., 1999). The hippocampus was sectioned exhaustively at 40 μm using a vibratome and every 20th section was labeled (10–12 sections per rat). All labeling was performed on free floating sections in PBS containing 0.3% Triton X-100 and 5% normal goat serum and sections were

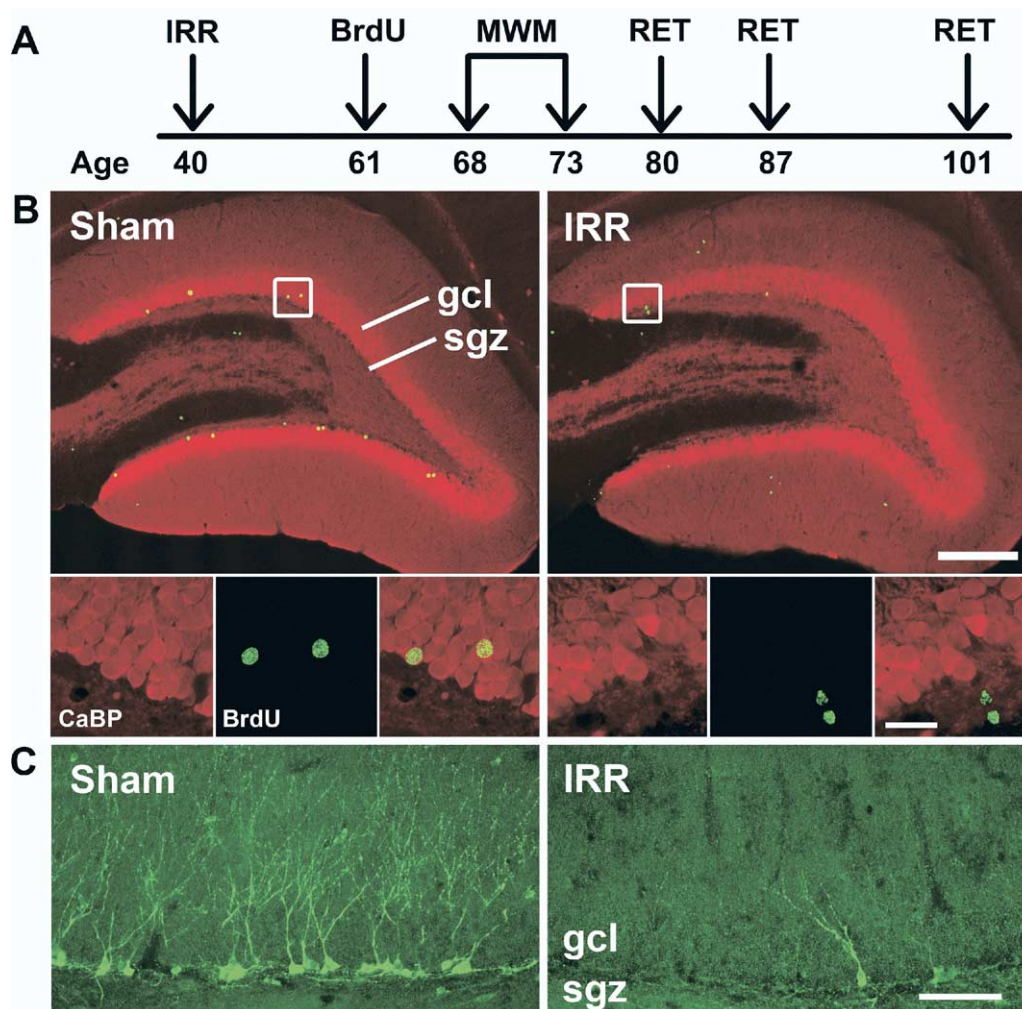


Fig. 1. Irradiation-learning protocol. (A) Experimental timeline as a function of rat age in days. Rats were irradiated (IRR) or sham-irradiated, trained in the MWM and subsequently tested for long-term memory in a probe trial retention test (RET). All rats received BrdU 1 week prior to the start of training. (B) While BrdU+/CaBP+ granule neurons could be found throughout the GCL and SGZ in cage controls, only non-neuronal cells (BrdU+/CaBP-) could be found in IRR-learners. (C) Representative samples of doublecortin stained tissue show that irradiation was effective in reducing neurogenesis throughout the entire experiment. At the time of death sham-irradiated rats typically had many phenotypically young, doublecortin expressing neurons. In irradiated rats these profiles were rare. Scale bar=200 μm for whole dentate gyrus pictures, close-ups=30 μm , doublecortin=100 μm .

washed with PBS before and after the addition of each antibody. Sections were incubated with rabbit anti-CaBP (1:100; Chemicon, Temecula, CA, USA) for 24 h at 4 °C and then Alexa-568 goat anti-rabbit secondary antibody (1:200; Molecular Probes, Eugene, OR, USA) for 2 h at room temperature. Sections were then incubated in 1 N HCl at 45 °C for 40 min to denature DNA and expose BrdU. Rat anti-BrdU (1:200; Accurate Chemical, Westbury, NY, USA) was added for 24 h at 4 °C followed by Alexa-488 goat anti-rat (1:200; Molecular Probes) for 2 h at room temperature. The sections were then washed with PBS, briefly rinsed in distilled water, mounted onto slides, and coverslipped with Permafluor (ThermoShandon, Pittsburgh, PA, USA). In order to assay precursor proliferation sections were also labeled for Ki67 (Kee et al., 2002; Dayer et al., 2003). The labeling procedure was similar to that of CaBP except that sections were labeled with a rabbit anti-Ki67 primary antibody (1:1000; Novocastra, Newcastle upon Tyne, UK) for 48 h instead of anti-CaBP, and were not double labeled with BrdU. To assay the presence of young neurons the microtubule associated protein doublecortin was also labeled, using goat

anti-doublecortin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Alexa-488 donkey anti-goat antibodies (1:200; Molecular Probes).

The number of cells positively labeled for BrdU alone (BrdU+/CaBP-), and BrdU plus CaBP (BrdU+/CaBP+), was determined for each section. Only cells in the combined granule cell layer (GCL) plus SGZ, the proliferative region defined as a 20 μm wide region below the GCL, were counted. Cell counting was performed on a Leica TCS-SL confocal microscope with a 63 \times objective (1.32 NA) using the optical fractionator technique. Each BrdU+ cell was analyzed for co-labeling with CaBP. Initially, in order to determine an efficient sampling scheme, surviving neurons were quantified in three animals from the 1-week learner and 4-week learner groups by sampling every 10th section and every 20th section. Both sampling schemes gave the same results, with equally low variance, and so the latter scheme was chosen (West et al., 1991). Quantification of Ki67 was performed with the same sampling scheme but instead used fluorescence microscopy.

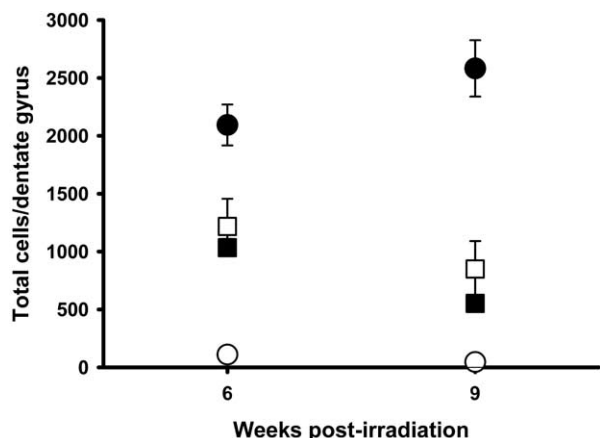


Fig. 2. Irradiation eliminates adult neurogenesis. IRR-learners (○) had greatly reduced numbers of BrdU+/CaBP+ neurons as compared with shams (●). The number of non-neuronal (BrdU+/CaBP−) cells in IRR-learners (□) did not differ from shams (■). Values=mean±S.E.

Electrophysiology

After perfusion the left hippocampus was extracted and placed on a tissue chopper. 400 μm thick slices were cut and placed in a holding chamber containing oxygenated ACSF for at least 1.5 h prior to recording. For recording, slices were placed in a recording chamber and continuously perfused with oxygenated artificial cerebrospinal fluid (approximately 2 ml/min, 30–32 °C). Stimulation of the medial perforant path was performed with bipolar tungsten electrodes and synaptic field responses were recorded in the same layer with glass micropipette electrodes. Stimulation intensity was used which produced half maximal responses (300–400 μA , 20 μs) and test pulses were delivered every 20 s. After at least half an hour of stable recordings the duration from the stimulus artifact to the peak of the field excitatory post-synaptic potential (fEPSP) interval was measured, and paired pulse ratios at 40 ms were obtained. Stimulus-response curves were also obtained where the stimulus intensity was increased in 0.05 mA intervals and the initial slope (0.4 ms) of the fEPSP was measured. Similarly, electrodes were placed in stratum radiatum in order to record responses from CA1 pyramidal neurons (Snyder et al., 2001).

RESULTS

Irradiation blocks adult neurogenesis

Irradiation has been shown to severely reduce adult hippocampal neurogenesis, while sparing the production of other cell types in the adult CNS (Monje et al., 2002). Using confocal microscopy we found that adult-born neurons dual-labeled for BrdU and CaBP (BrdU+/CaBP+) were virtually eliminated in irradiated rats but the number of adult born non-neuronal cells (BrdU+/CaBP−) was not different from sham-irradiated rats (Figs. 1B, 2). Numbers of surviving neurons at 3 and 6 weeks post-BrdU (6 and 9 weeks post-irradiation) were as follows: IRR-learners 110±46 and 46±46, shams 2094±176 and 2582±243 respectively (mean±S.E.; $F_{1,37}=65.35$; $P<0.001$). Shams represent pooled values from learners and cage controls since they did not differ from each other (see Fig. 7). There was no main effect of irradiation on the number of non-neuronal (BrdU+/CaBP−) cells: IRR-learners 1216±180

and 850±161, shams 943±147 and 531±149 at 3 and 6 weeks respectively ($F_{1,37}=3.66$, $P=0.064$). ANOVA with Holm-Sidak post hoc analysis.

The extent of the reduction was verified via immunolabeling for the cell proliferation marker Ki67 and the microtubule associated protein doublecortin, found in granule neurons up to 4 weeks of age (Brown et al., 2003). At the time of death the number of Ki67 expressing subgranular cells was drastically reduced in irradiated rats indicating a lasting effect of irradiation on neurogenesis (sham irradiated rats: 4338±179, irradiated rats: 1084±380; $F_{1,41}=60.04$, $P<0.001$, ANOVA, data pooled from 1, 2, and 4 week rats). Consistent with reduced proliferation, a qualitative examination of doublecortin expression revealed a pronounced reduction of labeled neurons in the dentate gyrus of three irradiated rats examined (Fig. 1C).

By qualitative observation, irradiated rats ate and drank normally. They also continued to gain weight although at a slightly reduced rate. Irradiated rats at 3 weeks post-irradiation (i.e. time of BrdU injection) weighed 305±6 g as compared with 330±4 g for sham-irradiated rats (mean±S.E.; $T_{48}=3.5$, $P<0.05$). Red and white blood cell counts were independently analyzed (Vita-Tech Canada) and compared between irradiated and non-irradiated animals. No differences were found in the numbers of red or white blood cells, or in the numbers of different white blood cell types (lymphocytes, monocytes, neutrophils, eosinophils, basophils; $n=20$, $P>0.18$ in all cases) suggesting no global effect of irradiation on the body's immune response or ability to produce blood cells.

Long-term spatial memory deficits in neurogenesis deficient rats

To assess changes in hippocampal function we trained rats in a spatial version of the MWM 4 weeks after irradiation, a time when no neurons aged 4 weeks or younger will be present to contribute to the task. Repeated measures ANOVA revealed that learners and IRR-learners learned the task equally well ($F_{1,220}=2.3$; $P=0.14$), showing a decrease in latency to find the hidden platform with successive days of training (Fig. 3A). This result agrees with other reports, which show that adult neurogenesis is not required for acquisition in this task (Shors et al., 2002; Madsen et al., 2003; Raber et al., 2004a,b). To test for a role of young neurons in long-term memory we gave learner and IRR-learner rats a probe trial at either 1, 2, or 4 weeks after training. Each rat was tested with a single probe trial, meaning different rats comprised the 1, 2, and 4 week groups. There was no performance difference between IRR-learners and learners in the 1-week probe trial ($F_{1,36}=0.07$; $P=0.79$). Both groups spent significant time in the target quadrant, indicating intact reference memory. However, IRR-learners performed significantly worse than learners on the 2 week ($F_{1,36}=17.97$; $P<0.001$) and 4 week ($F_{1,36}=5.81$; $P=0.02$) probe trials and spent only chance amounts of time in the target quadrant (Fig. 3B). ANOVA with Holm-Sidak post hoc analysis.

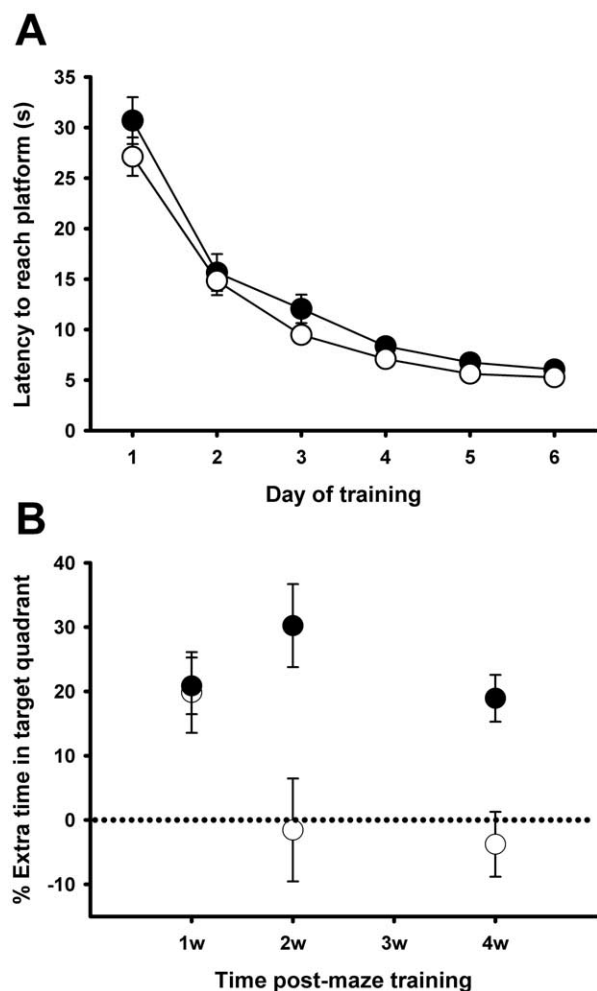


Fig. 3. Acquisition and retention in the MWM. (A) IRR-learners (○) performed as well as learners (●) in the acquisition of spatial information in the water maze. (B) There was no performance difference between IRR-learners and learners in the 1 week probe trial ($F_{1,36}=0.073$; $P=0.788$). Y axis shows the difference between the % time spent in the quadrant formerly containing the hidden platform (target) and the mean % time spent in the other three quadrants. On the 1 week probe trial, both groups spent significant time in the target quadrant, indicating intact reference memory. However, IRR-learners performed significantly worse than learners on the 2 week and 4 week probe trials and spent only chance amounts of time in the target quadrant (i.e. 0%). Values=mean±S.E.

Specificity of the irradiation-induced memory deficit

Behavioral and physiological tests for delayed irradiation damage. To confirm that the memory loss seen in IRR-learners was not due to delayed, non-specific irradiation damage we spatially trained seven rats 1 week later than in our first schedule. We reasoned that, if the memory deficit is due to a pathological process, which occurs at a fixed time after irradiation, training 1 week later should shift the memory deficit from the 2-week retention test to the 1-week retention test. However, these IRR-learners spent $45\pm 5\%$ extra time in the target quadrant on their 1-week probe trial ($F_{1,12}=140.9$; $P<0.001$), arguing against such an interpretation of the retention results. Swim speeds were

equivalent in irradiated and sham-irradiated rats during probe trial testing suggesting both groups were equally motivated and capable of swimming normally (pooled speeds from all animals in 1, 2, and 4 week probes: learners= 21.5 ± 1.1 cm/s, IRR-learners= 22.1 ± 1.0 cm/s, mean±S.E.; $F_{1,38}=0.48$, $P=0.62$; ANOVA).

Electrophysiological analysis revealed no differences in synaptic function in the dentate gyrus and CA1 of irradiated and non-irradiated rats. Whereas we have previously reported significant differences in long-term plasticity between young and mature neurons, the need to adhere to a strict irradiation-training-retention schedule made extensive electrophysiological testing unfeasible in this study. Input–output curves in the dentate gyrus of irradiated and sham irradiated rats were not significantly different ($F_{1,126}=2.36$, $P=0.13$; Fig. 4A) and latency from stimulus to peak fEPSP was similar between groups ($T_{10}=-0.17$, $P=0.87$) as was the paired-pulse ratio ($T_{20}=1.74$, $P=0.10$; Fig. 4B) indicating no effect of irradiation on axonal conduction velocity or synaptic release probability. Input–output curves in region CA1 of irradiated and sham-irradiated rats were also similar ($F_{1,42}=2.25$, $P=0.14$; Fig. 4C), and CA1 fEPSPs from irradiated and sham-irradiated rats did not differ in their stimulus to peak fEPSP interval ($T_6=2.04$, $P=0.09$), or in their paired pulse ratios ($T_6=1.29$, $P=0.24$; Fig. 4D).

Cued water maze task. An alternative explanation for our results could be that irradiation has a non-specific effect such that mature neurons throughout the brain are no longer capable of storing information for the long term. To test this hypothesis we trained learners and IRR-learners in a cued version of the water maze. Cued water maze learning occurs independent of the hippocampus but instead relies on the striatum (Packard and Teather, 1997), a structure that does not produce appreciable numbers of neurons in adulthood. As in the spatial task, both groups learned the acquisition portion of the task. However, unlike the spatial task, both groups remembered that the hidden platform was associated with the hanging cue, as seen by the strong bias for the quadrant containing the cue (paired t -test: $P<0.001$, $n=8$ for both groups). There was no difference between groups (t -test: $T_{14}=1.06$, $P=0.31$; Fig. 5). Intact long-term memory for cued relationships provides evidence that the long-term spatial memory deficit in IRR-learner rats is not due to a more general inability to form long-term memories.

Varying the irradiation-training interval

Thus far we can only conclude that neurons born from 4 weeks prior to training are important for long-term spatial memory. To examine the potential importance of neurogenesis solely during the post-training interval we irradiated one group of rats on the 2 days following training. In a 3-week retention test these irradiated rats showed a strong bias for the target quadrant, which was equal to that of sham-IRR-learners ($T_{14}=0.80$, $P=0.44$). An additional group was irradiated at 4 and 3 days prior to training and also showed long-term memory for the platform location,

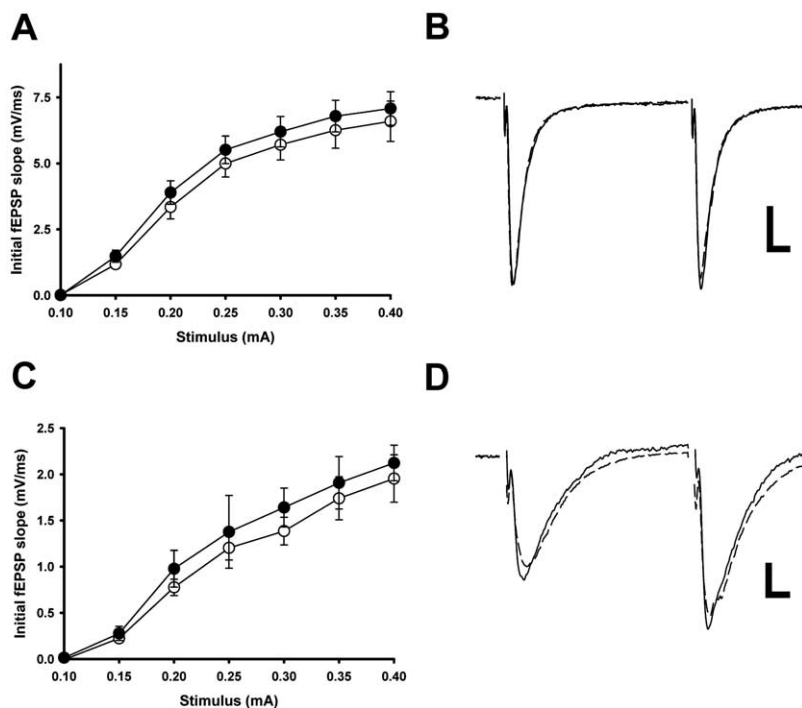


Fig. 4. Synaptic transmission in irradiated and non-irradiated rats. (A) Input–output curves in the dentate gyrus of irradiated (○) and sham-irradiated (●) rats were not significantly different. (B) Overlay of dentate gyrus fEPSP in irradiated (dashed line) and sham-irradiated (continuous line) rats. Latency from stimulus to peak fEPSP and paired-pulse ratios was not different between groups indicating no effect of irradiation on axonal conduction velocity or synaptic release probability. Scale bars=1 mV, 5 ms. (C) Input–output curves in region CA1 of irradiated (○) and sham-irradiated (●) rats were not significantly different. (D) Overlay of CA1 fEPSPs from irradiated (dashed) and sham-irradiated (continuous) rats. Stimulus to peak fEPSP interval and paired pulse ratios were not different between groups. Scale bars=0.5 mV, 5 ms. Values=mean±S.E.

which was equal to shams ($T_{14}=0.27$, $P=0.79$; Fig. 6). Neither group showed any effect of irradiation on rate of learning as indicated by performance during the acquisition phase of the MWM. Collectively, these results suggest that neurons aged 4–28 days old at the beginning of training are critical for long-term memory in the MWM.

Relations between learning and neuronal addition

Neurogenesis in learners vs. cage controls. To explore relationships between learning and neuron addition, learner and cage control rats were injected with BrdU 1 week prior to water maze training to label neurons aged 1 week old at the time of learning. There was no effect of learning on the survival of 1-week-old neurons ($F_{1,38}=0.10$; $P=0.75$; Fig. 7A). While a trend for greater survival in learners existed at 1 week this was not significant ($P>0.2$). Immunostaining for the cell proliferation marker Ki-67 also revealed no differences in total cell proliferation between learners and cage controls ($F_{1,30}=2.51$, $P=0.12$; Fig. 7B).

Learning as a function of neuronal survival, proliferation. Correlating the individual probe trial performances of all sham-IRR-learners, at all time points, with total BrdU+/CaBP+ cells revealed no significant relationships ($r=0.11$, $F_{1,24}=0.27$, $P=0.61$; Fig. 8A). Similar results were obtained when correlations were examined by group (1w: $r=0.39$, $P=0.18$; 2w: $r=0.28$, $P=0.59$; 4w:

$r=0.12$, $P=0.79$). Total surviving new neurons was also not predictive of performance on the acquisition portion of the maze, as measured by the total latency of all trials in all sham-IRR-learners ($r=0.18$, $F_{1,17}=0.48$, $P=0.50$). As with the survival data, there was no relationship between probe performance and total proliferating Ki-67+ cells (pooled data from all sham-IRR-learners: $r=0.01$, $F_{1,17}<0.01$, $P=0.96$; by group: 1w: $r=0.09$, $P=0.87$; 2w: $r=0.45$, $P=0.37$; 4w: $r=0.10$, $P=0.84$; Fig. 8B) and no correlation was found between acquisition performance and proliferating cells at the time of death ($r=0.08$, $F_{1,17}<0.1$, $P=0.78$).

DISCUSSION

Here we provide evidence that adult-born dentate granule neurons may be required for the formation of long-term spatial memories in the rat. Evidence for a contribution of neurogenesis to learning and to the behavioral effects of antidepressants has been presented (Shors et al., 2001, 2002; Madsen et al., 2003; Santarelli et al., 2003; Raber et al., 2004a,b), but this is the first evidence for a specific role in long-term memory. To test whether the memory deficits described were due to the removal of young neurons, and not a non-specific effect of irradiation, several control experiments were done. First, we trained one group of rats 5 weeks after irradiation instead of the usual 4. At 1 week post-training, these rats showed clear preference for the

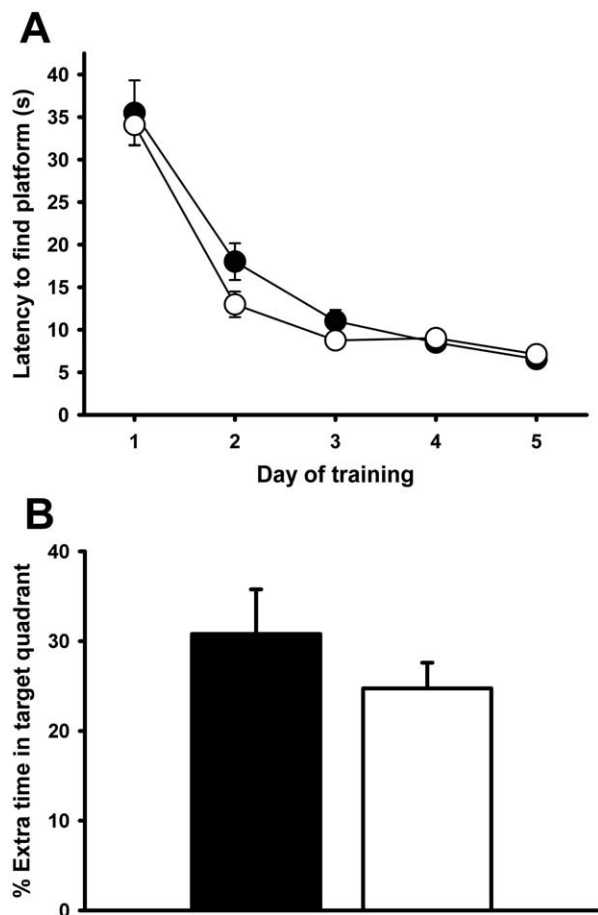


Fig. 5. Irradiation and learning in the cued MWM. (A) Learner (●) and IRR-learner (○) rats acquired the cue task equally well over 5 days of training. (B) Both groups remembered that the hidden platform was associated with the hanging cue, as seen by the strong bias for the quadrant containing the cue. Values=mean±S.E.

quadrant formerly containing the hidden platform, indicating irradiation had no delayed pathological effect. Second, irradiated rats had intact long-term memory for the location of a cued platform and irradiation just before, or after, training did not affect long-term spatial memory. This indicates that irradiation did not cause a general inability to maintain or retrieve memories. Third, we found no differences in basic synaptic properties or swim speeds between irradiated and sham-irradiated rats. These control experiments are consistent with a recent study showing that low dose irradiation leaves mature neurons unaffected, in terms of total cell number in the dentate gyrus and CA1. They also reported no effect of irradiation on the dendritic branching pattern of mature granule and cortical neurons (Raber et al., 2004b).

The water maze data are consistent with our previous results (Wang et al., 2000; Snyder et al., 2001) showing that young neuronal long-term potentiation (LTP), a proposed physiological mechanism of memory formation (Martin and Morris, 2002), has a lower induction threshold and is non-decremental over time as compared with mature neuronal LTP. This “young LTP” relies on the NR2B

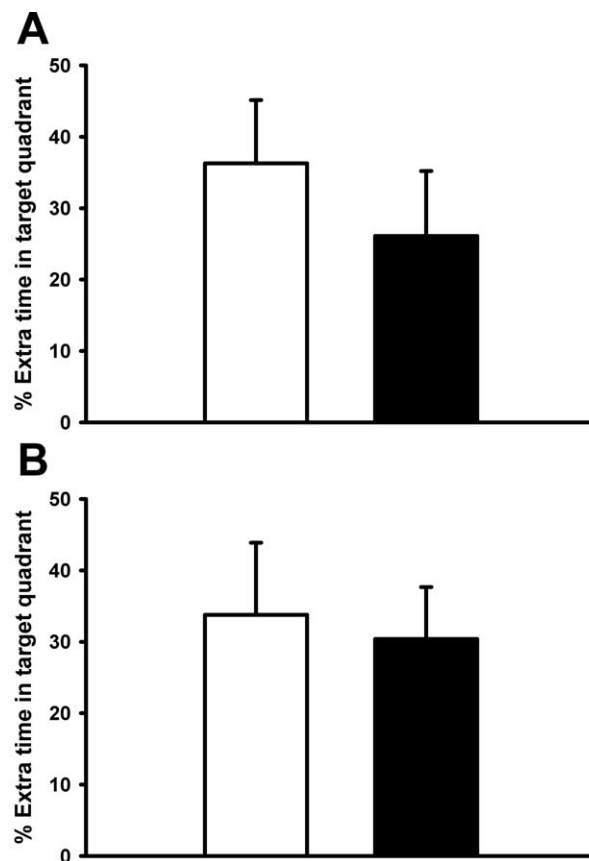


Fig. 6. Determining the age of neurons important for long-term memory. (A) Rats were irradiated on the 2 days following MWM training to eliminate neurogenesis in the weeks between training and the probe trial. Three weeks after training both learners (■) and IRR-learners (□) showed a strong bias for the correct quadrant. (B) Similarly, rats were irradiated at 4 and 3 days prior to training to test the importance of neurogenesis during, and immediately preceding, training. These IRR-learners also learned normally and had intact memory for the correct quadrant in their 3 week probe trial. Values=mean±S.E.

subunit of the NMDA receptor (Snyder et al., 2001), a critical link to molecular pathways required for long-term forms of plasticity and memory (Krapivinsky et al., 2003). As the dentate gyrus is essential for normal learning and memory (McNaughton et al., 1989; Ferbinteanu et al., 1999), it follows that the synaptic plasticity provided by the continual addition of new granule neurons (9000/day in juvenile rats; Cameron and McKay, 2001) could facilitate the acquisition and retention of new information. Mature neurons, with their higher threshold for information storage and limited capacity to sustain information over time, may support acquisition and short term retention of spatial information but apparently lack the long-term storage capability of younger granule neurons. Similarly, the capacity of mature neurons to store learned information may be sufficient for performing certain (easier) tasks and may explain why reductions in neurogenesis do not affect learning in all tasks (Shors et al., 2002; Madsen et al., 2003; Raber et al., 2004a).

By irradiating at different times relative to the acquisition and long-term retention tests we were able determine

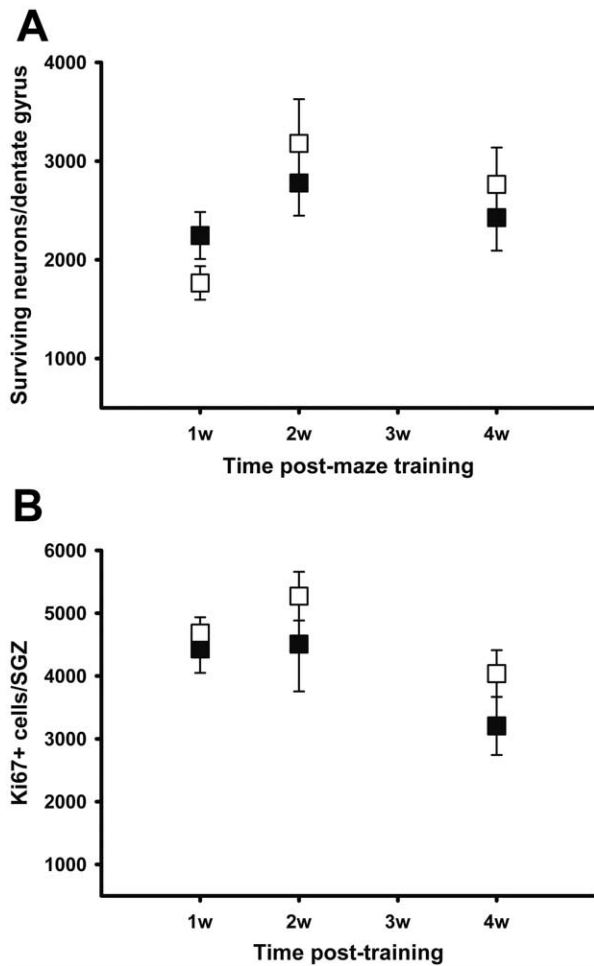


Fig. 7. No effect of learning on new neuron addition. (A) Learning in the MWM did not alter the survival of adult generated neurons in the dentate gyrus (learners (■) vs. cage controls (□)). (B) MWM training also did not cause any changes in the total number of proliferating, Ki-67 expressing, cells. Learners=■, cage controls=□. Values=mean±S.E.

that neurons aged 4–28 days old at the time of training are important for long-term spatial memory. This age bracket is consistent with data showing that new neurons do not extend axons to CA3 until 4–10 days of age (Hastings and Gould, 1999), are functional at 4 weeks of age (van Praag et al., 2002), contribute to trace eyeblink conditioning at 6–21 days of age (Shors et al., 2001), and possess an enhanced LTP when 4 weeks of age and younger (Snyder et al., 2001). The relative unimportance (for long-term memory) of neurons born during and after training would suggest that learning induced changes in neuronal proliferation (Dobrossy et al., 2003) may be more of an adaptive response designed to facilitate memory formation for future learning episodes. Since reductions in neurogenesis have been associated with deficits in short-term (acute) hippocampal learning (Shors et al., 2001, 2002; Madsen et al., 2003; Raber et al., 2004a) we propose that, in our study, the plasticity afforded by 4–28 day old neurons contributes to the efficient storage of spatial information at the time of initial learning, such that this information per-

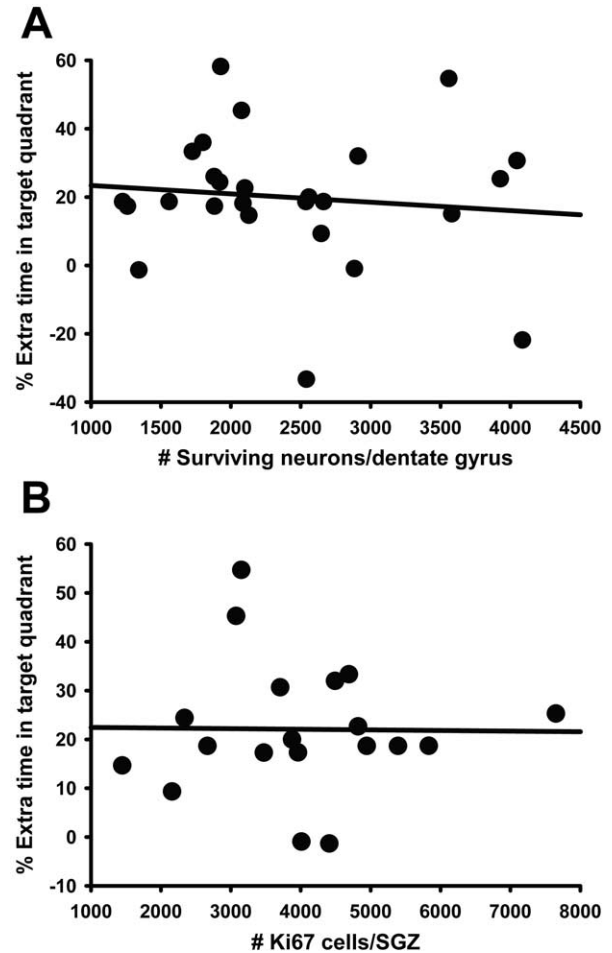


Fig. 8. Individual memory performances as a function of neuron addition. (A) Correlating individual long-term memory performances with neuronal survival revealed no significant relationships. Graph shows data pooled from all 1w, 2w, and 4w sham-IRR-learner animals. (B) Long-term memory scores also did not correlate with cell proliferation in the SGZ of the dentate gyrus. Data are pooled from all 1w, 2w, and 4w sham-IRR-learner animals.

sists for the long term. However, it is entirely possible that these 4–28 day old neurons are offline at the time of initial learning but have an important role solely in the post-training consolidation of spatial memories (if not being involved in both acquisition and consolidation). It also remains to be seen whether these neurons store this information within the hippocampal formation, or if they allow it to be transferred elsewhere. While some reports claim the hippocampal formation has a prolonged, if not permanent, role in supporting spatial memories in rodents (Sutherland et al., 2001), it remains possible that long-term spatial memories are ultimately consolidated in the neocortex (Maviel et al., 2004).

Memories are thought to exist as structural changes in the brain, the most documented locus being the synapse (Martin and Morris, 2002). However, in neurogenic regions, the loss of old cells and the addition of new cells must also be considered. An intriguing possibility is that neuronal turnover may provide a necessary plasticity for

information storage that more differentiated neurons cannot (Nottebohm, 2002). Consistent with this idea, the survival of young adult-born neurons can be increased by learning and enriched environments (Gould et al., 1999; Kempermann et al., 1997). One interpretation of activity-dependent survival is that these neurons are involved in the representation of learned information, and that the life cycle of a neuron equals the duration of the memory (memories) it supports. This has been demonstrated most convincingly in the songbird, where the birth and death of high vocal center neurons temporally parallels the acquisition and forgetting of different songs (Kirk et al., 1994). One might therefore expect greater neuronal survival when a memory still resides in the hippocampus as opposed to having been consolidated in the neocortex.

Against our expectations, we observed no effect of learning on new neuron survival to 1, 2, or 4 weeks post-training. When analyzed at the level of individual rats, survival did not correlate with long-term memory performance. Thus it would appear that the proposed temporary role of the hippocampus in memory storage (McClelland et al., 1995; Maviel et al., 2004) is not subserved by temporary neurons born 1 week before learning. While many adult born neurons have a temporary existence, a significant population survives for many months and may persist permanently (Kempermann et al., 2003; Dayer et al., 2003; Eriksson et al., 1998). Thus, an alternative to the hypothesis of a functional role for temporary neurons could be that the permanent addition of adult-born neurons relates to a possible permanent role of the hippocampus in storing memories (Rosenbaum et al., 2001; Sutherland et al., 2001). Of course, it also remains possible that neuronal persistence in the dentate gyrus is not directly proportional to the persistence of memories within this structure. Our survival results are in contrast to those obtained by Gould et al. (1999) who found new neuron survival more than doubled as a result of learning. Differences in animal strain, age and training protocol could account for this discrepancy in results. It must also be noted that experience, including water maze learning, can modulate the addition of a very specific subset of adult-born neurons depending on neuronal age and/or state of maturation (Wilbrecht et al., 2002; Dobrossy et al., 2003; Ambrogini et al., 2004). Thus, significant proliferation, survival or death of adult born neurons may have occurred in our learner-rats, yet was missed by the timing of our BrdU injections.

In summary, we present a new role for adult-born neurons in the formation of long-term hippocampal-based memories. Using radiation doses in the clinical range we provide a possible explanation for the memory deficits seen in irradiated patients (Roman and Sperduto, 1995), particularly those in children, who would have greater levels of neurogenesis than adults (Kuhn et al., 1996). The lack of activity-dependent survival, in view of the current literature (Gould et al., 1999; Dobrossy et al., 2003; Ambrogini et al., 2000, 2004), suggests that much work needs to be done to understand how rates of neuron addition/removal contribute to hippocampal learning.

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