Imaging activation of adult-generated granule cells in spatial memory

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New neurons are continuously generated in the subgranular zone of the hippocampus throughout adulthood, and there is increasing interest as to whether these new neurons become functionally integrated into memory circuits. This protocol describes the immunohistochemical procedures to visualize the recruitment of new neurons into circuits supporting spatial memory in intact mice. To label adult-generated granule cells, mice are injected with the proliferation marker 5-bromo-2′-deoxyuridine (BrdU). At different delays after BrdU treatment, mice are trained to locate a hidden platform in the Morris water maze, and spatial memory can then be tested in a probe test with the platform removed from the pool. Ninety minutes after this probe test, mice are perfused and tissue is sectioned. Immunohistochemical procedures are used to quantify BrdU-labeled cells and expression of the immediate early gene, *Fos*. Because Fos expression is regulated by neuronal activity, the degree of overlap between BrdU-labeled and Fos-labeled neurons provides an indication of whether adult-generated granule neurons have been incorporated into spatial memory circuits.

INTRODUCTION

It is well established that new neurons are continuously generated in the subgranular zone of the dentate gyrus throughout adulthood in all mammals[1]. Within a few days, newly generated neurons migrate into the granule cell layer, begin to extend dendritic processes toward the molecular layer and an axon toward the CA3 region. At this stage, immature neurons may receive γ-aminobutyric acid-mediated excitatory synaptic inputs. However, major glutamatergic synaptic activation from perforant path afferents does not occur until new neurons are at least 2 weeks old, when spines begin to form[2]. Because the hippocampus plays a key role in many forms of memory, there is considerable interest in how these new neurons might contribute to hippocampal memory processing[3].

One way this issue has been addressed is to examine the effects of suppressing adult neurogenesis on hippocampal learning. To suppress adult neurogenesis, the two most common strategies have been irradiation[4] (either whole brain or focal) or administering antimitotic agents such as methylazoxymethanol acetate[5]. However, the effects of these manipulations on hippocampal learning have been mixed[6]. In some cases these treatments disrupt hippocampal learning[5,7–9], whereas in others these treatments either have no effect[7,9–11] or even enhance some forms of learning[12]. When these treatments produce effects, it has been difficult to rule out nonspecific effects on mature cells or on general health[13,14]. The absence of treatment effects are equally difficult to interpret. Because these treatments typically produce an incomplete knockdown of adult neurogenesis (~80%)[13,15], it is difficult to rule out the possibility that residual adult-generated neurons are sufficient to support hippocampal learning or whether a sufficiently mature cohort of adult-generated neurons have been eliminated. Even if these treatments were to completely eliminate adult neurogenesis, it is still possible that hippocampal learning might be supported by the existing or mature granule cells[3].

An alternative strategy is to use immunohistochemical approaches to visualize the recruitment of adult-generated neurons into the memory circuits in intact mice[16–19]. The basic scheme is to inject mice with the proliferation marker 5-bromo-2′-deoxyuridine (BrdU) to 'birth-date' the dividing cell population[20–22]. Mice are subsequently trained in the Morris water maze, and spatial memory is assessed in a probe test with the platform removed from the pool. After this spatial memory test, expression of the immediate early gene *Fos* is quantified in order to identify the granule cells processing spatial memory[17]. The overlap between BrdU-labeled and Fos-labeled neurons then provides an indication of whether or not adult-generated granule neurons have been incorporated into spatial memory circuits. The protocol described here is for studying the contribution of adult-generated granule cells to water maze memory in mice. However, it could easily be adapted for use in rats, and with other types of hippocampus-dependent learning tasks.

Water maze

Developed in the early 1980s by Richard Morris[23], the water maze has become one of the most commonly used tasks to measure spatial learning in rodents, including normal and genetically modified mice[24]. In this task, training typically takes place over several days in a large, circular tank filled with opaque water. In each training trial, a mouse is given the opportunity to navigate to a platform submerged below the water surface. Because the platform is not visible, the mouse must locate it using an array of distal, visual cues surrounding the pool. After the completion of training, spatial memory can then be assessed at different retention delays in a probe test, where the platform is removed from the pool and the mouse is allowed to search for it using the distal cues.

For these studies, the water maze offers several important advantages. First, lesion, pharmacological, genetic and neuroimaging studies have established an essential role for the hippocampus in the formation of water maze memories[25]. Second, mice trained in this task exhibit robust and long-lasting spatial memory. While some previous studies have emphasized species differences in performance in the water maze[26,27], it is worth stressing that

mice learn this task very well and show robust retention up to several weeks[17,28] (**Fig. 1**). Third, expression of this memory remains dependent on the activity in the hippocampus, even at very long retention delays[29]. For example, pharmacological inactivation of the dorsal hippocampus blocks the expression of either a day-old or month-old water maze memory[28]. These latter two features are particularly advantageous in designing experiments, since it allows greater flexibility in the use of retention delays between training and testing.

There are several important methodological issues to consider in water maze studies. First, water maze performance is highly dependent on background strain of the mouse[30,31]. For example, mice in a pure C57B6 background are much poorer compared to hybrid C57B6/129 mice, requiring more training to produce robust long-term retention (**Fig. 2**). Second, anxiety can interfere with water maze performance. There are several ways to minimize the confounding effects of anxiety on learning. For example, the pool should be dimly lit, and mice should be handled extensively before the commencement of training. In our studies, mice are handled for 2 min d^{-1} for 7 d.

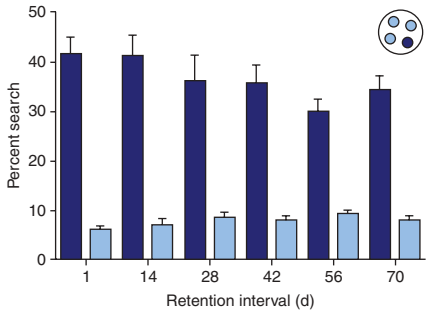

Figure 1 | Long-lasting water maze memory in mice. C57B6/129 mice were trained in the water maze (5 d, six trials d^{-1}) and tested 1 d ($n = 9$), 14 d ($n = 9$), 28 d ($n = 9$), 42 d ($n = 10$), 56 d ($n = 10$) or 70 d ($n = 9$) later. At each retention delay, mice spent significantly more time searching the target zone (dark blue) compared to other (light blue) zones, and there was little decline in performance even at longer retention delays. Adapted from Kee *et al.*[17].

BrdU

BrdU is used almost universally to detect proliferating cells in the studies of adult neurogenesis[20–22]. BrdU crosses the blood–brain barrier, and therefore can be administered systemically, either by injection or, somewhat less commonly, orally (e.g., via drinking water). As BrdU is a thymidine analog, it competes with endogenous thymidine for incorporation into dividing cells during S phase (DNA synthesis) of the cell cycle. Incorporation into the DNA of the cell is permanent, and BrdU may be detected weeks, months or even years later in fixed tissue using specific primary antibodies[32]. For visualization, the primary antibody may either be labeled with a secondary antibody tagged with a fluorescent compound or with an enzyme for diaminobenzidine as a substrate for visualization.

Until the mid-1990s, radiolabeled thymidine (^{3}H-thymidine) was used to label dividing cells[33]. In addition, more recent studies have used endogenous cell-cycle markers, such as Ki67 (ref. 34) and GFP-expressing retroviruses[35], to identify dividing cells. However, for this protocol the application of BrdU offers two chief advantages over these techniques. First, unlike ^{3}H-thymidine, immunohistochemical methods are used to detect incorporated BrdU. Therefore, immunostaining for markers associated with neuronal activation (such as *Fos*) makes it possible to determine whether a BrdU-labeled cell has been recently activated. Similarly, as newborn granule cells sequentially express different markers as they age, double- or triple-labeling makes it possible to determine the state of differentiation and maturation of BrdU-labeled cells. Second, Ki67 immunohistochemistry provides a 'snapshot' of proliferating cells at the time of perfusion[34]. In contrast, BrdU immunohistochemistry identifies cells that were undergoing division at the time of injection. By varying the delay between BrdU treatment and water maze training, it is therefore possible to assess the incorporation of different aged cohorts of BrdU-labeled cells into memory circuits. Retroviral GFP approaches also make it possible to 'birth-date' cells undergoing division. However, retroviruses must be microinfused directly into the dentate gyrus (rather than administered systemically) and are therefore more labor-intensive. Furthermore, retroviral approaches typically label fewer cells compared to BrdU, and GFP expression may be silenced over time[35,36]. Therefore, current retroviral-based approaches may not be optimal for these sorts of studies, but may be particularly suited to more detailed morphological[35,37] and electrophysiological[38–40] analyses.

There are several important methodological issues to consider when using BrdU. First, what dose of BrdU to use? In the young adult mouse, several thousand new neurons are generated daily, and, of these, only a small proportion will survive beyond 4 weeks[41] (**Fig. 3**). Since the total number of neurons in the mouse dentate gyrus is between 239,000 and 351,000 depending on the strain[42], this represents a relatively small subpopulation and so BrdU treatment protocols that maximize labeling are preferred. In mice, BrdU is typically injected at doses of 50–200 mg kg^{-1}. While doses at the higher end of this range have been associated with developmental abnormalities when given to neonatal mice (e.g., <4 weeks in age)[43,44], such effects are not observed in adult mice. We have found that labeling saturates at ~200 mg kg^{-1} in adult C57B6/129 mice, and so we recommend use of this dose.

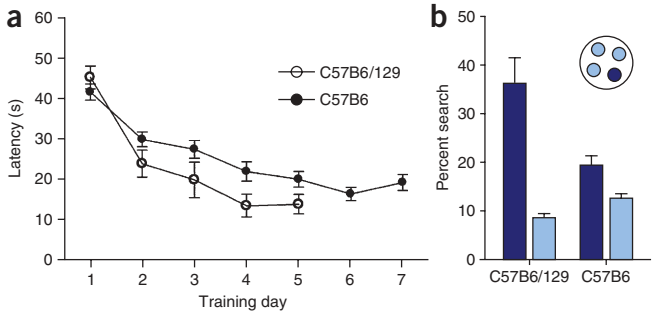

Figure 2 | Comparison of C57B6/129 and C57B6 mice in the water maze. (**a**) C57B6/129 ($n = 9$) and C57B6 ($n = 19$) mice were trained in the water maze (six trials d^{-1}) for 5 and 7 d, respectively. The mean daily escape latencies (± s.e.m.) for C57B6/129 (open circles) and C57B6 (closed circles) are plotted. C57B6/129 mice acquired the task more rapidly. (**b**) Thirty days after the completion of training, mice were given a probe test. In the probe test both C57B6/129 and C57B6 mice searched selectively, each spending significantly more time searching the target zone (dark blue) compared to other (light blue) zones. However, C57B6/129 spent significantly more time in the target zone compared to C57B6 mice.

BrdU is usually administered as a series of i.p. injections over the course of several days. The chief advantage of this approach is that the multiple injections ensure that sufficiently large numbers of cells are labeled for quantitative analyses. The disadvantage of this approach is that temporal resolution is compromised. However, maturation of new neurons to a stage where they begin to contribute to mnemonic function in the dentate gyrus takes weeks, rather than days, and so this degree of temporal resolution is appropriate[17].

A second concern is the specificity of BrdU labeling. Because BrdU is a thymidine analog, in principle it could also be incorporated into any cell undergoing DNA repair, in addition to those in mitosis[45]. However, whereas mitosis involves the synthesis of the entire genome, DNA repair typically involves the synthesis of relatively few nucleotides (e.g., <100). Because differences in synthesis rates are several orders of magnitude, these two processes should always be easy to discriminate. Consistent with this, several observations suggest that this risk of false-positive labeling associated with DNA repair is negligible. First, after BrdU treatment, BrdU-labeled cells are not found throughout the CNS, but rather are confined to the two major neurogeneic regions in the adult brain. This specificity is evident even when high doses of BrdU are used (>400 mg kg^{-1})[46,47]. Second, even under conditions that promote inflammation and apoptosis (e.g., irradiation), BrdU-labeling is limited to dividing cells[48].

A third major issue is that adult neurogenesis is highly regulated by a large number of environmental variables. Since recruitment of new neurons into memory circuits might be influenced by the availability of new neurons, it is important to ensure that different experimental groups have equivalent levels of BrdU-labeled neurons. In particular, both proliferation and survival are affected by exercise[49], enrichment[50] and stress[51], and so ensuring that these factors are held constant across conditions is essential in these types of studies. Another major influence on adult neurogenesis is age. As animals age, the generation of new neurons falls exponentially[52] and therefore it is important that animals in different experimental conditions are age-matched.

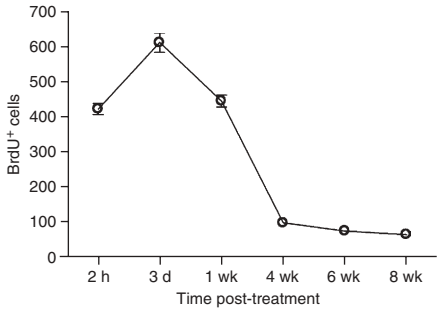

Figure 3 | Survival of adult-generated granule cells. Separate groups of C57B6/129 mice were given a single injection of 5-bromo-2′-deoxyuridine (BrdU) (200 mg kg^{-1}) and then perfused at different delays ($n \geq 3$ for all groups). Maximal numbers of BrdU-labeled cells were observed in the mice perfused 3 d after BrdU treatment, and declined thereafter. This indicates that the majority of adult-generated granule cells do not survive beyond 4 weeks.

Fos expression

Neuronal stimulation is associated with increases in intracellular calcium levels through N-methyl D-aspartate (NMDA) receptor activation or voltage-gated calcium channels. Because these increases in calcium lead to the rapid upregulation of immediate early genes such as *Fos*, measuring *Fos* levels allows for the detection of recently activated neurons[53]. For example, after stimulation, Fos protein may be detected immunohistochemically in fixed tissue using specific primary antibodies, and Fos-positive nuclei can be subsequently quantified visually or using automated software such as Image J (National Institutes of Health). Because these immunohistochemical methods offer single-cell resolution, double-labeling for BrdU makes it possible to determine whether adult-generated neurons have been recently activated.

Fos has been used as an activity marker in many different experimental situations. For example, Fos is induced in the hippocampus after seizures[54–56], NMDA-dependent long-term potentiation (LTP) induction[57–59], noxious stimulation[60], as well as after learning[61,62].

When using Fos expression to map circuits in behavioral studies, there are several important issues to consider. First, after stimulation, Fos protein levels peak between 1 and 2 h, decaying thereafter[63]. While basal levels of Fos are low in unstimulated animals (e.g., home cage), the relatively poor temporal resolution of Fos expression means that it is critical that the mice remain minimally stimulated both before and after behavioral testing. For example, extraneous noises or other stimulation that might stress the mice should be avoided. Perhaps as important, novelty is associated with elevated Fos expression in several brain regions, including the hippocampus[64]. Therefore, care should be taken to ensure that behavioral procedures on test days match, as much as possible, those used during training. Even subtle changes in procedures (e.g., transport, handling, lighting conditions) might elevate Fos levels above basal conditions. Despite these caveats, we find that Fos expression in the dentate gyrus remarkably specific after the water maze testing. In particular, within the dentate gyrus only 0.5–2.5% of neurons express Fos after behavioral testing[17]. This matches previous immediate early gene[65] and electrophysiological[66] studies, showing that a similarly small proportion of granule cells are activated during spatial exploration, and is consistent with the idea that spatial information is sparsely encoded in the dentate gyrus. Furthermore, Fos expression is reduced in mice carrying a point mutation in the α-CaMKII gene[67] that prevents spatial learning (but otherwise does not disrupt the regulation of Fos)[17]. This suggests that nonspecific aspects of the test procedures (e.g., swimming, physical exercise, stress or arousal) do not contribute significantly to Fos expression in neurons in the dentate gyrus.

Second, most behavioral studies have focused on the induction of Fos after learning[61,62]. This focus on learning-induced changes in Fos expression is motivated, in part, by the idea that Fos may play a key role in activity-dependent modification of circuits underlying memory formation[68]. Consistent with this, genetic deletion of *c-fos* impairs two forms of hippocampal-dependent learning (contextual and spatial) and the induction of NMDA receptor-mediated LTP[69]. However, as Fos is a general neuronal activity marker, it may also be used to map the circuits activated after memory recall. Using this approach, recent studies have mapped the circuits activated after recall of spatial[28,70], aversive[64,71], appetitive[72], olfactory[73,74] and gustatory[75] memories. Indeed, in the water maze we have found that levels of Fos expression in the dentate gyrus are similar following training and memory probe[17]. It is therefore likely that Fos mediates the stabilization of synaptic remodeling after

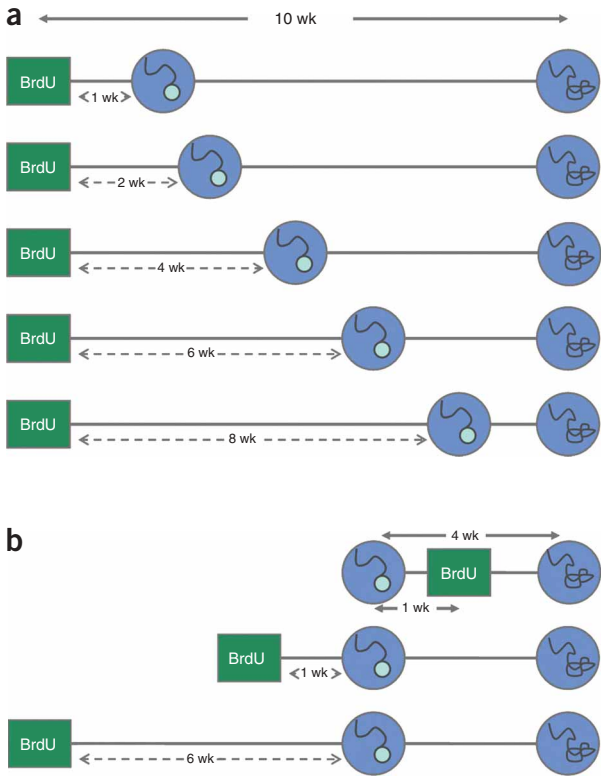

Figure 4 | Experimental designs. (**a**) To determine whether new neurons are incorporated into dentate gyrus, the circuits supporting spatial memory separate groups of mice are treated with 5-bromo-2′-deoxyuridine (BrdU) and then trained in the water maze 1, 2, 4, 6 or 8 weeks later. Spatial memory is assessed in a series of three probe tests 10 weeks after BrdU treatment. Because the BrdU-probe test delay is fixed, this ensures that all BrdU-labeled cells are the same age at the time of testing. (**b**) To control for age of memory, in this design a fixed retention delay of 4 weeks is used in all groups, while the delay between BrdU treatment and training is varied.

reactivation of existing memory circuits, as well as during memory formation[72].

A third concern is that Fos expression may be developmentally regulated. Therefore, neuronal activity may induce Fos expression in mature neurons, but not in newborn granule cells. Indeed, while seizure activity leads to Fos expression in ~80% of mature granule cells, this number falls to ~50% in 25-d-old adult-generated granule cells and 0% in 15-d-old adult-generated granule cells[16]. The absence of Fos expression in newborn cells might be either due to an inability of newborn granule cells to express Fos or because newborn granule cells are not yet completely integrated into hippocampal circuitry. In memory studies, one way to rule out the former possibility is to use a design with a fixed delay between BrdU labeling and memory testing (but varying the timing of the training)[17]. This design ensures that all BrdU-labeled cells are the same age at the time of testing, and therefore any differences in Fos expression cannot be accounted for developmental stage.

Expression of other immediate early genes such as *Activity-regulated cytoskeletal-associated protein* (*Arc*) (also known as *Arg3.1*) and *Egr1* (also known as *zif268*, *Krox-24*, *NGIF-A*, *ZENK*) is regulated by neuronal activity, and may be used either in addition, or as alternatives, to *Fos*[16–19]. Indeed, we have directly compared Fos and Arc expressions in tissue from the same mice after water maze testing, and found that these markers give remarkably similar pattern of results. Expression of Fos and Arc was limited to a similarly small proportion of granule cells after water maze testing, and both methods indicated that maximal recruitment of new neurons into spatial memory circuits does not occur until new neurons are 4 or more weeks in age[17]. The advantage of using multiple activity markers is that their expression relies on potentially different signal transduction cascades. Therefore, similar patterns of gene expression must reflect general neuronal activity rather than activation of a specific signaling cascade.

Experimental designs

The basic experimental protocol involves three main experimental steps: BrdU treatment, water maze training, water maze testing. Ninety minutes after this last step, mice are perfused; hippocampal tissue is processed for BrdU and Fos immunohistochemistry, and the numbers of $BrdU^+$ and Fos^+ and $BrdU^+/Fos^+$ cells in the dentate gyrus are quantified. The primary experimental manipulation is the delay between BrdU treatment and training. Because BrdU provides a 'birth-date', varying this delay makes it possible to study the integration of different aged cohorts of adult-generated granule cells into spatial memory circuits. The logic is that if BrdU-labeled cells are incorporated into memory networks at the time of training, then there should be overlap between BrdU-positive cells and Fos-positive or Arc-positive cells at the time of testing.

There are several variations on the experimental design (**Fig. 4**). In the first design, a fixed delay between BrdU treatment and mouse perfusion is used, and the delay between BrdU treatment and training varied. Importantly, this ensures that all BrdU-labeled cells are the same age at the time of testing, and so potential differences in Fos expression cannot be accounted for in terms of neurochemical maturation. Using this approach, it is therefore possible to evaluate whether week-old granule cells become incorporated into the circuits supporting spatial memory. The disadvantage of this design is that not only does the delay between BrdU treatment and training vary but so does the delay between training and testing. Since there is some evidence for time-dependent reorganization of circuits supporting water maze memory within the hippocampus[76], a second design uses a fixed retention delay, but varies the timing of the BrdU treatment with respect to training. This ensures that the age of the spatial memory is equivalent across groups. An important group in this design is the group in which training *precedes* BrdU treatment. Because new neurons are labeled after training, this group provides an estimate of Fos expression in BrdU-labeled cells associated with nonmnemonic aspects of the probe test (such as swimming, physical exercise, stress or arousal). In our studies, we have identified very few double-labeled cells in this condition, suggesting that these nonspecific aspects of the testing procedures do not contribute significantly to Fos expression in these new neurons[17].

Additional analyses and experiments may be used to control for a number of different factors. First, Fos and BrdU expression may be quantified in the olfactory bulb, the other major neurogenic region in the adult brain. Since the olfactory bulb would not be expected to play a major role in spatial memory, the same pattern of results would not be predicted. Second, nonlearning controls provide another way to assess the contribution of nonspecific aspects of the testing procedures on Fos expression in new and existing neurons in the dentate gyrus. Learning impaired mutant mice (e.g., $CaMKII^{T286A}$) or pharmacological manipulations that prevent spatial learning

(e.g., NMDA receptor or protein synthesis inhibitors) may also be used.

Finally, as the hippocampus is involved in both spatial and nonspatial forms of learning, this protocol may easily be adapted for other hippocampus-dependent tasks (e.g., contextual fear conditioning, social transmission of food preference, trace eye-blink conditioning, object recognition). In adapting this protocol, two issues in particular are worth considering. First, training conditions that produce long-lasting memory (e.g., >4 weeks) are desirable, since the use of longer retention delays allows for greater flexibility in experimental design. For example, while contextual fear memories may last many weeks (or even months), object recognition memories are typically less enduring. Second, whereas the hippocampus is permanently involved in the expression of water maze memories[28], its role may be more transient in other forms of hippocampus-dependent learning (such as contextual fear and trace eye-blink conditioning[77]). However, it is clearly of great theoretical interest whether or not adult-generated granule cells also contribute in a persistent manner to the expression of these types of memory.

MATERIALS

REAGENTS

- Mice (see REAGENT SETUP)
- Chloral hydrate (Sigma, cat. no. C-8383) or other approved anesthetic
- Sodium phosphate, monobasic anhydrous (NaH_2PO_4, 1 kg, $F_W = 120.0$; Sigma, cat. no. S-0751)
- Sodium phosphate, dibasic anhydrous (Na_2HPO_4, 1 kg, $F_W = 142.0$; Sigma, cat. no. S-0876)
- Normal goat serum (Sigma, cat. no. G9023)
- Triton X-100 (*t*-octylphenoxypolyethoxyethanol, 100 ml; Sigma, cat. no. T-9284)
- Trisodium citrate dihydrate ($F_W = 294.10$; Sigma, cat. no. W302600)
- Paraformaldehyde powder (1 kg; Sigma, cat. no. P6148) (see REAGENT SETUP) ! CAUTION Harmful and should be handled with care. Wear proper protective gear, laboratory coat and weigh under fume hood.
- (+) BrdU (97%; Sigma-Aldrich, cat. no. 858811) (see REAGENT SETUP) ! CAUTION A suspected mutagen and should be handled with care. Wear proper protective gear, laboratory coat and weigh under fume hood.
- Sucrose (EMD, cat. no. SX1075-3) (see REAGENT SETUP)
- Rat anti-BrdU primary monoclonal antibody (Clone BU1/75,ICR1; Accurate Chemical & Scientific, cat. no. OBT0030). Optimal dilution ratio 1:500
- Rabbit anti-Fos polyclonal primary antibody (e.g., Calbiochem, cat. no. PC 38). Optimal dilution 1:5,000
- Alexa Fluor 488 chicken anti-rat IgG (H + L) (e.g., Invitrogen, cat. no. A21470) secondary antibody. Optimal dilution ratio 1:1,000
- Alexa Fluor 568 goat anti-rabbit/mouse IgG (H + L) (e.g., Invitrogen, cat. no. A11011) secondary antibody. Optimal dilution ratio 1:1,000
- Mouse anti-NeuN monoclonal primary antibody (e.g., Chemicon International, cat. no. MAB377). Optimal dilution 1:1,000
- Mounting medium (e.g., Shandon PermaFluor; Thermo Scientific, cat. no. 434990)
- PBS (see REAGENT SETUP)
- Hydrochloric acid (HCl)

EQUIPMENT

- Syringes (1 ml; Becton Dickinson, cat. no. 309602)
- $27^1/_2$ G, $^1/_2$ inch needle for injections (Becton Dickinson, cat. no. 305109)
- Perfusion pump (e.g., Cole-Parmer Instrument, cat. no. 77200-60)
- Glass vials or centrifuge tube (15 ml, 17 × 120 mm^2, Sterile, PPN; Greiner Bio-One, cat. no. 188261)
- Cryostat (e.g., Leica CM1850; Leica)
- 24-Well tissue cell culture polystyrene plates (e.g., Sarstedt, cat. no. 83.1836)
- Micro cover glasses (24 × 60 mm^2) (VWR, cat. no. 48404 454)
- Epifluorescent microscope (e.g., Nikon Eclipse 80i; Nikon ACT-1; Nikon)
- Confocal microscope (e.g., Olympus IX81DUS, Olympus; Image-Pro AMS)
- Analysis software (e.g., Image J; NIH) or other commercially available analysis software
- Water maze pool (see EQUIPMENT SETUP)
- Water maze tracking software (e.g., WaterMaze, Actimetrics)

REAGENT SETUP

Mice In our experiments we use offspring from a cross between C57BL/6 NTacfBr [C57B6] and 129Svev [129] mice (Taconic). We breed these mice in our colony at The Hospital for Sick Children. This hybrid strain is particularly suited for these types of experiments, since they learn well in the water maze and exhibit long-lasting water maze memories[17,28,78]. Other strains of mice may be used (e.g., C57B6 mice), but the amount of training and durability of memory may differ between strains[31,78]. Mice should be maintained on a 12-h light/dark cycle with free access to food and water. Mice should be at least 8 weeks of age at the start of experiments, and behavioral procedures conducted at the same time each day. Experiments should be conducted blind to the treatment condition of the mouse. ! CAUTION Several factors including housing conditions and age influence adult neurogenesis, and so care should be taken to match these variables across groups. ! CAUTION All experiments must be performed in accordance with relevant authorities' guidelines and regulations.

PBS, 0.1 M, pH 7.4 In 1–2-l beaker, add 2.7 g of NaH_2PO_4, 11.5 g of Na_2HPO_4 and 9 g NaCl. Add 1,000 ml of distilled water and stir. Measure pH, which should be ~7.4.

BrdU solution Heat PBS solution to 40–50 °C, and slowly dissolve BrdU by vortexing. Allow the BrdU injection solution to cool to room temperature (18–22 °C), and use immediately or store at 4 °C and use within 3 d. Longer-term storage is not recommended due to the formation of white BrdU precipitates (crystals). ! CAUTION There is some evidence that BrdU may be carcinogenic and produce developmental abnormalities when given to prenatal and neonatal animals[1,15]. The compound should be handled in the fume hood. BrdU should be handled with gloves.

Blocking solution, 0.1 M PBS, 0.3% Triton X-100, 2% serum In a 1–2-l beaker add 20 ml of normal goat serum, 3 ml of Triton X-100, 0.1 M PBS up to 1,000 ml and stir. Store in 50-ml aliquots at −20 °C.

4% Paraformaldehyde in 0.1 M PBS In 1–2-l beaker, heat ~800 ml of 0.1 M PBS to 60–65 °C while stirring. Once at 60–65 °C, add 40 g of paraformaldehyde powder slowly while stirring. (*Note*: Adding a few drops of 1 N NaOH helps to keep the solution clear.) Continue to stir until paraformaldehyde powder is dissolved, making sure that the temperature is maintained at 60–65 °C. Let the solution cool to room temperature. Filter solution and store at 4 °C. ! CAUTION Prepare in the fumehood and handle with gloves. May be stored at 4 °C for up to 1 week before using.

30% Sucrose in 0.1 M PBS In 1–2-l beaker add ~800 ml of 0.1 M PBS. Weigh 300 g of sucrose and stir until fully dissolved. Fill the beaker to 1,000 ml with 0.1 M PBS.

Antibody dilution All antibodies are diluted in blocking solution.

EQUIPMENT SETUP

The water maze Large circular tanks are typically used as water mazes. The absolute dimensions of water mazes vary greatly across studies, and there is no single correct size. However, water mazes designed for use with mice are typically smaller (e.g., 100–150 cm in diameter) than those designed for use with rats (e.g., 120–200 cm in diameter). The pool that we have used is 120 cm in diameter and 50 cm deep. The pool is filled to a depth of 40 cm with water made opaque by adding white nontoxic paint. Water temperature is maintained at 28 ± 1 °C by a heating pad located beneath the pool. The escape or goal platform may be circular or square. Our platform is circular and 10 cm in diameter, and represents ~1/144th (or <1%) of the total pool surface. Our platform is submerged 0.5 cm below the water surface and placed in one of the quadrants. The maze should be located in a sufficiently large room so that there is at least 1 m between the perimeter of the pool and the surrounding walls. Distinct, visual cues should be placed on the walls where they are clearly visible to the mice. They must remain in a fixed position throughout the experiment. There are a large number of commercially available tracking systems, which output a large range of measures during both training and probe tests (e.g., WaterMaze (Actimetrics), HVS Image, Noldus). During training, latency to reach the platform is the most widely reported measure of performance (although path length is also often used). During the probe test, one or more of the following are usually reported: time spent in each quadrant (percent time or percent swim path), time spent in target zone versus other equivalent

zones in pool (percent time), platform crossings (number) or average proximity (distance) are usually reported. Platform crossings are not recommended since their relatively low frequency of occurrence increases variability, thereby decreasing their sensitivity[24]. ! CAUTION Some laboratories maintain water at a lower temperature (e.g., ~20 °C). However, lower temperatures may induce significant hypothermia in mice, especially if short intertrial intervals are used during training[79]. The disadvantage of higher temperatures is that they may encourage floating behavior. However, floating can usually be avoided by handling mice extensively before beginning the experiment. ! CAUTION The relationship between platform size and pool size will determine the difficulty of the task. Using a smaller platform or a larger pool will increase task difficulty, and mice may not learn due to the lack of opportunities for reinforcement. ! CAUTION When platforms are submerged at depths >0.5 cm, there is an increased tendency for mice to continue swimming even after finding the platform. This is likely because the submerged platform does not offer sufficient escape from the water and is therefore less reinforcing. ▲ CRITICAL Pools that are too small (e.g., <1 m in diameter for mice), contain proximal cues (e.g., markings on the inner wall of the tank) or distal cues that are too close to the pool perimeter, may render the task hippocampus-independent.

PROCEDURE

Housing and acclimation

1| If mice are imported from a commercial vendor or other external source, allow mice to acclimate to the vivarium for at least 1 week before beginning experiments.

! CAUTION Stress associated with travel may influence both adult neurogenesis[51] and learning[80], and therefore increase variability. Mice should be group housed with littermates (three to five per cage), since social isolation negatively regulates adult neurogenesis[81] and learning[82].

BrdU injections

2| Inject mice with BrdU (100–200 mg kg^{-1}; i.p.), two times per day for 5 d. In rodents, including mice, the S phase of the cell cycle lasts ~8 h (ref. 21,83). Therefore, injections should be spaced at least 8 h apart in order to label distinct populations of dividing cells. For an i.p. injection, the lower abdominal cavity should be isolated. In mice we use a $27^1/_2$-gauge needle and a 1-ml syringe.

! CAUTION Care should be taken not to stress the mice during the injection, since stress negatively regulates adult neurogenesis.

▲ CRITICAL STEP The goal of the BrdU administration is to label a subset of dividing cells in the dentate gyrus, rather than all dividing cells. However, it is important that a sufficiently large sample is labeled, and so multiple injections over days are necessary to label many cells undergoing mitosis.

■ PAUSE POINT The delay between BrdU injections and handling/water maze training depends on the experimental design (**Fig. 4**). Preferably, both short (e.g., 1 week) and long (e.g., 6 weeks) delays should be used. For example, in our study we found minimal incorporation of newborn neurons using short BrdU injection-training delays and maximal incorporation of newborn neurons when delays of 6 weeks or longer were used[17].

Handling and water maze training

3| Before the commencement of training, individually handle mice for 2 min d^{-1} for 7 d. Place mice in the palm of the hand, occasionally picking up and replacing into hand to mimic handling during water maze training procedures.

▲ CRITICAL STEP Handling is important since it allows mice to get used to experimenter, and minimizes potentially confounding effects of stress and anxiety on learning and adult neurogenesis (e.g., survival).

4| Before training, mark the tails of mice with an indelible marker (or some other easy-to-see identifier). Since mice are group housed, tail-marking before the experiment facilitates identification during the behavioral procedures, thereby reducing delays between mice and minimizing disturbances to other littermates.

5| To start a block of training trials, place mouse on the platform in the pool for 15 s. Once 15 s has elapsed, pick up the mouse and place in pool at one of the four start positions (e.g., N, S, E or W). When placing the mouse in the pool, release the mouse gently with the mouse facing the wall. Start timer.

▲ CRITICAL STEP In order to minimize stress associated with handling, when possible avoid picking the mouse up by its tail. For example, when picking the mouse up from platform, slide your hand under the mouse and allow the mouse to climb into the palm of your hand.

6| Finish the trial when the mouse finds the platform or 60 s have elapsed (whichever occurs first). During the first few trials, the majority of mice will fail to find the platform. When this happens, at the end of the trial guide the mouse to the platform (e.g., because mice have a tendency to swim toward the experimenter, placing your finger on center of platform is sufficient to guide the mouse toward it).

7| Initiate a new trial using a new start position and repeat Steps 5–6. There are many possible training protocols for mice in the water maze. Because the goal of this training is to produce a robust and long-lasting spatial memory, we recommend a relatively intensive training schedule (six trials per day, presented in two blocks of three trials with an interblock interval of ~1 h and an intertrial interval of ~15 s). The order of start locations should be pseudo-randomly varied throughout training. Using these procedures, C57B6/129 mice learn rapidly and are able to express a robust spatial memory for a remarkably long

time, with little decrement in performance even with retention delays of 9 weeks[17]. Even 3 d of training is sufficient to produce a memory lasting at least a month in C57B6/129 mice[28]. However, more prolonged or more intensive training may be necessary to produce enduring memories in other mouse strains such as C57B6 (ref. 28).

▲ CRITICAL STEP Spaced (e.g., two blocks of three trials), rather than massed (six trials in a row), presentation of training trials is more effective at producing stable long-term spatial memory[82,84], and so is recommended here.

? TROUBLESHOOTING

Water maze testing

8| Either days or weeks after the completion of training (delay depends on experiment) give mice a series of probe tests to assess their spatial memory. In the probe test, place mouse on the platform for 15 s, and then pick the mouse up from the platform, remove the platform from the pool, and place mouse in the pool in a unique start position. (We normally use a start position 180° opposite the platform location). Finish probe test once 60 s has elapsed by removing mouse from pool. For each mouse, repeat two more times, with an intertrial interval of ~3 min. Several measures of performance may be used to assess spatial memory. These include the time spent in quadrant (percent time or percent swim path), time spent in target zone versus equivalent zones in pool (percent time), platform crossings (number) or average proximity to site of platform (distance).

! CAUTION In our experiments, we give mice three probe tests to maximize the induction of Fos in dentate granule cells associated with memory recall. However, one potential disadvantage of using multiple probe trials is that this may lead to the extinction of spatial memory. Since extinction involves the formation of a new inhibitory memory that competes with the original memory for control of behavior[85], it might be difficult to distinguish the Fos expression associated with the reactivation of the original spatial memory from the Fos expression associated with formation of a new extinction memory. However, we have found that three probe tests produce minimal extinction (although this may vary in different mouse strains).

▲ CRITICAL STEP Before and after the probe tests, mice should remain minimally stimulated to avoid nonspecific induction of Fos. In addition, care should be taken to ensure that procedures (e.g., transport of mice, housing and handling of mice, lighting conditions) are, as much as possible, identical to those used during training to avoid Fos induction associated with novelty.

Anesthesia

9| Ninety minutes after the start of the probe tests, anesthetize mice with chloral hydrate (400 mg kg^{-1}) or other approved anesthetic. After ~1–2 min, check if mouse is fully anesthetized. A fully anesthetized mouse should display the following properties: respiratory (breathing) rate should be regular and relaxed; withdrawal reflexes should be absent (e.g., lightly pinch tail); responses to external stimuli should be absent (e.g., no response to blowing air on the eye). If mouse is not fully anesthetized, inject mice with 25% of original dose and repeat monitoring.

! CAUTION To prevent blood clotting, the heart should still be beating at the time of the perfusion. Therefore, care should be taken not to overdose the mouse.

Transcardial perfusion

10| Expose heart using dissecting tools.

11| Insert needle connected to pump into the left ventricle. Make an incision in the right atrium to allow blood to flow out of the mouse's body. Needle may be held in place with small clamps.

12| Perfuse the mouse with 0.1 M PBS (rate 10–12 ml min^{-1}).

13| Once the draining blood becomes clear, perfuse the mouse with 4% (wt/vol) paraformaldehyde in 0.1 M PBS (rate 10–12 ml min^{-1}).

! CAUTION When switching from PBS to 4% paraformaldehyde avoid air bubbles, since this may result in blood coagulation.

▲ CRITICAL STEP Poor-quality perfusions will likely lead to poor immunohistochemical results. For example, incomplete clearance of blood is associated with autofluoresence of tissue, and poor fixation will lead to degradation of antigens. There are several hallmarks of a good perfusion, including the rapid, jerky movement of the limbs, whitening of the liver and paws and stiffening of the body.

Dissection

14| Using appropriate dissecting tools, remove the head and then remove the muscle and membranous tissue from the top part of the skull and gently extract the brain from the skull.

15| Cut the trigeminal and optic nerves, and let the brain fall into a beaker of cold 4% paraformaldehyde in 0.1 M PBS.

Postfixation

16| Immerse brain in the 4% paraformaldehyde in 0.1 M PBS for 24–48 h at 4 °C.

▲ CRITICAL STEP Overfixation (>2 d) may result in the lack of staining due to unavailability of antigens.

■ PAUSE POINT Brain tissue can be left for 1–2 d in the 4% paraformaldehyde in 0.1 M phosphate buffer at 4 °C.

17| Remove brain from the 4% paraformaldehyde in 0.1 M PBS solution, and immerse the brain in 30% sucrose in 0.1 M PBS solution until the brain sinks to the bottom (usually takes 24–48 h).
■ **PAUSE POINT** Brain tissue can be left for 1–2 d in the 30% sucrose in 0.1 M PBS at 4 °C.
? TROUBLESHOOTING

Sectioning
18| Section tissue into 50-µm coronal slices using a cryostat. Transfer sections to 24-well plates loaded with 0.1 M PBS (pH 7.4), including 0.02% sodium azide. Collect four sets of sections per brain. Typically, we collect 40–48 sections per dentate gyrus, and so there are 10–12 sections per set.
▲ **CRITICAL STEP** Sections should be cut immediately following Step 17. Brain tissue kept in 30% sucrose in 0.1 M PBS solution for >1 week might affect the morphology and compromise analysis.
■ **PAUSE POINT** Sections may be stored at 4 °C for several weeks.

DNA denaturation
19| Rinse sections three times, 5 min each with 0.1 M PBS (pH 7.4) on a shaker.

20| Denature DNA by incubating sections in 1 N HCl for 30 min at 45 °C.
▲ **CRITICAL STEP** Denaturating the cellular DNA into single strands is necessary to expose incorporated BrdU to the BrdU antibody. Various denaturation procedures (ethanol treatment, enzyme treatment) may be used[45]. However, we recommend using the heated HCl treatment since it results in more effective exposure of the halogenated-nucleotide antigen. This step is critical, and careful adjustments may be necessary to optimize results. Incomplete denaturation makes it difficult to detect BrdU-labeled cells, while some harsher denaturation procedures may damage tissue. For example, incubation with high concentrations of HCl (>2 N HCl) at high temperatures (>65 °C) can be detrimental to other antigens (particularly surface antigens and receptors), and so will interfere with Fos immunohistochemistry.

21| Neutralize the acid by rinsing sections three times, 5 min each with 0.1 M PBS (pH 7.4) on a shaker.
? TROUBLESHOOTING

BrdU and Fos immunohistochemistry
22| Incubate sections with BrdU (1:500–1:1,000) and Fos (1:5,000–1:10,000) primary antibodies diluted in blocking solution for 48–72 h at 4 °C on a shaker. To control for nonspecific BrdU staining, BrdU immunohistochemistry may be conducted in control mice that were not injected with BrdU. To check for nonspecific cross-reactions, omit the addition of the primary antibody.
■ **PAUSE POINT** Sections can be left for 2–3 d at 4 °C.

23| Rinse sections three times, 5 min each in 0.1 M PBS (pH 7.4) on a shaker.

24| Incubate sections with fluorochrome-conjugated secondary antibodies (Alexa Fluor 488 chicken anti-Rat and Alexa Fluor 568 goat anti-Rabbit; 1:500 to 1:1,000) in the dark, diluted in 0.1 M PBS (pH 7.4) with 0.3% Triton-X solution for 2 h at room temperature on a shaker. Detergents such as Triton-X can be added to the secondary antibody diluent to reduce hydrophobic interaction between tissue and reagent proteins, thus reducing nonspecific binding of secondary antibodies.
! **CAUTION** Exposing tissue to light can lead to photobleaching. Wrap 24-well plates in aluminum foil to avoid exposure to light.

25| Rinse sections three times, 5 min each in 0.1 M PBS (pH 7.4) on a shaker.

26| Carefully transfer sections to slides using a soft brush.

27| Add antifade mounting medium (PermaFluor) and place coverslips. Allow to dry for 2 d.
■ **PAUSE POINT** Mounted brain sections at 4 °C may be stored in the dark for up to 2 months before imaging with microscope.

Quantification
28| Using epifluorescent and confocal microscopes, quantify numbers of $BrdU^+$ (option A), Fos^+ (option B) and $BrdU^+/Fos^+$ (option C) cells. Quantification may be limited to the granule cell layer[17] or additionally include cells in the subgranular zone (defined as a two-cell-body-wide zone bordering the granule cell layer and the hilus) (**Fig. 5a**). Because Fos expression in the subgranular zone is generally lower, quantification of BrdU-labeled cells in this region will lead to a modest reduction in the proportion of BrdU-labeled cells expressing Fos. We do not recommend quantifying BrdU-labeled cells in the hilus as the contribution of hilar cells to learning and memory is poorly understood. Numbers of Fos^+, $BrdU^+$ and $Fos^+/BrdU^+$ cells may be normalized per fixed area of the dentate gyrus or per section. Alternatively, absolute numbers for the entire dentate gyrus may be projected[86].
▲ **CRITICAL STEP** Quantification should be conducted blind to experimental condition. Where possible, counts should be verified by a second observer.

(A) Fos

(i) Using an epifluorescent microscope with a ×10 objective, take 2D images of sections containing the dentate gyrus.

(ii) Using threshold-based software (e.g., Image J), quantify Fos$^+$ cells in these sections. Gradually, increase the threshold so that all Fos$^+$ cells above background are selected.

(iii) Record total area of dentate gyrus for each section.

! CAUTION Sections are relatively thick (50 μm). Therefore, there is a possibility that exposure to the Fos antibody may not be uniform throughout the section, resulting in fainter labeling of the cells located in the middle of the section. The threshold should be carefully adjusted to include faintly labeled cells that are clearly distinguishable from background noise. Fos-labeled cells should be appropriately sized (~10 μm in diameter) and shaped (round).

? TROUBLESHOOTING

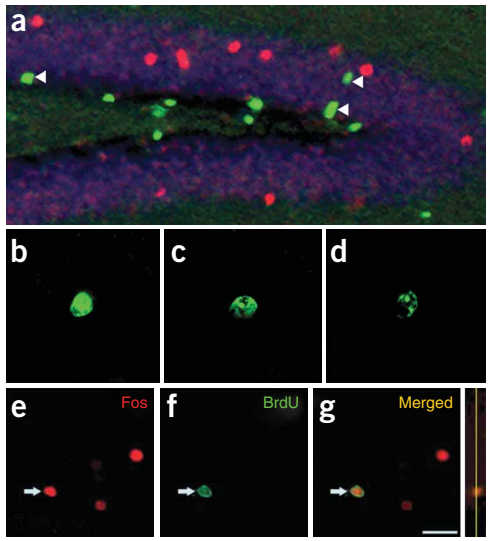

Figure 5 | Examples of Fos- and 5-bromo-2′-deoxyuridine (BrdU)-labeling in the dentate gyrus. (**a**) Low magnification merged image of the dentate gyrus showing Fos immunofluorescence (red), BrdU immunofluorescence (green) NeuN immunofluorescence (blue). Arrow heads mark three BrdU-labeled cells located within the granule cell layer. Within the granule cell layer, note that the majority of BrdU-labeled cells are located in the innermost third. In contrast, the majority of Fos-labeled cells are located in the outmost regions of the granule cell layer. (**b**–**d**) Examples of BrdU-labeled cells in the dentate gyrus. BrdU-labeling may be (**b**) uniform, or more patchy (**c**,**d**), possibly due to dilution of BrdU signal through redivision. Note that despite the patchy labeling, it is evident that the cell is appropriately shaped (round) and sized (~10 μm). Representative confocal images of (**e**) Fos$^+$ (red), (**f**) BrdU$^+$ (green) and (**g**) Fos$^+$/BrdU$^+$ (arrow) cells in the dentate gyrus after probe testing (scale bar = 20 μm). Adapted from Kee *et al.*[17].

(B) BrdU

(i) Using an epifluorescent microscope with a ×40 objective, quantify BrdU$^+$ cells manually, moving in and out of focus throughout the entire granule cell layer. Scrutinize putative BrdU$^+$ cells for size of nucleus (~10 μm in diameter) and shape (round). Note that for some BrdU$^+$ cells, labeling of nucleus may be nonuniform and appear patchy (**Fig. 5b–d**).

! CAUTION BrdU-labeled cells may redivide and this raises two concerns. First, redivision may compromise the temporal resolution of labeling. However, the vast majority of redivision is limited to a few days immediately after BrdU treatment, and therefore temporal resolution is only minimally compromised[35,39]. Second, multiple rounds of redivision may lead to dilution of the BrdU signal. While dilution of signal would be a problem in quantifying the absolute number of BrdU$^+$/Fos$^+$ cells, it will not affect quantification of the proportion of BrdU-labeled cells that express Fos (the primary dependent measure in these studies).

? TROUBLESHOOTING

(ii) *BrdU/Fos double-labeling*. Using an epifluorescent microscope with a ×40 objective, quantify the phenotype (Fos$^+$/Fos$^-$) of all identified BrdU$^+$ cells manually, moving in and out of focus throughout the entire granule cell layer.

(iii) Verify identified double-labeled cells on a confocal microscope with a ×40 objective (**Fig. 5e–g**).

! CAUTION To exclude false double-labeling due to the overlay of signals from different cells located in the same *z*-axis, analyze BrdU$^+$ cells by moving through the entire *z*-axis of each cell to ensure signals come from the same source. To avoid cross-detection of signals (e.g., Alexa 488 signals in the Alexa 568 channel or vise versa), the fluorescent images should be examined sequentially for each channel.

! CAUTION We recommend quantifying Fos$^+$, BrdU$^+$ and BrdU$^+$/Fos$^+$ cells through the entire anterior–posterior extent of the dentate gyrus for two reasons. First, the low frequency of BrdU$^+$/Fos$^+$ cells renders sampling approaches (e.g., stereological counting) less appropriate. Second, in the dentate gyrus Fos labeling is sparse after behavioral testing (typically <2.5 % of all cells). Therefore, overlap in the *z*-axis is rare, and permits 2D images to be used for quantification of Fos labeling.

? TROUBLESHOOTING

● TIMING

Step 1, housing and acclimation: 1 week
Step 2, BrdU injections: 5 d
Step 3, Handling: 1 week
Steps 4–7, water maze training: 5 d

Step 8, probe test and Steps 9–15, perfusion: 1 d
Steps 16 and 17, postfixation: 3–4 d
Step 18, sectioning: 2–3 d
Steps 19–21, DNA denaturation: 2–3 d
Steps 22–27, BrdU and Fos immunohistochemistry: 1 d
Step 28, quantification: 1–4 weeks

? TROUBLESHOOTING

Step 7, Morris water maze

Performance problems are somewhat more common in mice compared to rats. The most common of these are difficulty climbing onto the platform, a propensity not to remain on the platform during the intertrial interval and floating. The frequency of these performance problems varies greatly amongst strains, but extensive handling before the commencement of training is the best approach to reduce the incidence of these problems. If they do occur, then the important decision is how to treat these mice. The general rule is persistence. These problems occur more frequently on the first day of training, but, in most cases, will disappear without any experimenter intervention over subsequent days. For example, mice that choose to swim during the intertrial interval (rather than stay on the platform) eventually get tired and learn to stay on the platform once they find it. The one issue that may persist is floating. While some experimenter interventions effectively reduce floating (e.g., startling the mouse by clapping hands), they are difficult to apply uniformly. Since persistent floating is relatively uncommon, mice that persistently float should be removed from the experiment.

More troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
16–17	Fos: Poor positive staining and/or no positive staining with little or no background staining	Overfixation with 4% paraformaldehyde in 0.1 M PBS	Do not leave brain tissue in fixative for >24 h
19–21	BrdU: Poor positive staining and/or no positive staining with little or no background staining	Denaturation of DNA by hydrochloric acid (HCl) was not sufficient	Use higher concentration of HCl and/or longer incubation time. Also use increased temperature with HCl incubation
22–28	5-Bromo-2′-deoxyuridine (BrdU)/Fos: Poor positive staining and/or no positive staining with little or no background staining	BrdU/Fos primary and secondary antibody concentration was not optimal	Titrate BrdU/Fos primary antibody upon arrival as concentrations may vary across batches
		Incubation time with primary and/or secondary antibody was too short	Use a longer incubation time for primary antibody and/or secondary antibodies
		The primary antibody does not recognize the antigen due to incorrect fixation or overfixation	Include antigen retrieval step. *Note*: For BrdU primary antibody, overfixation does not affect BrdU immunostaining
		Brain sections were left to dry	Do not let sections dry out and keep wet at all times during the staining procedure
	BrdU/Fos: Nonspecific and/or high background staining	Nonspecific binding of primary or secondary antibody	Increase the number and time of washes in between steps
		Incubation time with BrdU/Fos primary antibody was too long	Reduce BrdU/Fos primary antibody incubation time
		Blocking reaction failed to prevent nonspecific binding of the secondary antibodies	Increase length and/or concentration of incubation with blocking solution made from the same species as the host of the secondary antibody
		Aggregates binding	Centrifuge antibody stock briefly in microcentrifuge at high speed to remove aggregates
		Cross-reactivity between secondary antibodies	Source of both secondary antibodies should ideally come from same host (e.g., goat anti-rat and goat anti-rabbit)

ANTICIPATED RESULTS

The outlined protocol utilizes immunohistochemical approaches to visualize the recruitment of new neurons into the circuits supporting water maze memory in intact animals. BrdU treatment should result in robust labeling of new neurons in the dentate gyrus. These should be predominantly located in the innermost layers of both the upper and lower blade of the dentate gyrus. Furthermore, numbers of BrdU-labeled cells should decrease as the post-BrdU treatment survival times lengthen. After water maze testing, induction of Fos should be expected in ~0.5–2.5% of granule cells. Fos expression is typically more prevalent in the outermost layers of both the upper and lower blade of the dentate gyrus. The overlap between these two populations should be sensitive to the delay between BrdU treatment and training in the water maze. At delays beyond 4 weeks, maximal numbers of $BrdU^+/Fos^+$ cells are expected, indicating that as new neurons mature they become increasingly likely to be incorporated into the circuits supporting spatial memory in the dentate gyrus.

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