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ARTICLES

Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain

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Neurogenesis occurs continuously in the forebrain of adult mammals, but the functional importance of adult neurogenesis is still unclear. Here, using a genetic labeling method in adult mice, we found that continuous neurogenesis results in the replacement of the majority of granule neurons in the olfactory bulb and a substantial addition of granule neurons to the hippocampal dentate gyrus. Genetic ablation of newly formed neurons in adult mice led to a gradual decrease in the number of granule cells in the olfactory bulb, inhibition of increases in the granule cell number in the dentate gyrus and impairment of behaviors in contextual and spatial memory, which are known to depend on hippocampus. These results suggest that continuous neurogenesis is required for the maintenance and reorganization of the whole interneuron system in the olfactory bulb, the modulation and refinement of the existing neuronal circuits in the dentate gyrus and the normal behaviors involved in hippocampal-dependent memory.

Neurogenesis occurs continuously in two brain regions of adult rodents, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus[1–6]. A huge number of neurons born in the SVZ migrate into the olfactory bulb, form a rostral migratory stream and differentiate into local interneurons (granule and periglomerular cells), whereas neurons born in the SGZ become granule cells of the dentate gyrus. It has been suggested that old neurons are replaced by new neurons in the olfactory bulb, as the size of the olfactory bulb does not substantially change throughout life[7–10], whereas neurogenesis contributes to the increase in neuronal number in the dentate gyrus in adult rats[11–15]. Furthermore, it has been shown that newly formed neurons are incorporated into the functional networks of both the olfactory bulb and the dentate gyrus, suggesting important roles for adult neurogenesis in brain functions[16–18].

Despite these extensive studies, the importance of adult neurogenesis is still obscure. It has been extensively analyzed by labeling neural stem cells (NSCs) and neural progenitors with [^{3}H]-thymidine, BrdU or retrovirus, and by ablating these cells using irradiation or with anti-mitotic drugs. However, these methods have only limited specificity and/or efficiency[19], and the extent to which neurons are replaced and added, and whether neurogenesis is required for the normal structure and function of the adult brain, remains to be determined. For example, no definitive evidence is available concerning whether the majority of granule cells are replaced or whether only subsets of granule cells are repeatedly replaced in the olfactory bulb, and it is essential to determine how newly formed neurons are integrated into the pre-existing neural circuit to understand the functional importance of adult neurogenesis. Furthermore, although it has been suggested that adult neurogenesis is involved in spatial memory formation[18,20,21], disorders in such brain functions were not observed in rats, 129/Sv mice or mixed background mice whose neurogenesis had been reduced by treatment with anti-mitotic drugs or irradiation[22,23]. These discrepancies could be the results of differences in species, mouse strains and/or the efficiency of neurogenesis blockade.

To address these issues, we used genetic approaches that specifically and efficiently labeled NSCs and ablated newly formed neurons in the adult brain of mice in the C57BL/6 background, a standard transgenic mouse strain for behavioral tests[24]. Similar labeling analyses have been recently reported, but only short periods (about 2–3 months) were examined and no ablation experiments were performed[25,26]. Our long-term labeling and ablation study provides evidence that continuous neurogenesis is required for the normal structure of the forebrain and behaviors in contextual and spatial memory tests.

RESULTS

Genetic labeling of NSCs in the SVZ

To mark NSCs and their progeny, we used Nes-CreER^{T2} mice, in which a tamoxifen-inducible Cre recombinase (CreER^{T2}) is expressed under the control of a nestin promoter and enhancer[27]. In line 5-1 of Nes-CreER^{T2} mice, the CreER^{T2} protein was expressed in the SVZ of the lateral ventricles (**Fig. 1a**). Of the CreER^{T2}-positive cells, 85.1 ± 0.4%

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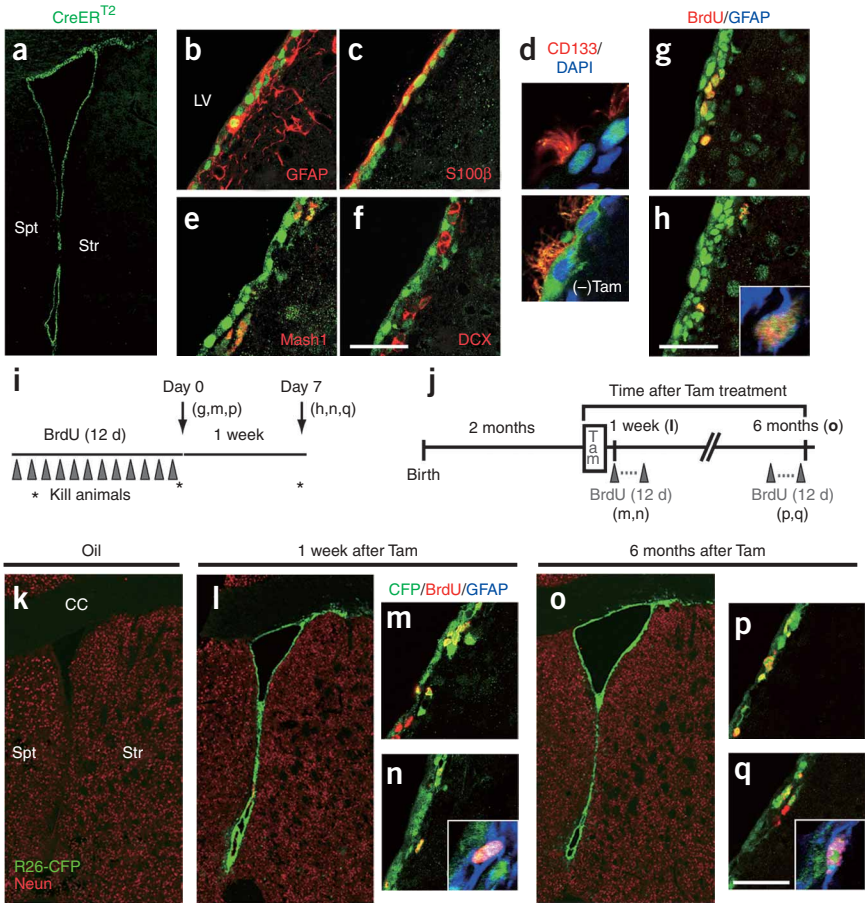

Figure 1 Tamoxifen-induced Cre recombinase activity in adult NSCs in the SVZ of the lateral ventricles of Nes-CreER^{T2} mice. (**a**–**f**) Immunostaining of coronal sections of the SVZ of the lateral ventricles (LV) in Nes-CreER^{T2} mice. Sections were examined 24 h after tamoxifen (Tam) treatment, except for the lower panel in **d**. CreER^{T2}-positive cells (green) expressed GFAP (**b**), S100β (**c**), CD133 (**d**) and Mash1 (**e**), but not DCX (**f**). CreER^{T2} protein (green) moved efficiently from the cytoplasm to the nuclei after tamoxifen treatment (**d**). (**g**,**h**) We double labeled cells for CreER^{T2} (green) and BrdU immediately (**g**) or 1 week (**h**) after BrdU treatment. Inset shows colocalization of CreER^{T2} protein, BrdU and GFAP. (**i**,**j**) Experimental designs. (**k**) No CFP expression occurred in oil-treated Line 5-1/R26-CFP mice. (**l**–**q**) Tamoxifen treatment led to CFP expression in 1 week (**l**), which was maintained for 6 months (**o**). BrdU was incorporated and retained in CFP$^+$ cells of Line 5-1/R26-CFP mice 1 week (**m**,**n**) and 6 months (**p**,**q**) after tamoxifen treatment. cc, corpus callosum; Spt, septum; Str, striatum. Scale bars represent 50 μm.

expressed glial fibrillary acidic protein (GFAP; **Fig. 1b** and **Supplementary Table 1** online), which marks NSCs (Type B cells), as well as mature astrocytes and ependymal cells[28]. About a half of the CreER^{T2}-positive cells abutted the lateral ventricles and expressed the ependymal cell markers S100β and CD133 (**Fig. 1c**,**d**), whereas the other half were present in the SVZ and did not express S100β and CD133, suggesting that the latter half included NSCs. Subsets of the CreER^{T2}-positive cells were located internally in the SVZ and expressed Mash1, a marker for transit-amplifying cells (Type C cells; **Fig. 1e**)[29]. However, the CreER^{T2}-positive cells mostly did not express neuroblast marker doublecortin (DCX) (**Fig. 1f**). To determine whether the CreER^{T2}-positive cells were dividing, we examined BrdU uptake (**Fig. 1g–i**). At day 0 after a 12-d administration of BrdU, all dividing cells, including slowly dividing NSCs, were labeled, whereas slowly dividing cells such as NSCs selectively retained BrdU at day 7, as fast-cycling cells dilute out the incorporated BrdU. We designated BrdU$^+$ cells at day 0 as dividing progenitors and BrdU- and GFAP-positive cells at day 7 as long-retaining cells. Nearly all of the dividing progenitors and long-retaining cells expressed CreER^{T2} (**Fig. 1g**,**h** and **Supplementary Table 1**). These results indicate that NSCs and transit-amplifying cells express CreER^{T2}.

We next examined the efficiency of tamoxifen-induced recombination by using Line 5-1/R26-CFP or Line 5-1/R26-LacZ mice, in which activation of CreER^{T2} results in permanent expression of cyan fluorescent protein (CFP) or LacZ from the Rosa26 locus in NSCs and their progeny. We treated 2-month-old adult mice with tamoxifen and examined their recombination efficiency 1 week or 6 months later (**Fig. 1j**). Oil was administered as a negative control and did not show any recombination (**Fig. 1k**). Within 1 week of tamoxifen treatment, CFP was expressed in the same pattern as CreER^{T2} protein, and this CFP expression was maintained for at least 6 months (**Fig. 1l–q**). Furthermore, electron-microscopic analysis revealed deposits of X-gal reaction products in Type B cells near the lateral ventricles of Line 5-1/R26-LacZ mice, which were treated with tamoxifen 1 week before (**Supplementary Fig. 1** online). CreER^{T2}-mediated recombination occurred in about 60–70% of BrdU-positive dividing progenitors and BrdU-positive long-retaining cells both 1 week and 6 months after tamoxifen treatment (**Fig. 1m**,**n**,**p**,**q**). Thus, the NSCs that were genetically labeled at 2 months of age were maintained and were dividing in the SVZ, even after 6 months, and therefore 60–70% of all progeny born after 2 months of age were also labeled. CreER^{T2} expression and CreER^{T2}-mediated recombination occurred similarly along the entire anterior-posterior and dorsal-ventral axes of the SVZ (**Supplementary Fig. 1**), suggesting that there is no regional difference in recombination efficiency in the SVZ. We also examined another line of Nes-CreER^{T2} mice (Line 4) and obtained the same results (data not shown).

Replacement of granule cells in the adult olfactory bulb

We followed the fate of NSCs of Line 5-1/R26-CFP mice, which were treated with tamoxifen for 4 d consecutively at 2 months of age. Progressive CFP labeling was observed in DCX$^+$ neuroblasts in the SVZ, the rostral migratory stream and the core region of the olfactory bulb in 10 d of tamoxifen treatment, suggesting that neuroblasts born from labeled NSCs migrated from the SVZ into the olfactory bulb (**Supplementary Fig. 2** online). However, more mature neurons (CFP$^+$NeuN$^+$) did not appear in the olfactory bulb at this stage (**Supplementary Fig. 2**). When BrdU was administered at day 10 after tamoxifen treatment, newly formed neuroblasts (CFP$^+$BrdU$^+$DCX$^+$) appeared in the core region in 7 d (by day 17), whereas more mature neurons (CFP$^+$BrdU$^+$NeuN$^+$) appeared in the granule cell layer in 14 d (by day 24) (**Supplementary Fig. 3** online). By day 31, these newly formed neurons had many synaptic spines on their dendrites and expressed GAD67 and calretinin, suggesting

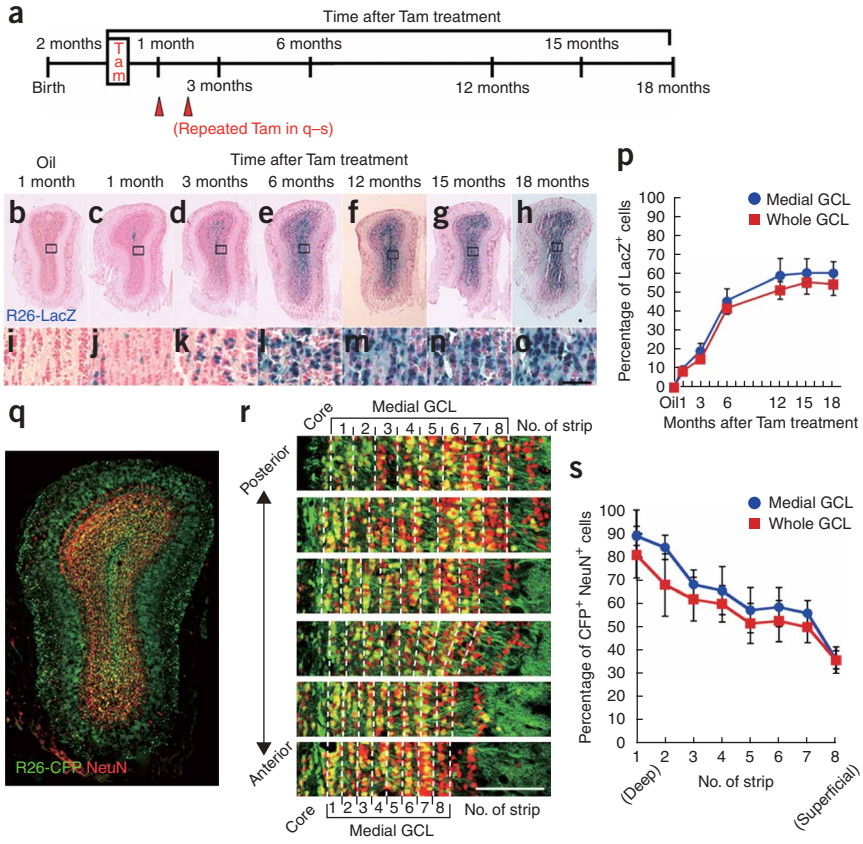

Figure 2 Continuous adult neurogenesis in the olfactory bulb visualized by long-term labeling of NSCs. (**a**) Experimental design. (**b**–**p**) The olfactory bulb of oil-treated (**b**,**i**) and tamoxifen-treated (**c**–**h**,**j**–**p**) Line 5-1/R26-LacZ mice. Granule cells in the olfactory bulb were progressively labeled with lacZ after tamoxifen treatment. (**i**–**o**) A higher magnification of boxed regions in **b**–**h**. (**q**–**s**) The olfactory bulb of Line 5-1/R26-CFP mice, which received repeated administration of tamoxifen, was examined 12 months later. Posterior-to-anterior coronal sections of the granule cell layer were divided into eight strips, and the proportions of CFP labeling (green) per NeuN+ granule cells (red) were determined (**r**,**s**). Note that CFP labeling of granule cell dendrites was observed outside of the granule cell layer. Scale bars represent 50 μm in **i**–**o** and 100 μm in **r**.

that they had become mature granule cells (**Supplementary Fig. 3**). We also treated Line 5-1/R26-LacZ mice with tamoxifen at 2 months of age and analyzed the long-term fate of the marked cells (**Fig. 2**). This analysis revealed a gradual increase in labeled cells in the granule cell layer of the olfactory bulb (**Fig. 2c–h,j–o**). Similarly, labeled periglomerular cells were increased in number (data not shown). In contrast, no such labeled cells were detectable in oil-treated mice (**Fig. 2b,i** and **Supplementary Fig. 3**).

The proportion of labeled granule cells in the olfactory bulb was about 40% after 6 months and increased to about 50–60% after 12–18 months, indicating that the labeling was saturated between 6 and 12 months after tamoxifen treatment (**Fig. 2p** and **Supplementary Table 2** online). This labeling efficiency in the olfactory bulb was mostly the same as the recombination efficiency of NSCs in the SVZ. On the basis of the recombination efficiency, we estimated that the majority of pre-existing granule cells in the olfactory bulb were replaced by new neurons by 12 months after tamoxifen treatment, although it is possible that a small population of granule cells are maintained stably without replacement. To analyze this point further, we repeatedly administered tamoxifen to achieve a higher degree of recombination. The recombination rate in the SVZ of Line 5-1/R26-CFP mice reached 86.8 ± 2.5% after three rounds of tamoxifen injections. As expected, most NeuN$^+$ granule cells became CFP$^+$ in the deep region of the olfactory bulb by 12 months after tamoxifen treatment (**Fig. 2q–s** and **Supplementary Table 3** online). These results suggest that in the deep region nearly all pre-existing granule cells are replaced by new neurons. In contrast, only a half became CFP$^+$ in the superficial region (**Fig. 2q–s** and **Supplementary Table 3**). It was reported that granule cells born during an early postnatal stage predominantly settle in the superficial region of the granule cell layer and survive longer[30]. Thus, it is likely that about half of the granule cells in this region are not replaced, but instead persist throughout life.

Ablation of newly born neurons affects the olfactory bulb

Our data indicated that the majority of granule cells have a short lifetime and are replaced by newly formed neurons in the olfactory bulb, especially in the deep regions. We next asked whether the death of old neurons is induced by a supply of new neurons or whether old neurons die even in the absence of new neurons. To address this question, we genetically ablated newly generated neurons using NSE-DTA mice[31]. These mice carry the loxP-STOP-loxP-IRES-diphtheria toxin fragment A (DTA) gene cassette, which was knocked into the 3′-noncoding region of the neuron-specific *enolase 2* (*Eno2*) gene (referred to here as NSE; **Fig. 3**). Crossing NSE-DTA mice with Nes-CreER^{T2} mice and administering tamoxifen led to deletion of the STOP region in the DTA cassette in NSCs. When these NSCs began neuronal differentiation, DTA was expressed from the *Eno2* locus, thereby killing the cells (**Fig. 3a**).

Line 5-1/NSE-DTA mice were treated with oil or tamoxifen at 2 months of age, and the subsequent defect was examined. Oil treatment did not induce cell death in these mice (**Supplementary Fig. 4** online). In contrast, tamoxifen treatment induced cell death that was specific to the neuronal lineage (**Supplementary Fig. 5** online). Many neuroblasts became TUNEL positive at the entry site of the rostral migratory stream in 7 d (**Supplementary Fig. 6** online). Furthermore, the number of DCX$^+$ neuroblasts started to decrease at the entry site and in the rostral migratory stream at day 7 and beyond (**Supplementary Fig. 6**), although no substantial defect was observed in the olfactory bulb at this stage (**Fig. 3g,h,q,r**). Around 3 weeks after tamoxifen treatment, many newly formed neurons (BrdU$^+$NeuN$^+$) were ablated in the olfactory bulb (**Supplementary Fig. 7** online), and granule cells started to decrease in number in the olfactory bulb (**Fig. 3i,s** and **Supplementary Table 4** online). The reduction in neuronal number in the olfactory bulb became more severe 6 and 12 weeks after tamoxifen treatment (**Fig. 3j,k,t–v** and **Supplementary Table 4**). In this experiment, more granule cells were lost in the deep region, whereas many neurons were preserved in the superficial region, consistent with the above labeling experiment, which indicates that

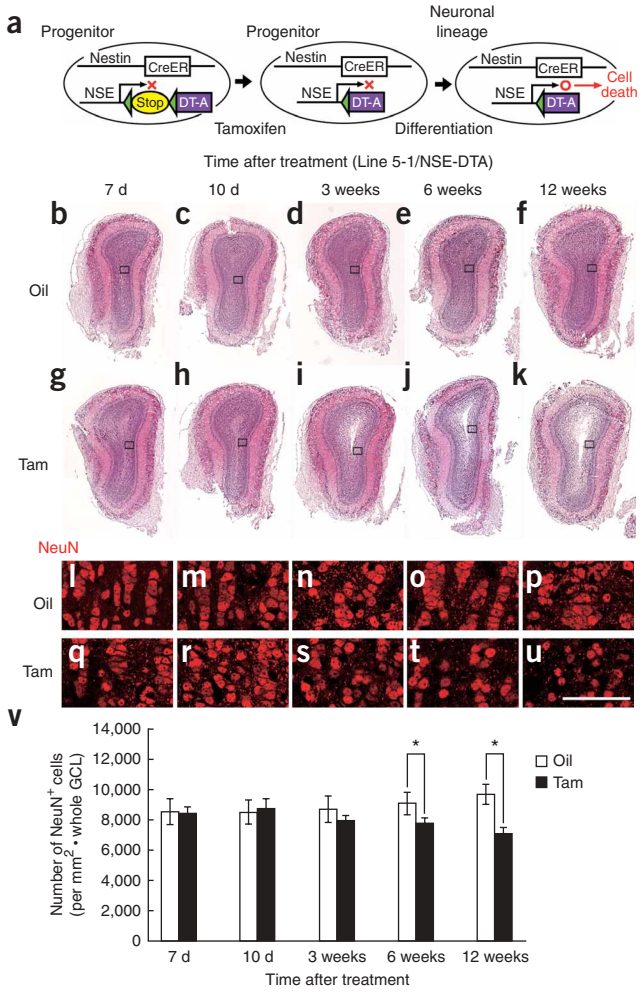

Figure 3 Ablation of neurogenesis decreases the number of granule cells in the olfactory bulb. (**a**) Experimental design. (**b**–**k**) Hematoxylin-Eosin (HE) staining of coronal sections of the olfactory bulb in oil- and tamoxifen-treated Line 5-1/NSE-DTA mice. (**l**–**u**) Boxed regions in **b**–**k** are enlarged and were stained for NeuN. (**v**) Quantification of the number of NeuN$^+$ granule cells in the olfactory bulb of oil- and tamoxifen-treated Line 5-1/NSE-DTA mice. * $P < 0.05$, *t* test. Scale bars represent 50 μm.

more profound neuronal replacement occurs in the deep region than in the superficial region. Similar phenotypes were observed in tamoxifen-treated Line 4 crossed with NSE-DTA mice (data not shown).

We next examined whether DTA expression in newly formed neuroblasts and neurons affects the survival of pre-existing cells. In the olfactory bulb, there was no substantial difference in the number of TUNEL-positive cells between oil- and tamoxifen-treated Line 5-1/NSE-DTA mice (**Supplementary Fig. 7**), indicating that the loss of neurons in the olfactory bulb was not the result of an increase in the death of old neurons, but probably resulted from the lack of supply of new neurons from the SVZ. We also labeled NeuN$^+$ granule cells with BrdU before oil or tamoxifen treatment and compared their survival between DTA-expressing (tamoxifen-treated) and non–DTA-expressing (oil-treated) mice (**Fig. 4**). Pre-existing NeuN$^+$ granule cells labeled with BrdU were found to survive similarly in oil- and tamoxifen-treated Line 5-1/NSE-DTA mice (**Fig. 4b–j,l–t**). Even after 24 weeks, the number of BrdU$^+$NeuN$^+$ neurons was similar between oil- and tamoxifen-treated mice, indicating that tamoxifen treatment did not abnormally induce death of pre-existing cells (**Fig. 4e,i,j**). Some neurons were born from nonrecombined NSCs (**Supplementary Fig. 6**), but they did not seem to reach the olfactory bulb efficiently, probably because chain migration was disrupted. These data indicate that old granule cells die irrespective of a supply of new neurons and that continuous neurogenesis is essential for maintenance of the granule cell number in the olfactory bulb.

Ablation of newly born neurons does not affect olfaction

We next examined whether the supply of new neurons is required for discrimination and memory of odors. Oil-treated Line 4/NSE-DTA mice showed similar behaviors to wild-type mice in all of the tests that we examined and thus were used as a control. Tamoxifen treatment 3 to 4 weeks prior did not affect spontaneous discrimination and innate olfactory preference between two different odors (**Supplementary Fig. 8** online), suggesting that blockade of neurogenesis in the adult brain does not affect the odor discrimination ability or innate olfactory response. Line 4/NSE-DTA mice that were treated with oil or tamoxifen 3 to 4 weeks prior were next trained for 4 d to associate one of two related odorants (enantiomers) with the sugar reward. On day 5, we separately placed both odors without sugar below bedding (5 cm depth) and measured the digging time spent near each odor. Both oil- and tamoxifen-treated Line 4/NSE-DTA mice spent significantly more time near the odorants associated with sugar rewards ($P < 0.05$; **Fig. 5a,b**). These results indicate that both oil- and tamoxifen-treated mice were well able to acquire odor-associated memory.

These trained mice were subjected to the same trials 1 week and 2 months later without further training. Again, both oil- and tamoxifen-treated Line 4/NSE-DTA mice spent substantially more time near the odorants that were associated with sugar rewards (**Fig. 5c–f**). Similar results were obtained with other sets of enantiomers, 2-butanol and 2-octanol (data not shown). Thus, adult neurogenesis is not required for long-term retention of odor-associated memory. Furthermore, even after 6 months, both oil- and tamoxifen-treated Line 4/NSE-DTA mice were able to acquire odor-associated memory (**Supplementary Fig. 9** online). We obtained the same results with oil- and tamoxifen-treated Line 5-1/NSE-DTA mice (data not shown). Thus, adult neurogenesis is not required for acquisition of odor-associated memory, although more difficult tasks about odor-associated memory could depend on neurogenesis.

Addition of granule cells in the dentate gyrus

We next examined neurogenesis in the dentate gyrus. R26-CFP mice were crossed with Line 4, which have a higher recombination efficiency in the SGZ than Line 5-1. In Line 4, CreER^{T2} was expressed by many GFAP$^+$ NSCs and by a few neuroblasts (DCX$^+$) in the SGZ, but not by mature granule cells (NeuN$^+$) (**Supplementary Fig. 10** online). Tamoxifen was administered at 2 months of age and the fate of labeled cells was examined. BrdU uptake indicated that 66.8 ± 5.8% of the dividing cells showed recombination in the SGZ (**Supplementary Fig. 10**). Labeled granule cells gradually increased in number and expanded into the granular cell layer, mostly in its inner side, as previously described[14,32,33], of the dentate gyrus only after tamoxifen treatment (**Fig. 6a–j** and **Supplementary Fig. 10**). A fraction of new neurons (NeuN$^+$, 5.02 ± 0.93%) were labeled at 3 months, whereas about 10% were labeled at 6, 9 and 12 months after tamoxifen treatment (**Fig. 6j** and **Supplementary Table 5** online). Distribution of the CFP-labeled cells was rather uniform along the anterior-posterior and dorsal-ventral axes (**Fig. 6k–o**). Thus, the proportion of labeled neurons increased to about 10% of the total neurons in the dentate gyrus, but this increase stopped at around 6 months after treatment.

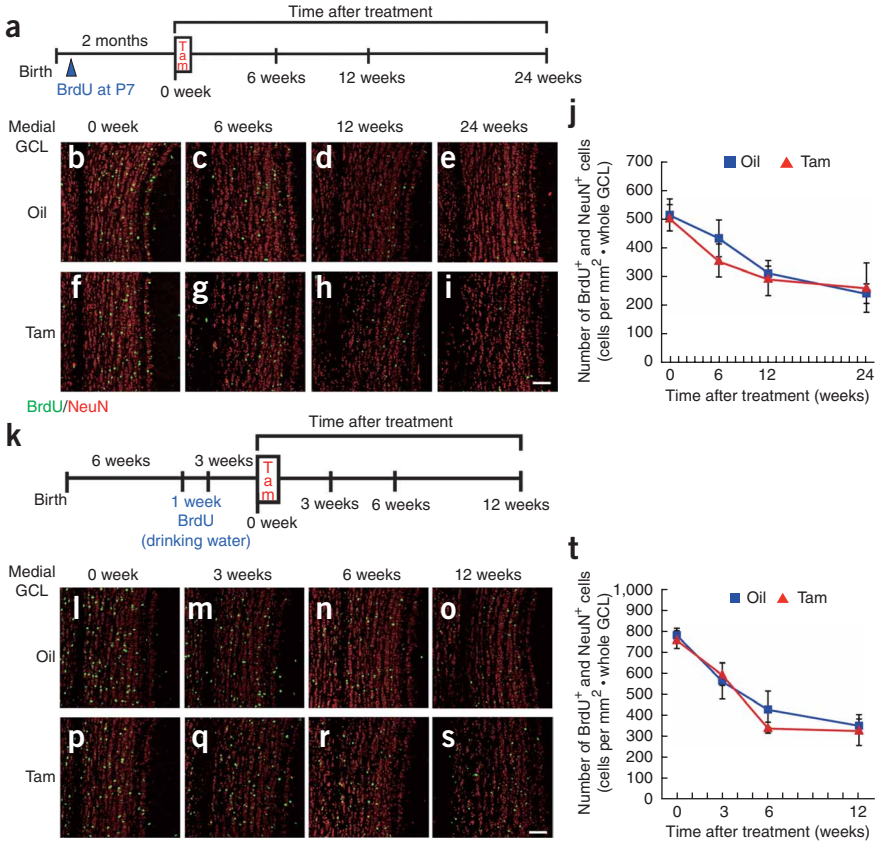

Figure 4 Similar survival of early born granule cells in oil- and tamoxifen-treated Line 5-1/NSE-DTA mice. (**a**) Experimental design for **b**–**j**. (**b**–**j**) Early-born NeuN+ granule cells labeled with BrdU survived similarly in oil- and tamoxifen-treated Line 5-1/NSE-DTA mice. (**k**) Experimental design for **l**–**t**. (**l**–**t**) NeuN+ granule cells born 3 weeks before oil or tamoxifen treatment (labeled with BrdU) survived similarly in oil- and tamoxifen-treated Line 5-1/NSE-DTA mice. Scale bars represent 50 μm.

This saturation is probably the result of a decrease of neurogenesis in the SGZ of aged mice[34]. Because the recombination efficiency was 66.8 ± 5.8%, the real proportion of new neurons would be higher than 10%.

We next examined the effect of ablation of neurogenesis in the dentate gyrus by using Line 4/NSE-DTA mice (**Fig. 7**). Production of DCX^+ neuroblasts and $BrdU^+NeuN^+$ neurons was substantially reduced by tamoxifen treatment (**Fig. 7d**,**e**, compare with **Fig. 7b**,**c**). In tamoxifen-treated mice, the histological structure of the dentate gyrus was mostly normal, but the density of granule cells seemed to be somewhat lower than in oil-treated mice at all time points examined (**Fig. 7g**–**r**). In oil-treated mice, the density of granule neurons substantially increased, whereas the density of granule neurons was almost constant during a 24-week period in tamoxifen-treated mice (**Fig. 7f** and **Supplementary Table 5**). Previous studies have shown that relatively few neurons die in the dentate gyrus and that the total number of granule cells of the dentate gyrus increases during adulthood in rats[9,11–15]. Thus, we next counted the total cell number of the mouse dentate gyrus. Similar to rats, granule cells significantly increased in number in the mouse dentate gyrus ($P < 0.05$; **Supplementary Fig. 11** online), unlike in the olfactory bulb (**Supplementary Fig. 11**). This increase of the total cell number paralleled the increase in cell density (**Supplementary Fig. 11**). In tamoxifen-treated Line 4/NSE-DTA mice, granule cell density of the dentate gyrus did not change (**Fig. 7f**). Furthermore, early born neurons ($NeuN^+$) labeled with BrdU did not die, but persisted for at least 24 weeks in both oil- and tamoxifen-treated Line 4/NSE-DTA mice (**Fig. 7s**–**u** and **Supplementary Fig. 12** online). These results suggest that blockade of neurogenesis does not substantially affect the existing neuronal circuit, but inhibits increases in the number of neurons in the dentate gyrus. This is in sharp contrast with the olfactory bulb, where neurogenesis mostly contributes to neuronal replacement.

Ablation of newly formed neurons impairs spatial memory

Although it has been suggested that hippocampal neurogenesis is involved in spatial memory[20,35,36], defects in such behaviors were not observed in rats or mice whose neurogenesis had been reduced by

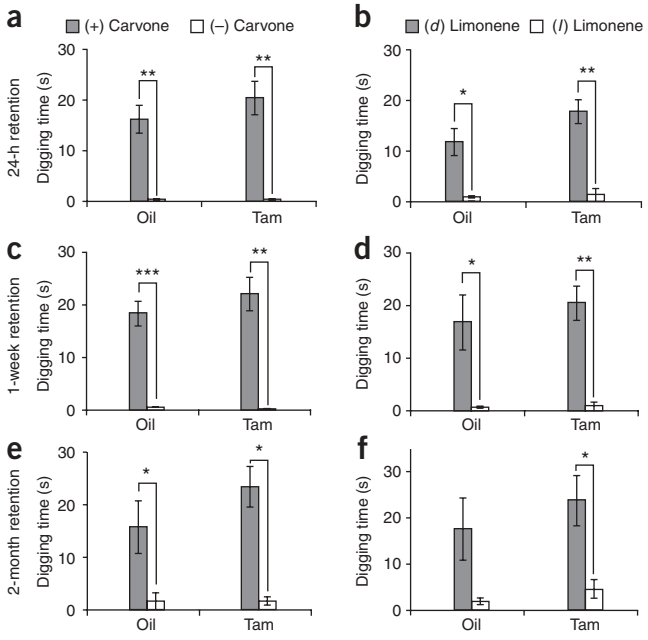

Figure 5 Ablation of newly formed neurons does not affect discrimination and memory of odors. (**a**,**b**) Oil- and tamoxifen-treated Line 4/NSE-DTA mice were trained for 4 d to associate a reward (sugar grains) with either of the two related odorants (enantiomers). On day 5, the sugar reward was removed from the bed and digging time was measured for each pair of related odorants. Both oil- and tamoxifen-treated mice spent significantly more time near the odorants associated with the reward. (**c**–**f**) The trained mice were subjected to the same trial 7 d (**c**,**d**) and 2 months later (**e**,**f**) without further training. Again, both oil- and tamoxifen-treated mice spent significantly more time near the odorants that were associated with the reward. Mean digging times ± s.e.m. during 2-min test periods are shown as bar graphs for odorant pairs with the reward (gray bars) and for unpaired odorants (white bars). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

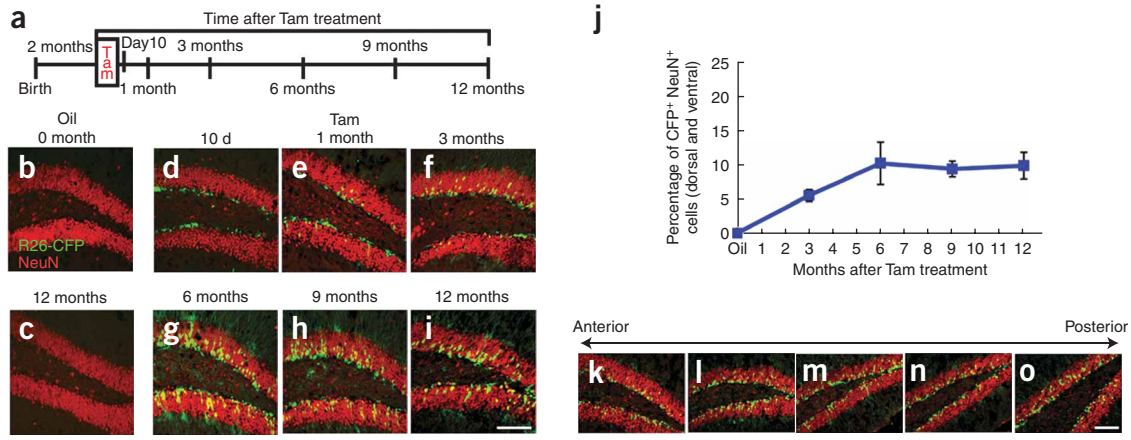

Figure 6 Continuous adult neurogenesis in the dentate gyrus visualized by long-term labeling of NSCs. (**a**) Experimental design. (**b**–**i**) The dentate gyrus of oil-treated and tamoxifen-treated Line 4/R26-CFP mice. Granule cells (NeuN$^+$, red) in the dentate gyrus were progressively labeled with CFP (green) after tamoxifen treatment. (**j**) Quantification of CFP labeling per NeuN$^+$ granule cells in the dentate gyrus of tamoxifen-treated Line 4/R26-CFP mice. (**k**–**o**) The similar numbers of CFP-labeled NeuN$^+$ granule cells were present along the anterior-posterior axis of the dentate gyrus 12 months after tamoxifen treatment. Scale bars represent 100 μm.

treatment with anti-mitotic drugs or irradiation[22,23]. Because these discrepancies could be the results of differences in species, mouse strains and/or efficiency of blockade of neurogenesis, we carried out hippocampal-dependent memory tests on oil- and tamoxifen-treated Line 4/NSE-DTA mice in the C57BL/6J background, a standard strain for behavioral tests. Oil-treated mice showed similar behaviors to wild-type mice and thus were used as control.

Line 4/NSE-DTA mice that had been treated with oil or tamoxifen more than 1 month before were subjected to the Barnes maze spatial memory test. It has been previously reported that newly formed neurons are incorporated into the neural circuit in 1 month[37], and thus these tamoxifen-treated mice should show behavioral defects if neurogenesis is required. Oil- and tamoxifen-treated mice moved at similar speeds (data not shown), but tamoxifen-treated mice tended to make more errors than oil-treated mice in locating the hidden platform (**Fig. 8a**,**b**). However, both mice were well able to remember the right target at day 7 (**Fig. 8c**). We assessed the maintenance of the spatial memory 1 week later and found that there was significant difference ($P < 0.05$) between the two groups. Oil-treated mice still remembered the right target, whereas tamoxifen-treated mice did not (**Fig. 8d**). These results indicate that blockade of adult neurogenesis impairs the retention of spatial memory.

Oil- and tamoxifen-treated mice were further tested for contextual fear conditioning. Tamoxifen-treated mice showed significantly less freezing responses during contextual fear conditioning ($P < 0.0001$; **Supplementary Fig. 13** online), but showed normal freezing responses

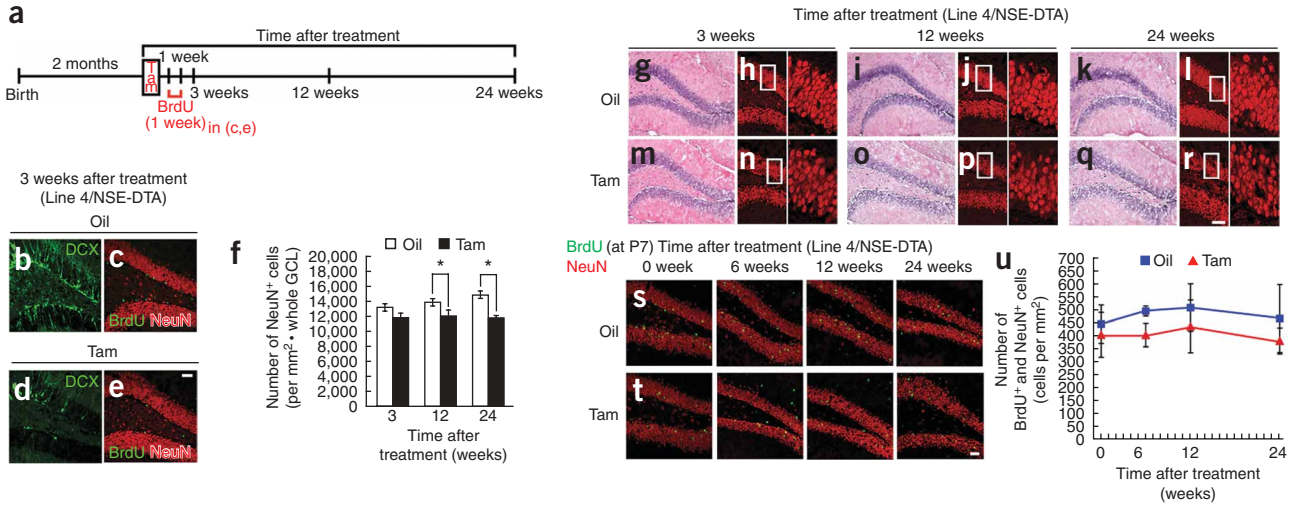

Figure 7 Ablation of neurogenesis inhibits the increase of the granule cell number in the dentate gyrus. (**a**) Experimental design. (**b**–**e**) The number of newly formed granule cells (DCX$^+$ and BrdU$^+$NeuN$^+$) decreased in the dentate gyrus of tamoxifen-treated Line 4/NSE-DTA mice compared with the oil-treated mice. (**f**) Quantification of the number of NeuN$^+$ granule cells in the dentate gyrus of oil- and tamoxifen-treated Line 4/NSE-DTA mice. (**g**–**r**) HE staining (**g**,**i**,**k**,**m**,**o**,**q**) and immunostaining for NeuN (**h**,**j**,**l**,**n**,**p**,**r**) of the dentate gyrus of oil- and tamoxifen-treated Line 4/NSE-DTA mice. (**s**,**t**) Early-born NeuN$^+$ granule cells labeled with BrdU survived similarly in oil- and tamoxifen-treated Line 4/NSE-DTA mice. (**u**) Quantification of the number of NeuN$^+$BrdU$^+$ granule cells in the dentate gyrus of oil- and tamoxifen-treated Line 4/NSE-DTA mice. NeuN$^+$ granule cells were labeled with BrdU before oil or tamoxifen treatment. *$P < 0.05$, *t* test. Scale bars represent 50 μm.

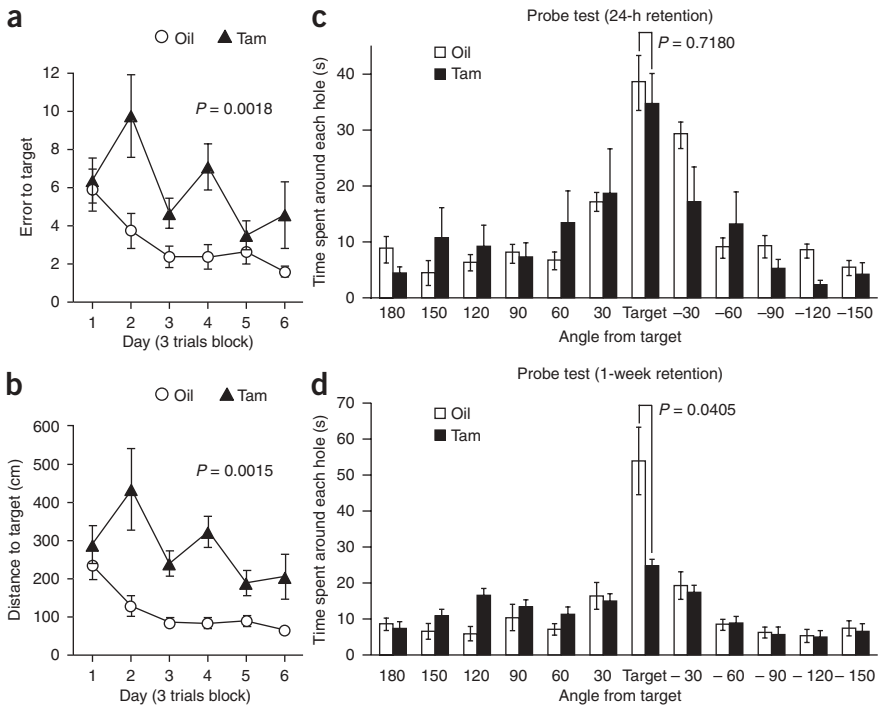

Figure 8 Ablation of newly formed neurons impairs retention of spatial memory. Oil- and tamoxifen-treated Line 4/NSE-DTA mice were subjected to the Barnes maze test. (**a**,**b**) The number of error trials (**a**) and the moving distance to target (**b**) during 6-d training were measured. (**c**) The time spent at each target area at day 7 was measured. Although tamoxifen-treated mice tended to make more errors than oil-treated mice in locating the hidden platform, both mice learned the position at day 7. (**d**) Oil-treated mice still remembered the right target 1 week after the training, whereas tamoxifen-treated mice did not.

during cued fear conditioning (with tone; **Supplementary Fig. 13**). These results are consistent with previous reports[23]. All or some of these behavioral defects could result from blockade of neurogenesis not only in the dentate gyrus, but also in the olfactory bulb, although these tamoxifen-treated mice discriminated different odors and acquired odor-associated memory at this stage (**Fig. 5**).

DISCUSSION

Here, we reported a genetic method for labeling NSCs and ablating newly formed neurons very efficiently in the adult brain. With this method, we found unique roles for neurogenesis in the adult brain and discovered that inhibition of neurogenesis leads to different outcomes in different tissues (for example, olfactory bulb versus dentate gyrus). Neurogenesis led to the replacement of the majority of granule cells in the olfactory bulb, and inhibition of neurogenesis resulted in a substantial reduction of the granule cell number. Previous analyses have not clearly shown whether newly generated neurons constitute a small population of neurons that are repeatedly replaced or whether they constitute a large population[38]. Our long-term labeling analysis revealed that most old neurons are replaced by new neurons in the deep regions, whereas about half of the population is replaced in the superficial regions of the olfactory bulb. Thus, subsets of granule cells are not replaced, but persist throughout life, and it is possible that these cells regulate the long-term memory of the smell. It was previously shown that about 40% of granule cells born at two months of age survived for up to 19 months[39], suggesting that the replacement of new neurons occurs only once or twice, even in deep regions of the adult olfactory bulb. In contrast, neurogenesis led to the addition of neurons to the existing system in the dentate gyrus, and although the structure was maintained without neurogenesis, the increase in the granule cell number was inhibited. Thus, in the dentate gyrus, most neurons are maintained and neurogenesis seems to contribute to an increase in the whole granule cell number during adulthood. These results suggest different roles and integration modes for adult neurogenesis: maintenance and reorganization of the whole system in the olfactory bulb, and modulation and refinement of existing neuronal circuits in the dentate gyrus.

The role of hippocampal neurogenesis in spatial learning and memory is rather controversial. Previous studies have reported that hippocampal neurogenesis is involved in spatial memory[20,21,35,36] and that newly formed neurons seem to be preferentially incorporated into circuits of the dentate gyrus during water-maze memory tasks[18]. In other studies, however, defects in spatial memory were not observed in rats or mice whose neurogenesis had been reduced by treatment with antimitotic drugs or irradiation[22,23]. These discrepancies could result from differences in species, mouse strains and/or efficiency of blockade of neurogenesis. We found that tamoxifen-treated Line 4/NSE-DTA mice in the C57BL/6J background showed defects in the retention of spatial memory, supporting the idea that newly formed granule cells indeed contribute to spatial memory formation.

Previous reports have shown that olfactory discrimination learning increases the survival of new neurons[40] and that sensory experience during a critical period is important for the survival of new neurons[41]. These observations led to the hypothesis that continual integration of new neurons is important in olfactory functions, such as olfactory discrimination and plasticity of olfactory circuits[38]. The olfactory bulb is known to send projections to the hippocampus through the lateral entorhinal cortex and the hippocampus is involved in several olfactory memory tasks[42]. In this regard, it is notable that blockade of neurogenesis in the adult brain did not inhibit discrimination and memory of odors, although it affected hippocampus-dependent contextual and spatial memory. These results suggest that new granule cells in both the olfactory bulb and the dentate gyrus are not required for discrimination and memory of odors, at least in the examined conditions. This result is somewhat different from the previous report about NCAM-null mice. These mice have a reduced number of granule cells in the olfactory bulb as a result of deficits of neuronal migration and have reduced odor-discrimination capabilities[43]. This discrepancy could be the result of different experimental conditions; NCAM-null mice were examined at 10–14 months of age and thus could have more severe defects by long-lasting blockade of neuronal migration, whereas we examined the mutant mice 3–4 weeks and 6 months after tamoxifen treatment. Thus, it would be interesting to examine the tamoxifen-treated mice more than 6 months later to determine whether these mice are fully capable of odor discrimination and memory at later stages. It would be also interesting to examine whether olfactory neurogenesis is involved in odor memory in different contexts, such as male pheromones during mating, which are known to affect pregnancy[44].

It has been claimed that neurogenesis occurs in the neocortex of adult rats[45] and can be induced by injury in the neocortex of adult mice[46]. However, we found very few labeled cells in the neocortex and other forebrain regions such as the septum and the striatum of tamoxifen-treated Nes-CreER^{T2}/Rosa26 reporter mice even after 1 year (data not shown), suggesting that neurogenesis is limited to the olfactory bulb and the dentate gyrus. It has been reported that NSCs in the SVZ of the lateral ventricles give rise to oligodendrocytes as well as neurons[47]. However, there were very few labeled oligodendrocytes in tamoxifen-treated Nes-CreER^{T2}/Rosa26 reporter mice (data not shown). Thus, nestin-positive NSCs in the SVZ of the lateral ventricles mostly give rise to granule cells and periglomerular cells in the olfactory bulb and their contribution to other cell types seems to be very small.

METHODS

Detailed procedures for staining and quantification are described in the **Supplementary Methods** online.

Animals. Nes-CreER^{T2} Line 5-1 and Line 4 mice have been described previously[27]. Detailed information of NSE-DTA mice[31] (T.I. and S.I., unpublished observations) will be described elsewhere. Nes-CreER^{T2}, R26-LacZ[48] and NSE-DTA mice were maintained on a C57BL/6J background. R26-CFP[49] and ZEG mice[50] were maintained on a mixed background of C57BL/6J and ICR. The genotype of each mouse was determined by PCR, as previously described[27]. All animals were handled in accordance with the Kyoto University *Guide for the Care and Use of Laboratory Animals*.

BrdU administration. BrdU (Sigma) was dissolved in 0.1 M phosphate-buffered saline, pH 7.4. We administered 50 or 200 μg per g of body weight of BrdU by a single intraperitoneal injection at a concentration of 10 mg ml^{-1}.

Tamoxifen treatment. For activation of CreER^{T2}, 10 mg of tamoxifen (Sigma) in corn oil (Sigma) were orally administered to 2-month-old mice once a day for 4 consecutive days. For a higher degree of Cre-mediated recombination, further rounds of injections were administered to Line 5-1/R26-CFP mice 1 and 2 months later (**Fig. 2a**). In ablation experiments, corn oil–treated Nes-CreER^{T2}/NSE-DTA mice were apparently normal and were used as control.

Analysis of brain tissues. Brains of at least two male and two female mice were analyzed at all time points. LacZ$^+$, CFP$^+$NeuN$^+$, BrdU$^+$NeuN$^+$ or NeuN$^+$ cells were analyzed throughout the anterior-posterior extent of the granule cell layer of the main olfactory bulb and the dentate gyrus. We took serial sections of the olfactory bulb and the dentate gyrus from the anterior end to the posterior end and stained every ten sections to determine their average scores. These average scores from the same groups were pooled together to determine the total average and standard deviation. *P* values were determined with unpaired Student's *t* tests. To analyze the marker expression profile of the CreER^{T2}-positive cells, we administered 10 mg of tamoxifen 24 h prior to killing the animals.

Behavioral analysis. Detailed procedures are described in the **Supplementary Methods**.

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

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AUTHOR CONTRIBUTIONS

I.I. conducted the experiments. I.I., M.S., M.Y. and K.M. carried out analysis on odorant discrimination and memory. I.I., K.T. and T.M. performed analysis on other behavioral tests. T.I. and S.I. provided the NSE-DTA mice. T.O. and R.K. supervised the project. I.I. and R.K. wrote the manuscript.

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