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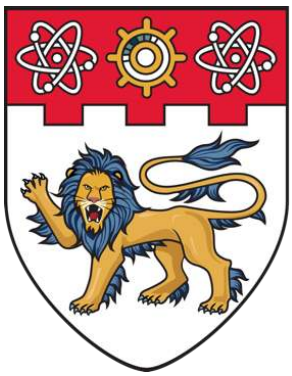

NANYANG TECHNOLOGICAL UNIVERSITY

SINGAPORE

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**SCHOOL OF BIOLOGICAL SCIENCES**

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A thesis submitted to the Nanyang Technological University in partial fulfilment of the requirement for the degree of Doctor of Philosophy
2021

## Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

Takuma Yamaguchi

22 Jan 2021 Takuma Yamaguchi

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I have reviewed the content and presentation style of this thesis and declare it of sufficient grammatical clarity to be examined. To the best of my knowledge, the thesis is free of plagiarism and the research and writing are those of the candidate's except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Ayumu Tashiro

## Authorship Attribution Statement

This thesis contains material from a paper published in the following peer-reviewed journal in which I am listed as an author.

Alessandro Luchetti*, Takuma Yamaguchi*, Masato Uemura, Glen Yovianto, Luka Čulig, Ming Yang, Wei Zhou, Franziska Oschmann, MinFeng Lua and Ayumu Tashiro, *equally contributions "Within-trial persistence of learned behavior as a dissociable behavioral component in conventional hippocampus-dependent memory tasks: a potential post-learning role of immature neurons in the adult dentate gyrus" in press *eNeuro*, accepted on 21st Jun 2021. https://doi.org/10.1523/ENEURO.0195-21.2021

The contributions of the co-authors are as follows:
Author contributions: A.L., T.Y., M.U., and A.T. designed research; A.L., T.Y., M.U., G.Y., and F.O. performed research; A.L., T.Y., G.Y., L._C., M.Y., W.Z., M.L., and A.T. analyzed data; A.L., T.Y., and A.T. wrote the paper.

Takuma Yamaguchi

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How I acquired data here is attributed to mostly what I learned during this PhD study. In contrast, how I analyzed and interpreted data especially in relation to persistency of learned behavior is attributed to mostly research attitude acquired in my former career in Japan.

# Abstract

Granule cells in the dentate gyrus (DGCs) are continuously produced through adult neurogenesis. The early (1-3 weeks old) and late (4-6 weeks old) maturational stages of adult born DGCs have different morphological and physiological properties. The early maturation stage developing their functional inputs and outputs corresponds to a critical period for making learning-dependent survival-or-death decision of adult born DGCs. Previous studies using pre-acquisition ablation suggest that adult born DGCs at the early and late maturational stage are involved in memory. However, with post-acquisition manipulation, it remains unclear whether adult-born DGCs at the early maturation stage (immature DGCs) are involved in memory after acquisition process, while adult born DGCs at a late maturational stage have been shown to have a post-acquisition role in memory. To investigate a post-acquisition role of immature DGCs in memory, we established a genetic method for ablation of immature DGCs which can be induced after acquisition session of spatial or fear memory tasks. Using this method, we then examined the effect of post-acquisition ablation in retrieval tests. The ablation showed impairment in the retrieval tests, suggesting that immature DGCs have a role in memory after acquisition process. Further analysis on impaired performance showed that retrieval in the initial phase of trial was intact, but later learned behavior was discontinued. Thus, our studies suggest that immature DGCs have a post-acquisition role in persistency of learned behavior. This finding unravelling the post-acquisition role of immature DGCs in memory contributes to understanding of neurogenesis in memory.

## Organization of the thesis

# 1. Introduction

### *1.1 Hippocampus*

The hippocampus is a brain region located at the medial temporal lobe of the cerebrum. There are two hippocampi in the brain, and each hemisphere has one hippocampus. Human hippocampus is a curved tube shape and an early anatomist described its shape with a seahorse which is “hippocampus” in Latin. The hippocampus conserves among mammals and consists of the dentate gyrus and Cornu ammonis region (CA, means horn of Amun, an ancient Egyptian god) including CA1, 2, 3 and 4. The principal cells of CA and the dentate gyrus are pyramidal neurons and granule cells, respectively. The major pathway of the hippocampus makes loops with entorhinal cortex (EC); EC layer II has axons projecting the dentate gyrus and CA3; EC layer III has axons projecting CA1; Granule cells in the dentate gyrus projects to CA3; CA3 pyramidal neurons project to CA1; CA1 pyramidal neurons project to EC. In addition to the major EC inputs, the hippocampus receives minor inputs from the perirhinal and postrhinal cortex and many other subcortical brain regions: the amygdala, the medial septum, the claustrum, the substantia innominata and the basal nucleus of Meynert, the thalamus, the hypothalamus, the ventral tegmental area, the raphe nuclei, the reticulotegmental nucleus, the periaqueductal gray, the laterodorsal tegmental nucleus, and the locus coeruleus (Amaral and Cowan, 1980; Montero-Crespo *et al.*, 2020). The hippocampus is thought to be the central for learning and memory, and the role in memory is introduced in the next section.

### *1.2 Hippocampus and memory*

A lot of perspectives about the role of the hippocampus in memory came from an epileptic patient Henry Molaison (HM). HM suffered from severe seizures and received operation for removing medial temporal lobes on both hemispheres to cure his epilepsy. The removed part of brain contains the hippocampus, while there was some remained

portion of the hippocampus (Annese *et al.*, 2014). HM showed anterograde amnesia which is the loss of the ability to create new memory, and short-term retrograde amnesia which is the loss of the ability to recall past memory (his cases retrograde amnesia within three years after the surgery (Squire, 2009)). He lacked a specific type of memory, that is declarative memory which is a type of memory that can be recalled and declared the content of memory on consciousness as image or language (Annese *et al.*, 2014). Declarative memory consists of episodic memories which is the specific contents of individual episodes or events, and semantic memories which includes knowledge of the meanings of words, factual information, and encyclopedic memories. On the other hand, some types of memory were spared in HM, that is non-declarative memory which is a type of memory that cannot be declared the content on the consciousness (Annese *et al.*, 2014). Non-declarative memory consists of procedural memory, priming, and some form of classical conditioning. An example for non-declarative memory (procedural memory) is remembering how to ride bicycle.

After perspectives from HM suggested the importance of hippocampus in memory, accumulating evidences from studies with humans and other animals such as rodents suggest important roles of the hippocampus in memory. To capture hippocampal functions in memory, many theoretical frameworks have been advocated; cognitive map theory (O'Keefe, 1978; Eichenbaum, 2017), memory indexing theory (Teyler and Rudy, 2007; Goode *et al.*, 2020), pattern separation and completion (Lee, GoodSmith and Knierim, 2020), novelty detection (Lisman and Otmakhova, 2001). In section 1.5, I introduce some of them which are suggested as a role of the dentate gyrus.

### *1.3 Hippocampus-dependent memory and distinct memory processes*

When recalling memory, there must be several memory processes until recalling: acquisition, the process to acquire memory, and retrieval, the process to recall memory. Between acquisition and retrieval, memory is thought to be stored and maintained. In the brain, it is thought to be that memory is stored in a subset of neural circuits with synaptic modifications during acquisition, the modifications are maintained during retention, and memory is represented by the activation of the circuits through the changed synaptic

connections during retrieval. The hippocampus is thought to be important for those memory processes, and memory tests have been used for investigating hippocampal role in memory. One of well known and used memory tests with rodent is water maze experiment which examines spatial learning and memory. In this test, a circular pool (~1-2m diameter) filled with water which works for aversive stimuli for rodent is used. A subject is placed into the pool and eventually reaches a platform which is located at a fixed place under the water not to be visible for a subject. Over multiple trials subjects learn how to reach platform using visual cues outside the pool. This spatial memory task is known to be hippocampus-dependent (Vorhees and Williams, 2006). Another well known memory test is fear conditioning. This test examines the ability to associate intrinsic aversive stimuli such as electrical foot-shocks with environmental components such as contexts and tones in a small chamber. If remembering the association, subjects will show freezing behavior when conditioned components are provided even without foot-shock. The hippocampus is involved in some form of fear conditioning: contextual fear conditioning where foot-shock and context are associated, and trace fear conditioning where foot-shock and tone with time interval in-between are associated (Misane *et al.*, 2005). In contrast, hippocampus seems not to be involved in delayed fear conditioning where foot-shock is co-terminated with tone or generated after tone without time interval (Misane *et al.*, 2005).

Memory processes such as acquisition, storage and retrieval seem to be regulated differently. Different neural circuits for acquisition and retrieval have been suggested (Roy, Kitamura, *et al.*, 2017). Different circuits for acquisition and retrieval may be suitable for updating memory when recalling memory while a new information is added (Roy, Kitamura, *et al.*, 2017). Different genetic modification in the hippocampus after acquisition and retrieval have been suggested (Peixoto *et al.*, 2015). For example, with the use of contextual fear conditioning, they found that expression of histone 2A variant H2AB were reduced only after acquisition, while splicing factor Rbfox1 and NMDA receptor-dependent microRNA miR-219 were reduced only after retrieval, followed by an increase in the expression of CAMKIIγ which is a target of miR-219. Previous studies showed that amnesia induced by protein-synthesis inhibitor or in mouse models of

Alzheimer's disease is primarily due to compromised memory retrieval but not storage (Roy *et al.*, 2016; Roy, Muralidhar, *et al.*, 2017). They showed that optogenetic activation of "engram cells" which are labelled with an immediate-early gene expression during training induced retrieval in a new context which did not induce memory recall naturally, suggesting that engram cells store the information. Despite those findings, it remains to be understood how memory processes during acquisition or after acquisition (storage) regulate retrieval later.

### *1.4 Dentate gyrus*

The dentate gyrus, a subregion of the hippocampus, consists of three distinct layers: molecular layer, granule cell layer, and polymorphic layer (=hilus). The principal cells in the dentate gyrus are granule cells composed of cell bodies, dendrites and axons. Their cell bodies are packed and located at granule cell layer. Their dendrites are located at molecular layer. Their axons, named mossy fibers, are extended to the hilus and reach CA3. The dentate gyrus is the first place at the hippocampal trisynaptic circuit to receive cortical input from medial or lateral entorhinal cortex via perforant path. The output of the dentate gyrus is sent to pyramidal cells and interneurons in CA3 and mossy cells and interneurons in the hilus. In rat hippocampus, single DGCs form synapses on 11–15 CA3 pyramidal cells and 7-12 hilus mossy cells with large mossy terminals (Acsády *et al.*, 1998). DGCs contact GABAergic interneurons with filopodial extensions of the mossy terminals and small *en passant* synaptic varicosities. The estimated ratio of interneurons to CA3 pyramidal cells or mossy cells as the targets of mossy fibers is 4:1 to 6:1 (Acsády *et al.*, 1998).

### *1.5 Dentate gyrus and memory*

Various functions of the dentate gyrus in memory have been proposed such as pattern separation (Treves and Rolls, 1994), pattern completion (Nakashiba *et al.*, 2012), novelty detection (Hunsaker, Rosenberg and Kesner, 2008), and working memory (Xavier, Oliveira-Filho and Santos, 1999). I introduce each of them below.

Pattern separation is the process that minimizes overlap between patterns of representations. Since pattern separation was proposed as a role of the dentate gyrus by David Mar 1971, it seems to have been paid attention most as a role of the dentate gyrus by researchers among other advocated functions until the present time (according to PubMed search (https://pubmed.ncbi.nlm.nih.gov/) with words “dentate gyrus pattern separation” hit 564 results, “dentate gyrus pattern completion” hit 374, “dentate gyrus novelty detection” hit 39 and “dentate gyrus working memory” hit 545 at 14th Jul 2021). Nowadays, many features of the dentate gyrus are thought to be suitable for pattern separation; in anatomy, numerous numbers of granule cells exist compared with EC layer II and CA3 pyramidal cells (~1,200,000 granule cells, ~112,000 EC layer II cells, ~250,000 CA3 pyramidal cells in rat (Mulders, West and Slomianka, 1997)), possibly creating divergence of cortical input at the dentate gyrus and convergence at CA3 (Schmidt, Marrone and Markus, 2012); in electrophysiology, the dentate gyrus shows sparse activity (sparseness in both the number of active cells and the activity itself), possibly facilitating patter separation (Madar, Ewell and Jones, 2019). Experimentally, lesions in rat dentate gyrus or NMDA receptor knockout in mouse dentate gyrus impaired the ability to discriminate two closely spaced locations or similar contexts (Gilbert, Kesner and Lee, 2001; McHugh *et al.*, 2007).

Pattern completion is the process that reconstructs complete representations from partial representations. Mouse behavioral experiments with inhibition of DGCs output have suggested their role in pattern completion (Nakashiba *et al.*, 2012). They found improvement on discrimination between two similar contexts with the inhibition of old adult-born DGCs output, suggesting that they normally work not to discriminate, but treat two contexts as the same, which is a concept of pattern completion.

Novelty detection has been proposed as a role of the dentate gyrus. Detecting change in object location or environment was impaired by lesion of rat dentate gyrus, suggesting that the dentate gyrus is involved in detecting novelty? (Hunsaker, Rosenberg and Kesner, 2008).

Working memory is the ability to hold the information in mind. The dentate gyrus has been specifically proposed to be involved in spatial working memory. This has been studied with delayed match-to-place task or non-match-to-place task using T-maze, plus maze, radial maze and water maze (Hainmueller and Bartos, 2020). For example, Xavier et al. showed that lesion in the dentate gyrus impaired the performance in match-to-place working memory task of water maze (Xavier, Oliveira-Filho and Santos, 1999).

### *1.6 Adult neurogenesis in the dentate gyrus*

The dentate gyrus has a unique feature, that is adult neurogenesis. In 1962 it was discovered that neurons are produced in the adult hippocampus of rats using tritiated thymidine (Altman, 1962). Later, proliferated cells were found with administration of the thymidine analogue in adult mouse hippocampus too (Autoradiographic, 1965; Caviness, 1973; Stanfield, Caviness and Cowan, 1979; Reznikov, 1991). The subregion of hippocampus where adult neurogenesis occurs is called the dentate gyrus. Through adult neurogenesis, granule cells in the dentate gyrus (DGCs), a principal cell type of the dentate gyrus, are continuously produced (Deng, Aimone and Gage, 2010). Neural stem cells, called radial glial cells, are located in the subgranular zone (SGZ) of the dentate gyrus (Dayer *et al.*, 2003; Seri *et al.*, 2004). They divide to generate neuroblasts which have a potential to differentiate into DGCs (Kempermann, Song and Gage, 2015). After differentiation, many newborn DGCs die before they mature (van Praag *et al.*, 2002; Dayer *et al.*, 2003). Surviving newborn DGCs pass through a long process of morphological and physiological maturation and integrate into existing neural circuits of the dentate gyrus as details are explained in the next sections (section 1.2 and 1.3). Neurogenesis is thought to be important for learning and memory (section 1.5).

In 1998 adult neurogenesis was found in the dentate gyrus of human hippocampus (Eriksson *et al.*, 1998). The discovery was made from cancer patients who received the intravenous infusion of bromodeoxyuridine (BrdU) for diagnostic purposes. Nowadays the existence of adult neurogenesis in human hippocampus is supported by some studies (Knoth *et al.*, 2010; Spalding *et al.*, 2013; Dennis *et al.*, 2016; Boldrini *et al.*, 2018;

Moreno-Jiménez, Flor-García and Terreros-Roncal, 2019) and questioned by other studies (Cipriani *et al.*, 2018; Sorrells *et al.*, 2018). Failure of detection of adult neurogenesis in human hippocampus in the latter studies may be due to the low quality of histological pretreatment and fixation conditions of the human brain. Moreno-Jiménez et al. demonstrated that the prolonged or uncontrolled fixation conditions lead to a sharp reduction in detection of immature DGCs with antibodies against a microtubule binding protein, doublecortin (DCX) in the adult hippocampus (Moreno-Jiménez, Flor-García and Terreros-Roncal, 2019).

Adult neurogenesis in the hippocampus is preserved among most of mammalian species including human beings and rodents (Amrein, 2015). In addition to the hippocampus, adult neurogenesis was reported in other brain regions. The subventricular zone in the lateral ventricle is well known to have adult neurogenesis in some mammalian species, although in humans studies some reported and others failed to detect adult neurogenesis in the subventricular zone (Cutler and Kokovay, 2020). Accumulating evidences suggest adult neurogenesis in mammalian hypothalamus (Yoo and Blackshaw, 2018), despite very low rate of neurogenesis under physiological conditions (Yuan and Arias-Carrion, 2012).

In other brain areas, neurons born in infancy or earlier may remain immature for long such as decades (Sorrells *et al.*, 2019; La Rosa *et al.*, 2020). Neurons with immature features have been found using antibodies against DCX or polysialylated neuronal cell adhesion molecule in the human hypothalamus, the human amygdala and the neocortex layer II of diverse mammalian species including rodents and non-human primates (Batailler *et al.*, 2014; Sorrells *et al.*, 2019; La Rosa *et al.*, 2020). Cells with progenitor cell features have been found in the adult human amygdala, although those cells sharply decrease in infants (Sorrells *et al.*, 2019). No cells with progenitor cell features were found in the adult neocortex (La Rosa *et al.*, 2020). Potentially, cortical immature neurons are migrated from subventricular zone in infants (Sanai *et al.*, 2011).

### *1.7 Morphological properties of adult-born dentate granule cells*

After adult neurogenesis, newborn DGCs go through maturation and change their morphological and physiological properties. Here I introduce maturational change of morphological properties of adult-born DGCs. Although the time duration of DGC maturation in the adult mammalian brain is species dependent (Deng, Aimone and Gage, 2010), I describe time duration in mice, which have been most extensively characterized. The studies below are from mice if not otherwise mentioned. The early (~1-3 weeks old) and the late (~ 4-6 weeks old) maturation stage of adult-born DGCs are distinguished. They could be differentiated by the expression of multiple marker proteins, one of which is DCX; the early stage expresses DCX while the late stage does not. In addition, DGCs in the two stages have different morphological properties. Morphological studies suggest that DGCs at the early maturation stage have significantly less amount of inputs than those at the late maturation stage. In up to one week after birth of DGCs in adult brain, adult-born DGCs migrate a short distance into the inner granule cell layer of the dentate gyrus. These cells do not have cellular processes long enough to be synaptically connected with the CA3 and perforant pathway network (Espósito *et al.*, 2005; Zhao *et al.*, 2006). Around two weeks after birth of adult-born DGCs, their axon terminals are found at the hilus and CA3 (Zhao *et al.*, 2006; Toni *et al.*, 2008). Their axons have smaller presynaptic boutons than mature DGCs (Hastings and Gould, 1999; Toni *et al.*, 2008). Their dendrites reach the molecular layer, but spines in their dendrites are rarely seen (Zhao *et al.*, 2006). Around three weeks after birth of adult-born DGCs, afferent and efferent connections are increased (Zhao *et al.*, 2006; Toni *et al.*, 2008). Afferent sources are medial and lateral EC excitatory neurons, interneurons, mossy cells, CA3 pyramidal cells, mature DGCs and septal cholinergic cells (Vivar *et al.*, 2012; Woods *et al.*, 2018). Projection from mature DGCs are transiently formed with adult-born DGCs < 8-week-old (Vivar *et al.*, 2012). More spines are seen in 3-week-old DGCs than 2-week-old DGCs (Zhao *et al.*, 2006). However, the spine density at up to 3-week-old DGCs are still lower than mature DGCs (~20% compared with 8-week-old DGCs) (Zhao *et al.*, 2006). In contrast, 4-week-old DGCs show a sharp increase in spine density (~80% compared with 8-week-old DGCs) and in the size of synaptic boutons (Zhao *et al.*, 2006; Toni *et al.*, 2008). Spine density gradually increases until around 8-week-old DGCs

(Zhao *et al.*, 2006). These suggest that DGCs at the early maturation stage have significantly less amount of inputs than the late maturation stage. 4-week-old DGCs have the highest number of connections with CA3 inhibitory neurons among 2-, 4-, 6- or 8-week-old DGCs (Restivo *et al.*, 2015).

## *1.8 Physiological properties of adult-born dentate granule cells*

### *1.8.1 Development of inputs to adult-born dentate granule cells*

Physiological maturation of adult-born DGCs is introduced below. Functional inputs to adult-born DGCs are formed over time after their birth. The early (~1-3 weeks old) and the late (~4-6 weeks old) maturation stages of adult-born DGCs have different physiological properties too. The early stage has smaller number of glutamatergic and GABAergic functional inputs and excitatory effects of GABAergic inputs, compared with the late maturation stage. 1-week-old adult-born DGCs do not show postsynaptic current even though neurotransmitter receptors exist on them, suggesting that they do not receive functional synaptic inputs (Espósito *et al.*, 2005). 1-week-old DGCs start to form NMDA receptor-only silent synapses, as pairing highly depolarized membrane potential (holding at +40mV) with stimulation at the inner molecular layer where mossy cells project causes postsynaptic currents at 10-11-day-old DGCs, which are blocked by NMDA receptor antagonist (Chancey *et al.*, 2013, 2014). Majority of 2-3-week-old adult-born DGCs receives functional inputs from only GABAergic inputs, while a scant proportion of them receives both GABAergic and glutamatergic inputs, which is the property of mature DGCs (Espósito *et al.*, 2005). From 2 weeks after birth, adult-born DGCs start receiving? inputs from both medial and lateral EC (Kumamoto *et al.*, 2012). 3-week-old DGCs have notably dominated inputs from lateral EC; 3-week-old DGCs receive around 10-fold higher numbers of? lateral EC inputs than medial EC, while mature DGCs (DGCs located at the outer one-third of the granule cell layer) receive both inputs to a similar extent (Woods *et al.*, 2018). GABAergic inputs to up to 3-week-old DGCs possibly work on both activation and inhibition of these young DGCs due to high intracellular concentration of $Cl^-$ which cause relatively depolarized reversal potential for

GABA, $E_{GABA}$ (~ -30 to -40 mV) compared with $E_{GABA}$ of mature DGCs (~ -65 to -80 mV) (Pathak *et al.*, 2007; Heigele *et al.*, 2016). If receiving weak GABAergic inputs (~1.5 nS), DCX+ immature DGCs (correspond to around 1-3-week-old DGCs) depolarize and evoke action potentials, while strong GABAergic inputs (>4 nS) block induction of action potentials of DCX+ immature DGCs (Heigele *et al.*, 2016). Notably, in around 4-week-old DGCs, the proportion which receives both GABAergic and glutamatergic inputs is acutely increased (Espósito *et al.*, 2005), as mature DGCs receive both inputs. These may imply that immature DGCs up to 3-week-old have unique inputs and regulations with local activity in the dentate gyrus compared with DGCs at 4 weeks old or older than that.

How developing DGCs receive GABAergic inputs changes depending on their age and the properties are distinguished between the early (~1-3 weeks old) and late (~4-6 weeks old) maturation stages of adult-born DGCs. There are two distinct GABAergic inputs: slow dendritic currents originating from somatostatin-expressing interneurons projecting distal dendrites, and fast perisomatic GABAergic currents originating from either parvalbumin-expressing interneurons projecting to somata or somatostatin-expressing interneurons projecting to proximal dendrites (Groisman, Yang and Schinder, 2020). GABAergic inputs to newborn DGCs up to 2-week-old are only slow dendritic responses (Espósito *et al.*, 2005). Newborn DGCs at 3-week-old start to have fast perisomatic/proximal GABAergic currents in addition to slow distal dendritic GABAergic currents, although a scant population (~20%) receives both types of currents (Espósito *et al.*, 2005). Notably, at around 4-week-old, a population which receives only fast GABAergic current or both fast and slow GABAergic currents sharply increases (~80%) (Espósito *et al.*, 2005). In short, 1-3-week-old DGCs have distinct properties of inhibitory inputs compared with older DGCs; 1-3-week-old DGCs have less amount of inhibitory inputs which is dominated by inputs received at distal dendrites.

Properties of synaptic plasticity are also distinguished between early and late maturation stage. Long-term potentiation (LTP) is more easily induced in newborn DGCs up to 6 weeks old by stimulating their inputs with tetanus burst stimulation compared with mature DGCs (Schmidt-Hieber, Jones and Bischofberger, 2004; Ge *et al.*, 2007). 4-6-week-old DGCs have higher amplitude of LTP and lower threshold for inducing LTP

from medial perforant path than mature ones (Ge *et al.*, 2007). 2-3-week-old DGCs also show lower threshold for inducing LTP from medial perforant path than mature ones, but have comparable or smaller amplitude of LTP compared with mature ones (Ge *et al.*, 2007). The reason why both 2-3- and 4-6-week-old DGCs have lower threshold for inducing LTP than mature DGCs may be because these 2-6-week-old DGCs have higher excitation/inhibition ratio than mature DGCs, and thus may be more easily excited. In accordance with the idea, 4-week-old DGCs were reported to have enhanced excitation/inhibition balance compared with mature DGCs (Marín-Burgin *et al.*, 2012). In contrast, theta burst stimulation of lateral perforant pathway induced lower magnitude of LTP at 4-6-week-old DGCs than 3-4-months-old DGCs (Vyleta and Snyder, 2021), suggesting that heighten synaptic plasticity at immature DGCs is medial perforant path-dependent.

### *1.8.2 Development of outputs from adult-born dentate granule cells*

Functional outputs from adult-born DGCs are also formed over time after their birth, making the early and late maturation stages of DGCs have distinct properties on their outputs. The functional outputs are created to CA3 pyramidal neurons, mossy cells in the dentate gyrus and interneurons in the dentate gyrus, and potentially in the CA3 which has not been investigated. The outputs to CA3 pyramidal neurons are formed in the earlier stage of maturation than the outputs to local interneurons. The outputs from several ages of adult-born DGCs to CA3 pyramidal neurons were systematically studied with optogenetic activation and whole-cell recording (Gu *et al.*, 2012). Based on this study, activation of DGCs aged 1, 2 or 3 weeks induces excitatory postsynaptic currents (EPSCs) on CA3 pyramidal neurons with EPSC amplitude ranging 0-40% of mature DGCs, while activation of 4-week-old DGCs induces EPSC with comparable EPSC amplitude with mature DGCs (Gu *et al.*, 2012). In other words, adult-born DGCs gradually form functional glutamatergic synapses with CA3 pyramidal neurons, and those synapses are strengthened and reach the mature level in around 4-week-old DGCs (Gu *et al.*, 2012). Optogenetic activation of 4-week-old DGCs can induce LTP in the CA3 more easily than activation of 3- and 8-week-old DGCs (Gu *et al.*, 2012).

Outputs from adult-born DGCs to local interneurons increase with age and the early maturation stage has distinct properties on outputs to interneurons. 3-week-old DGCs were reported to have no functional outputs to parvalbumin-expressing interneurons (Groisman, Yang and Schinder, 2020), suggesting that DGCs up to 3 weeks old do not activate inhibitory local network through parvalbumin-expressing interneurons. At 4 weeks old, DGCs start to have functional outputs to parvalbumin-expressing interneurons, and at around 6-8 weeks old, the proportion of DGCs which have functional outputs to parvalbumin-expressing interneurons become as high as mature DGCs (Groisman, Yang and Schinder, 2020). A scant proportion of 4-week-old DGCs were reported to have output to somatostatin-expressing interneurons (Groisman, Yang and Schinder, 2020), implying that DGCs younger than 4-week-old do not activate inhibitory local network through somatostatin-expressing interneurons either. The number of DGCs which have functional outputs to somatostatin-expressing interneurons also increases with age of DGCs and becomes similar to the one of mature DGCs when they are older than 8 weeks old (Groisman, Yang and Schinder, 2020).

Outputs from adult-born DGCs to mossy cells have been found, although the features of the outputs at different neuronal ages have not been characterized in contrast to outputs to CA3 pyramidal neurons and local interneurons. Toni et al. showed postsynaptic currents of mossy cells when optogenetically activating 3-4-month-old DGCs (Toni *et al.*, 2008), suggesting that adult-born DGCs have functional outputs to mossy cells. Outputs from adult-born DGCs to CA3 interneurons have been found. Optogenetic activation of 4-week-old DGCs caused more activation of CA3 interneurons than activation of 6- or 8-week-old DGCs (Restivo *et al.*, 2015).

### *1.9 The regulation of neurogenesis with various factors*

The extent of adult neurogenesis in the dentate gyrus changes depending on physical conditions of animals, surrounding environments, and activities of the dentate gyrus. When mice receive opportunities of running, they increase progenitor cell proliferation.

In contrast, when mice are placed into enriched environments or receive some forms of memory tasks, they promote survival of newborn DGCs (Kempermann, Kuhn and Gage, 1997; Gould *et al.*, 1999; van Praag, Kempermann and Fred H. Gage, 1999; Aasebø *et al.*, 2018). Enriched environments presumably give more opportunities for learning than conventional housing conditions. The second week after the birth of DGCs is a period most susceptible to the effect of exposure to the enriched environment on cell survival (Tashiro, Makino and Gage, 2007). Thus, these studies suggest that survival/death regulation of DGCs at the early maturation stage make the dentate gyrus plastic upon learning opportunities. In addition, learning opportunities promote maturation of dendrites, dendritic spines and functional excitatory and inhibitory inputs (Tronel *et al.*, 2010; Lemaire *et al.*, 2012; Alvarez *et al.*, 2016; Gonçalves *et al.*, 2016), suggesting that the integration of immature DGCs into neural circuits is regulated by learning opportunities. The quiescence and activation of neural stem cells are regulated by the activity of granule cells, local GABAergic interneurons, and mossy cells (Alvarez *et al.*, 2016; Yeh *et al.*, 2018; Dong *et al.*, 2020).

Adult neurogenesis in the dentate gyrus also changes according to various neurological conditions. Chronic stress is a risk factor for depression and negatively regulates neurogenesis (Mahar *et al.*, 2014). In contrast, antidepressant fluoxetine is known to increase neurogenesis in the dentate gyrus and its antidepressant effect depends on increased neurogenesis (Malberg *et al.*, 2000; Santarelli *et al.*, 2003; Navailles, Hof and Schmauss, 2008; Wang *et al.*, 2008; Kong *et al.*, 2009; Menalled *et al.*, 2009; David *et al.*, 2010; Mateus-Pinheiro *et al.*, 2013). More specifically, fluoxetine accelerates maturation of 1-2-week-old DGCs (Åmellem *et al.*, 2017), and the age susceptible to fluoxetine corresponds to a critical period for the survival of newborn DGCs (Tashiro, Makino and Gage, 2007).

The implication of adult hippocampal neurogenesis in neurodegenerative disorders was suggested. In accordance with findings of decreased adult neurogenesis in Alzheimer's disease patients as mentioned above (in section 1.1), accumulating pieces of evidence indicate that both proliferation of progenitor cells and survival of newborn

DGCs are impaired at the early stage of Alzheimer's disease progression in mouse models of Alzheimer's disease (Lazarov and Marr, 2010; Mu and Gage, 2011).

In short, neurogenesis is regulated with external stimuli such as running and enriched environment as well as with internal neurological conditions in the brain such as chronic stress and neurodegenerative disorders. Memory impairments accompanied with such disorders may be caused by disruption of adult neurogenesis in the dentate gyrus. In the next section, the role of neurogenesis in memory is introduced.

## *1.10 Neurogenesis and learning and memory*

### *1.10.1 Importance of neurogenesis in learning and memory*

In 2001, the causal link between adult neurogenesis and memory was first shown by Shors et al. (Shors *et al.*, 2001). They ablated adult neurogenesis in rat hippocampus with a DNA methylating agent methylazoxy-methanol acetate (MAM) and found impairment in memory formation with a trace eye blink conditioning task which is a hippocampus-dependent memory task. Later studies also suggest roles of adult neurogenesis in memories using hippocampus-dependent memory tasks such as spatial memory tasks including the Morris water maze (MWM) and fear memory tasks including trace or contextual fear conditioning, with chemical, genetical or radiational methods to ablate or inhibit neurogenesis; they found impairment in acquisition, retrieval, extinction, forgetting and cognitive flexibility (table1) (Saxe *et al.*, 2006; Zhang *et al.*, 2008; Dupret *et al.*, 2008; Imayoshi *et al.*, 2008; Deng *et al.*, 2009; Garthe, Behr and Kempermann, 2009; Arruda-Carvalho *et al.*, 2011; Burghardt *et al.*, 2012; Gu *et al.*, 2012; Vukovic *et al.*, 2013; Akers *et al.*, 2014; Seo *et al.*, 2015; Epp *et al.*, 2016). Many of these studies ablated the broad ranges of ages of adult born DGCs, so that they did not specify which ages of adult born DGCs are involved in memory. As introduced above (section1.2 and 1.3), adult born DGCs have different physiological and morphological features depending on their maturational stages, which makes one expect that they are involved in

memory differently depending on their maturational stages as well (Deng, Aimone and Gage, 2010). Thus, it is important to narrow the range of ages of adult born DGCs when investigating their roles in memory.

In accordance with the idea, I introduce some studies investigating the role of more specific ages of DGCs. Denny et al. and Gu et al. showed that the late maturation stage (4-6 week-old) of adult-born DGCs have roles in spatial memory and fear memory (Denny *et al.*, 2012; Gu *et al.*, 2012). Vukovic et al. showed that DCX+ immature DGCs (<3 week-old DGCs) have roles in acquisition of spatial memory (Vukovic *et al.*, 2013). Deng *et al.* and Seo *et al.* showed that immature DGCs up to 3 week-old have roles in spatial memory and fear memory (Deng *et al.*, 2009; Seo *et al.*, 2015). Thus, we could conclude from these studies that both immature DGCs (<3 weeks old at the time of memory acquisition) and more matured adult born DGCs (4-6 weeks old) have roles in hippocampus-dependent memory.

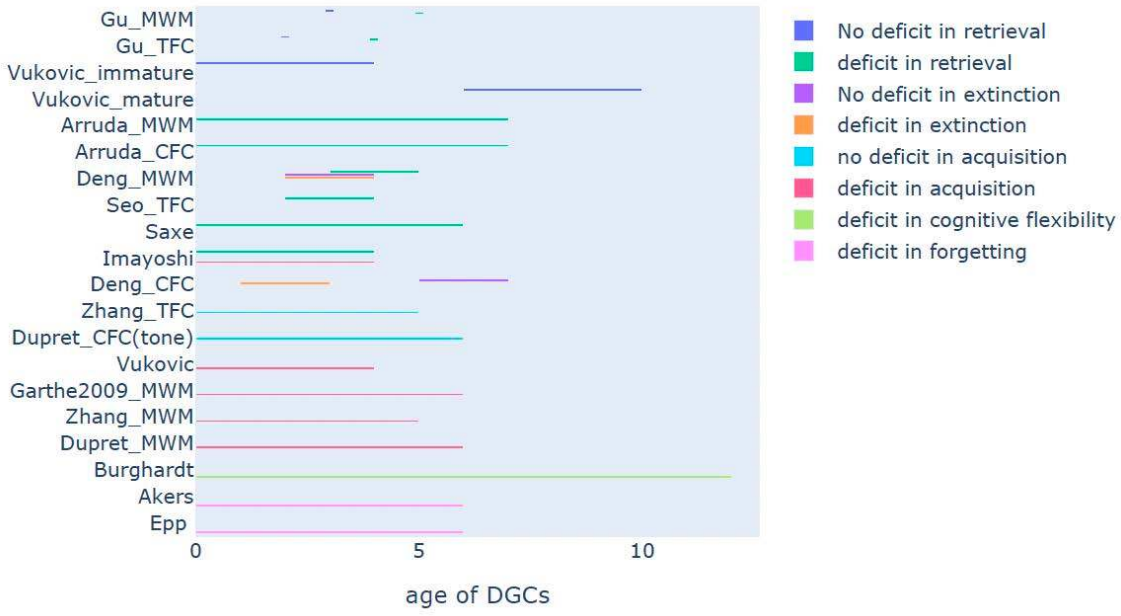



**Table 1. The effect of ablation/inhibition of adult-born DGCs at estimated ages on various memory processes**

### *1.10.2 Findings with pre- vs. post-acquisition manipulation*

Memory retention and retrieval depend on memory acquisition which occurs before retention and retrieval. Thus, to investigate the role of adult born DGCs in memory retention and retrieval, we need be careful in which timing in memory processes adult born DGCs is manipulated. If the manipulation is conducted before memory acquisition, the study investigates a role of adult-born DGCs in memory in general. There is a possibility that the pre-acquisition ablation firstly disturbed memory process during acquisition despite seemingly intact acquisition of learned behavior (Deng *et al.*, 2009; Seo *et al.*, 2015), so that the effect of ablation on retention was secondary. Under some conditions, such deficits in performance during acquisition sessions have been clearly detected with pre-acquisition ablation of adult-born DGCs (Dupret *et al.*, 2008; Zhang *et al.*, 2008; Garthe, Behr and Kempermann, 2009; Vukovic *et al.*, 2013).

In contrast, some studies were conducted with manipulation of adult born DGCs after acquisition process. Arruda-Carvalho et al. did posttraining ablation of up to 7-week-old adult-born DGCs and found impairment in retrieval after MWM training and contextual fear conditioning (Arruda-Carvalho *et al.*, 2011). Vukovic et al. did genetic ablation of DCX+ immature DGCs after the training of active place avoidance test (Vukovic *et al.*, 2013). Nevertheless, they did not find the effect of post-acquisition ablation in retrieval of spatial memory. Gu et al. used optogenetic inhibition during retention tests of MWM and fear conditioning (Gu *et al.*, 2012). Although they found impaired retrieval of both spatial memory and fear memory when inhibiting 4-5-week-old DGCs (correspond to DGCs at the late maturation stage), they did not find deficits in either retrieval tests when inhibiting 2-3-week-old DGCs (correspond to DCX+ immature DGCs at the early maturation stage). Thus, based on these studies it remains unclear whether DCX+ immature DGCs have roles in memory after acquisition process, while they seem to be involved in memory after acquisition process when their maturation stage passes over the early immature period.

### *1.11 Research objective: investigating post-acquisition role of immature dentate granule cells in memory retrieval*

During the early maturation stage, the functional inputs and outputs of immature DGCs develop, and survival-or-death decision of adult born DGCs happens. As described above, those processes are learning-dependent. Previous studies of pre-acquisition ablation of immature DGCs suggest their role in memory. Thus, I hypothesize that adult born DGCs at the early maturational stage (1-3-week-old DCX+ immature DGCs) may have post-acquisition role in memory. As described above (section 1.5.2), post-acquisition role of those immature DGCs remains unclear. Therefore, this study aims to reveal the post-acquisition roles of those immature DGCs in memory retrieval. To do this we selectively ablated DCX+ immature DGCs after normal memory acquisition; we first induced the expression of diphtheria toxin receptor (DTR) in immature DGCs under the control of DCX promoter using a lentiviral vector, and then injected diphtheria toxin (DT) to ablate those immature DGCs after mice received acquisition sessions of hippocampus-dependent memory tasks, such as MWM, trace fear conditioning and contextual fear conditioning. We evaluated the effect of post-acquisition ablation on performance during memory retrieval sessions in these tasks.

# 2. Materials and Methods

### 2.1 Lentiviral vector construction

A lentiviral transfer vector was designed to express DTR under the control of the human DCX promoter (Fig. 1A). cDNA encoding human DTR (pcDNA3 proHB-EGF WT, Addgene, USA) was inserted into a lentiviral transfer plasmid under the control of the human DCX promoter (Karl *et al.*, 2005). To visualize the transduced cells, cDNA encoding GFP was inserted 3' to the DTR gene following an internal ribosome entry site sequence. According to Karl et al., 2005, human, rat and mouse DCX genes have sequence similarity of above 85% only in the fragment between the start codon and the 3.5 kb upstream of the latter. They showed 3509-bp human regulatory region drives the gene expression in mouse tissues which endogenously express DCX.

Lentiviral particles were produced using a protocol modified from a previously described method (Tashiro *et al.*, 2015b). After the centrifugation steps previously described, the suspension was purified using Lenti-XTM Concentrator purification columns (Clontech, USA). Amicon® Ultra-4 Centrifugal Filter Units (EMD Millipore, Germany) were used to perform buffer exchange to sterile Dulbecco's phosphate-buffered saline (DPBS, Cat# 14040-174, Life Technologies, UK) and to concentrate the solution to a final volume of 100-150 µl. Aliquots of the lentiviral solution were stored at -80 °C until use.

### 2.2 Subjects

We used male and female C57BL/6 mice bred in our local facility in Norway or Singapore or purchased from Charles River (Germany) or InVivos (Singapore). For water maze and fear conditioning tasks, we used female C57BL/6 mice purchased from the two sources above. The mice were 7-12 weeks old at the start of the experiments and were housed in acrylic cages with access to food and water ad libitum under a 12:12-h light/dark cycle. All experiments were approved by the Norwegian Animal Research Authority and/or Institutional animal care and use committee (IACUC) at Nanyang Technological University.

**2.3 Surgical procedure**

The solution containing the lentiviral vector was stereotaxically injected into the dentate gyrus followed by a previously described method (Tashiro *et al.*, 2015b). The mice were anesthetized with 5% isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1- trifluoroethane) in air at a flow rate of 1,000 ml/min. The concentration of isoflurane was gradually reduced to 0.75% or higher, at which concentration deep anesthesia was maintained. The mice were subcutaneously injected with an analgesic (0.15 g/kg Temgesic). For the experiment described in Fig. S3 and Fig. 4/5/S2B-G, a local anesthetic, Marcaine (AstraZeneca, UK) and lignocaine (1.33 mg/ml, 0.1 ml), respectively, was additionally injected subcutaneously above the skull before performing the skin incision. For the experiment described in Fig. 4/5/S2B-G, buprenorphine (0.2 ml, 0.3 mg/ml) was additionally given intraperitoneally before surgery, and meloxicam and baytril were additionally given in drinking water (8 and 85 mg/l, respectively) for three days after the surgery.

The lentiviral solution was drawn into a microsyringe equipped with a blunt-end 33-gauge needle (Hamilton Company, USA). After the skull was exposed, one or two holes were drilled at the antero-posterior (AP) coordinate: -1.8 mm; medial-lateral (ML): +/-1.8 mm from the bregma. For the experiment described in Fig. 4/5/S2B-G, the coordinate of AP: -2.0 mm, ML: -1.3 mm was used. The tip of the needle was inserted into the brain at the same AP and ML coordinates and lowered to 2.3 mm ventrally from the skull surface. In each injection, 1.5 μl of lentiviral solution was infused at a rate of 2.5 nl per second (or 3.33 nl per second for the experiment described in Fig. 4/5/S2B-G). The titer of the lentiviral solution was 0.1-0.5 x $10^5$ GFP+ colony-forming units/ml in HEK 293FT cells (Life Technologies, UK). In the experiments described in Fig. 1, 2, S1, S2A, S3, S4 and S6, the viral vector was injected into one hemisphere. For Fig. 2, S1, S2A, S3, S4 and S6, the other hemisphere was used as a control. In the experiments described in Fig. 1, control mice were injected with PBS. In the experiments described in Fig. 3-5, S2B-G, S5, S7, S8), the vector was injected into both hemispheres.

**2.4 Diphtheria toxin injection**

DT (Calbiochem, Germany) was dissolved in DPBS at a final concentration of 5 μg/ml. The mice received a single dose of DT (50 ng/g body weight) intraperitoneally seven days after viral injection.

**2.5 BrdU injection**

We dissolved BrdU (Cat#B5002, Sigma, USA) in 0.9% saline at a concentration of 10 mg/ml, and the solution was filter sterilized. For each mouse, one dose of BrdU (100 μg/g body weight) was intraperitoneally injected.

**2.6 Novel environment exploration**

To induce the activation of granule cells in the dentate gyrus, we placed the mice in a novel environment (an open field consisting of a 32 x 28 x 28 cm plastic box with black- and white- striped walls). The mice were allowed to explore the novel environment for 15 minutes. The mice underwent perfusion fixation 90 minutes after the novel environment exposure.

**2.7 Histology**

The way to prepare fixed brain sections has been previously described (Tashiro *et al.*, 2015a). 40 μm-thick coronal sections were collected from the antero-posterior level covering the entire hippocampus (approximately 1.22 mm to 3.80 mm posterior to the bregma). The sections were stored in a cryoprotectant solution (20% glycerin, 30% ethylene glycol in 0.1 M PB) at -20 °C until use. After being rinsed with 0.1 M Tris-buffered saline (TBS), the sections were incubated with a blocking solution [0.25% Triton X-100 (Sigma, USA) and 3% donkey serum (Sigma, USA) in 0.1 M TBS] for 60 minutes to minimize non-specific immunoreactions. The sections were incubated with primary antibodies in the blocking solution for 2 days at 4 °C. After washing sections with 0.1 M TBS, and the sections were then incubated with secondary antibodies in the blocking solution for 4 hours at room temperature. Nuclear staining with 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI, MERCK, Germany) was performed after treatment with secondary antibodies. The primary antibodies used were rabbit anti-c-fos (1:500; Santa Cruz Biotechnology Cat# sc-52), goat anti- DCX (1:500 or 1:400; Santa Cruz Biotechnology Cat# sc-8066), rat anti-GFP (1:500; Nacalai Tesque Cat# 04404-84), rabbit anti-GFP (1:500; Life Technologies Cat# A11122), mouse anti-NeuN (1:500; Millipore Cat# MAB377), rat anti-BrdU (1:1000; AbD Serotec Cat# OBT0030G) and goat anti-Iba1 (1:500; Abcam Cat# ab5076). All secondary antibodies

were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and used at 1:600 or 1:250 dilutions. The secondary antibodies used were donkey anti-rabbit-DyLight 488 (Cat# 711-485- 152), donkey anti-rat-Alexa 488 (Cat# 712-545-153), donkey anti-rat-Cy3 (Cat# 712-165-153), donkey anti-goat-DyLight 649 (Cat# 705-495-147), donkey anti-mouse-DyLight 649 (Cat# 715- 495-151) and donkey anti-goat DyLight 549 (Cat# 705-505-147). Golgi staining was performed using FD Rapid GolgiStainTM kit (Cat# PK401, FD NeuroTechnologies, Inc.).

**2.8 Image analysis**

Epifluorescence imaging and cell counting were performed with Axio Scope A1 or Axio Imager M1 microscopes (Zeiss, Germany) with 5x, 10x and 20x objective lenses. Confocal imaging was conducted using an LSM710 confocal microscope (Zeiss, Germany) equipped with 488-, 543- and 633-nm laser lines and ZEN image-acquisition software. ImageJ software (National Institutes of Health, USA) was used to count cells in confocal images and measure the area of granule cell layers defined by DAPI staining. The volume of the granule cell layer for individual sections was calculated by multiplying the area of the granule cell layer by the thickness of the section (40 μm). Cell density was calculated by dividing the cell number by the volume of the granule cell layer. The normalized cell densities indicated in Fig. 2 and S3 were calculated as the densities in the ablated hemisphere divided by the densities in the contralateral non-injected hemisphere of each mouse.

The proportion of GFP+/DCX- cells in the total granule cell population was estimated using previously reported estimates of granule cell density in the granule cell layer (3.3 x $10^6$ cells/$mm^3$) (Abusaad *et al.*, 1999). The number of GFP+/DCX- cells and the volume of the granule cell layer were measured from confocal images covering the entire granule cell layer of three sections per mouse. These sections were selected from comparable rostro-caudal levels between mice.

The normalized densities of c-fos+ or DCX+ cells were calculated by dividing the density in the injected hemispheres by the densities in non-injected hemispheres in the same mice (Fig. S3C and E).

Confocal z-stacks for Golgi-stained granule cells were acquired, and the dendrites of Golgi- stained granule cells were traced using Neuromantic software (Myatt *et al.*, 2012).

Sholl analysis was performed in ImageJ software with Fiji package (Schindelin *et al.*, 2012). Dendritic spines were counted under Axio Scope A1 microscope with a 100x objective lens (Zeiss, Germany).

**2.9 Water maze task**

Training in a series of water maze tasks was performed in a 1-m diameter pool placed in a dimly lit room. Dark blue curtains displaying two visual cues covered the area around the pool. The pool was filled with 17-21 °C water approximately 30 cm deep. The water was made opaque with white, non-toxic paint. For all trials except the probe trials, the mice were moved to the water maze room in Plexiglas cages and allowed to habituate to the room for 5-10 minutes before the beginning of the experiments. Then were placed in the pool facing the wall and left to swim until they located a transparent Plexiglas circular platform (11 cm in diameter). The mice were removed from the pool after 30 seconds on the platform for the first trial of the day or 15 seconds in subsequent trials. When the mice failed to locate the platform within 60 seconds, they were directed toward the platform by the experimenter using a finger to point at the platform's position, and the experimenter waited for the mice to climb onto the platform. Mice were dried with paper towels after each trial.

"Pre-training" using a visible platform was performed over 4 days prior to surgery. The platform was made visible to the mice by adjusting the water level 1-2 cm below the platform. At the beginning of each day, the platform was moved to a new position 10 cm from the wall and distributed in four quadrants (NW, NE, SW and SE). The mice underwent three trials per day. The starting positions were pseudorandomly determined from the three locations at 90, 180 and 270 degrees from the platform.

One to three days after the completion of pre-training, viral vector injections into both hemispheres were performed. After 7 days of recovery, "training" using a hidden platform was performed in 1 day. The platform was submerged 1 cm below the water surface. The starting positions were pseudorandomly determined from the four locations at 45, 135, 225 and 315 degrees from the platform. Four blocks of three trials were performed in both the morning and the afternoon (a total of 24 trials), with a 6-hour interval between the morning and afternoon sessions. The platform position was identical throughout the 24 trials. Between 5 and 10 minutes from the end of the last training trial,

the mice were injected intraperitoneally with either DT (50 μg/kg) or an equal volume of vehicle (DPBS).

After 7 days, spatial memory for the platform position used during training was tested in probe trials. The platform was removed from the pool, and the mice in Plexiglas cages were moved individually to the water maze room before their trials. Then, the mice were left alone in the room for 3 minutes. The mice were then placed into the pool facing the wall and allowed to swim for 1 minute in the pool. Three probe trials were conducted for each mouse with inter-trial intervals of 1 minute.

Starting 1 day after the probe trials, the mice underwent "re-training" with eight trials per day for three consecutive days. The platform was submerged 1 cm below the water surface. The platform was moved to a new position each day in one of three quadrants that had not been used during training or previous re-training days. The starting position was pseudo-randomly chosen from four locations at 45, 135, 225 and 315 degrees from the platform. After the final re-training trial, the mice were perfusion fixed with 4% paraformaldehyde in 0.1M PB.

All trials were recorded with a video camera placed over the pool and analyzed with the ANY-maze software (Stoelting, USA). For Fig. 3, the first probe trial of one control mouse was accidentally not recorded, and data for this mouse were removed from the probe trial analysis. For pre-training, training and re-training, swim speed and latency to reach the platform were measured. For the probe trials, the time that the mice spent in proximity to the platform position (within 14 cm from the center of the platform), the number of entries into the platform position and swim speed were measured. The latency to reach the platform was measured as the time from when the mouse was placed in the pool to when the mouse climbed onto the platform, which was defined as when 80% of the mouse's body was located inside the platform zone. The chance level of time spent near the platform was calculated based on the area ratio of near-platform zone to the whole pool. For occupancy plots, the time series of position data extracted from the ANY-maze software were smoothed with a two-dimensional Gaussian filter and used to calculate the time spent in each position of the pool using custom-made programs written in Matlab software (Mathworks, USA).

**2.10 Fear conditioning**

Fear conditioning was performed in a conditioning chamber (24 cm wide × 20 cm deep × 30 cm high, Ugo Basile, Italy). Shortly after mice were moved into the chamber, the behavior of mice started being recorded with an overhead infrared red light camera. The floor and wall were cleaned with 70% ethanol before training for each trial. The data were analyzed for freezing behavior using EthoVision software (Noldus, USA). After virus injection, mice were handled for 10 min per day for 5 days.

Training for trace tone fear conditioning was conducted in context A, which had a floor made of steel rods and acrylic walls and was scented with acetic acid. The floor and wall were cleaned with 70% ethanol before training for each mouse. Training was performed in a trace conditioning protocol, which was consisted of five pairings of a tone (5 kHz, 75 dB, 20 sec) and an electrical foot-shock (0.4 mA, 2 sec) with a 20-s interval. Tone delivery started at 180, 370, 620, 900, and 1060 sec after the start of recording. Foot-shock delivery started at 220, 410, 660, 940, and 1100 sec after the start of recording. 1 week after the training the tone tests were conducted in context B, which had a white plastic floor and stripe-pattern walls. The floor and wall were cleaned with Clorox Fresh Scent before a test for each mouse. Tone delivery (5 kHz, 75 dB, 20 sec) started at 180, 280, 390, 510, and 620 sec after the start of recording without pairing with a foot-shock. Data from the first tone test were analyzed. Because of accidental cessation of the task, abnormal morphology of the dentate gyrus or failure of DT or virus injections, we removed 7 mice. The total numbers of mice included in the analysis were 13 and 12 mice for the control and ablated groups, respectively.

Training, reminder training and contextual tests for contextual fear conditioning were conducted in context A. During training, mice received an electrical foot-shock (0.7 mA, 2 sec) 180 sec after the start of recording. The mice stayed there for additional 120 sec before being removed from the chamber. During reminder training conducted next day, the mice received an electrical foot-shock (0.7mA, 0.2 sec) 180 sec after the start of recording. The mice stayed there for an additional 120 sec before being removed from the chamber. During contextual tests, the mice stayed in the chamber for 180 sec after the start of recording. No foot-shock was given. Data from the first contextual test were

analyzed. Because of failure of virus injections, we removed 2 mice. The total numbers of mice included in the analysis were 20 and 18 mice for the control and ablated groups, respectively.

**2.11 Statistical analyses**

Statistical analyses were performed with Excel (Microsoft, USA) and SPSS software (IBM, USA). For independent t tests, we first performed "F-test two sample for variances" in Excel or "Levene's test for equality of variances" in SPSS. When $p < 0.05$, a t-test without the assumption of unequal variances was performed. When $p > 0.05$, a t-test with the assumption of equal variances was performed. For repeated-measures ANOVA, we first performed Mauchly's test of sphericity. When $p < 0.05$ for a given parameter, the Huynh-Feldt correction was applied. All data in text and figures were presented as the mean ± s.e.m. Sample size was determined considering previous studies using similar water maze tasks and fear conditioning tasks.

# 3. Results

### *3.1 Virus-mediated ablation of immature granule cells in the dentate gyrus*

To selectively ablate immature granule cells in the dentate gyrus (DGCs), we developed a lentiviral vector bicistronically expressing diphtheria toxin receptor (DTR) and enhanced green fluorescent protein (GFP) under the control of the DCX promoter (Fig. 1A, B). DCX is expressed in neuronal progenitors and immature DGCs in the dentate gyrus (Brown *et al.*, 2003). In adult mice, DCX expression starts decreasing < 2 weeks after neuronal birth, and then further decreases along neuronal maturation (Kempermann *et al.*, 2003; Snyder *et al.*, 2009). Therefore, DTR expression is expected to be limited to neuronal progenitors and immature DGCs. GFP expression was localized predominantly along the hilar border of the granule cell layer across the antero-posterior axis of the dentate gyrus, where adult-born DGCs are located (SI Appendix, Fig. S1). Immunostaining for DCX revealed that up to 40.7% of DCX+ cells expressed GFP, indicating that viral transduction was achieved in a large proportion of immature DGCs.

As a proof of principle for the ablation method, we injected the viral vector into the dentate gyrus, and 1 week later, we intraperitoneally injected diphtheria toxin (DT) for ablation or PBS as a control. After an additional 1-week survival period, we perfused the mice and examined DCX+ cells in the dentate gyrus. We observed a clear reduction in the density of DCX+ cells in DT-injected mice compared to that in PBS-injected controls (Fig. 1C; quantitative results of larger cohorts in SI Appendix, Fig. S7B, S8B, D), while the volume (SI Appendix, Fig. S2A, B; quantitative results of larger cohorts in SI Appendix, Fig. S7C, S8C, E) and cell density (SI Appendix, Fig. S2C) of the granule cell layer and the morphology of mature granule cells (SI Appendix, Fig. S2D-G) remained intact. These observations indicate that DT injection ablated DCX+ cells without inducing the large-scale elimination or damage of mature DGCs in the granule cell layer. We found that the ablation of immature DGCs gradually occurred over multiple days (SI Appendix, Fig. S3A-C) and that the ablation technique did not affect neurogenesis either in the olfactory bulb or in the subventricular zone (SI Appendix, Fig. S4). As observed in a previous study (Arruda-Carvalho *et al.*, 2011), an increase in the number of Iba1+ cells was detected after the induction of ablation by a combination of the viral vector and DT

Figure 1

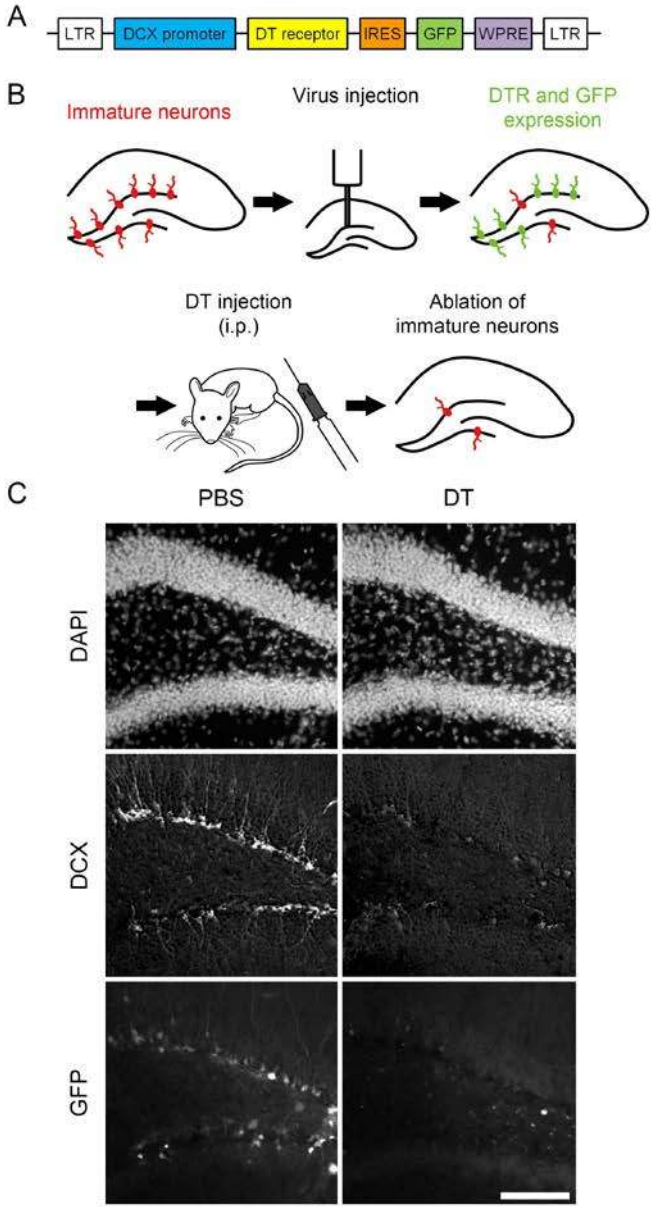



**Figure 1. Diphtheria toxin-induced ablation of DCX+ cells in the dentate gyrus using lentiviral transduction of diphtheria toxin receptor.**

**A**, Schematic representation of the recombinant lentiviral vector construct. LTR: long terminal repeat; DCX: doublecortin; DT: diphtheria toxin; IRES: internal ribosome entry site; GFP: green fluorescent protein; WPRE: Woodchuck hepatitis virus post-transcription regulatory element.

**B**, Diagram of the ablation technique specific for immature DGCs.

**C**, Fluorescent images of the virus-injected dentate gyrus after the injection of PBS or DT. (Top) The granule cell layer visualized by DAPI staining was intact after DT-induced ablation. (Middle and Bottom) DT injection led to an obvious reduction in DCX+ and GFP+ cells. Scale bar: 100 μm.

## Figure S1

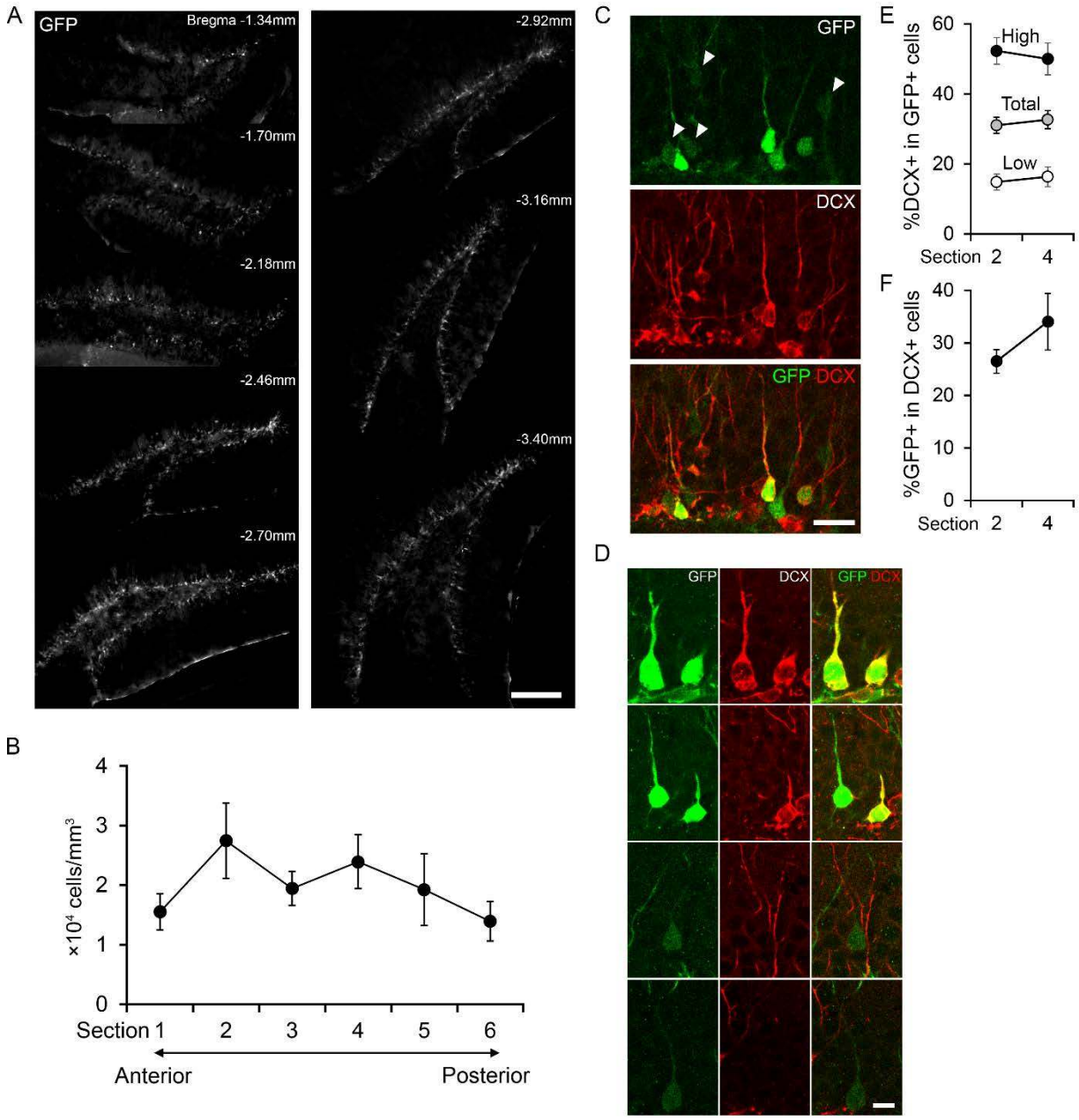



**Fig. S1. The distribution and specificity of viral transduction.**

**A-C**, Historical characteristics of virus-driven GFP expression 7 days after virus injection, which corresponds to the time when DT was injected in behavioral experiments (Fig. 3-5).

**A**, Representative images showing the distribution of virus-transduced cells (GFP+ cells) over the antero-posterior axis of the dentate gyrus.

**B**, Density of GFP+ cells across the antero-posterior axis of the dentate gyrus (9 mice). GFP+ cells were quantified in every 12 coronal sections covering the antero-posterior axis of dentate gyrus. Sections 1 and 6 correspond to approximately 1.06-1.34 and 3.52-3.80 mm posterior to the bregma, respectively.

**C**, A representative confocal image of the granule cell layer in a section immunostained against GFP and DCX. Among 460 DCX+ cells examined (from one hemisphere in three sections each of two mice), 40.7% of DCX+ cells expressed GFP, indicating that viral transduction was achieved in a large proportion of

immature DGCs. Among 455 GFP+ cells examined (from one hemisphere in three sections each of two mice), 43.8% of GFP+ cells were DCX+. We noted that GFP expression level was highly variable between GFP+ cells (low GFP-expressing cells are highlighted by arrowheads).
**D-F**, Further quantification 17 days after virus injection (9 control mice after the behavioral experiment described in Fig. 3).
**D**, Representative confocal images showing colocalization of DCX and GFP in DGCs in the granule cell layer.
**E**, Proportion of GFP+ cells expressing DCX. Percentage within total population, high and low GFP-expressing cell populations were plotted separately.
**F**, Proportion of DCX+ cells expressing GFP. We observed some GFP+DCX- cells which appeared to include non-neuronal and granule cells based on their morphology. Without an active degradation mechanism, GFP protein is highly stable even after the transcription activity of the promoter is shut off (Andersen *et al.*, 1998). Therefore, GFP protein is expected to be maintained in new DGCs that expressed DCX at the time of virus injection but thereafter lost the expression of DCX (and presumably DTR) during the survival time. Thus, some of GFP+DCX- cells were likely to be new DGCs that had just lost DCX expression. In consistent with this possibility, the proportion expressing DCX was higher in high GFP-expressing cells (>50%) than that of low-expressing cells (<20%). Nonetheless, we cannot completely exclude the possibility that some of GFP+ cells were mature DGCs. Our estimation revealed that the proportion of GFP+/DCX- cells among the total granule cell population in the dentate gyrus would be small (0.6%, see the Materials and Methods). This is consistent with our observation of no ablation of mature granule cells (9 weeks old), shown in Fig. 2. Scale bars: 200 μm in A, 20 μm in C, and 10 μm in D.

## Figure S2

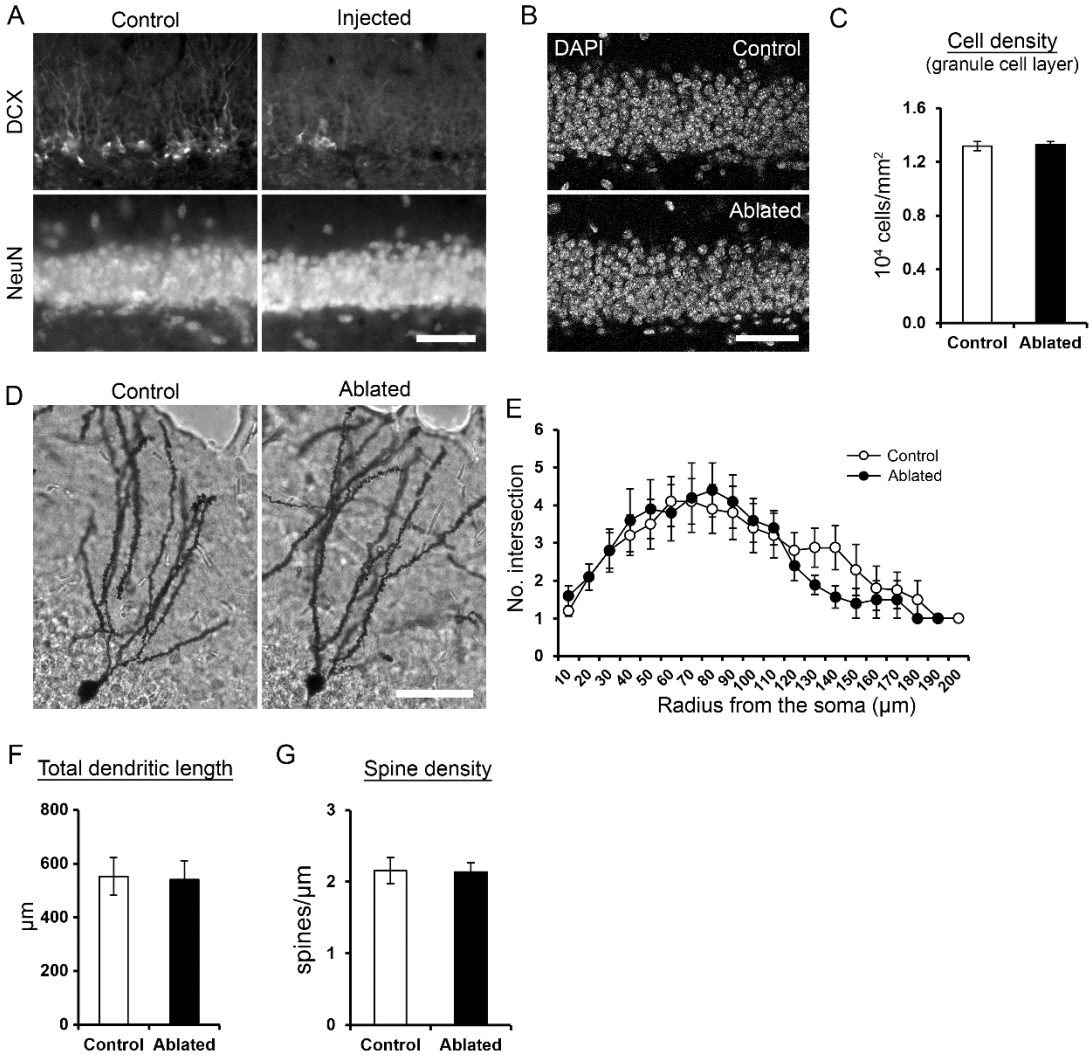



**Fig. S2. The overall integrity of the granule cell layer and mature granule cells is intact after DT-induced ablation.**

**A**, NeuN immunostaining showed the integrity of granule cell layer after DT-induced ablation. The lentiviral vector was injected into the dentate gyrus in one hemisphere. Seven days later, DT was injected. After another 7-day survival, brain sections were prepared and immunostained with DCX or NeuN. The images were from virus-injected (Injected) and non-injected (control) hemispheres. Scale bar: 50 μm.

**B**, DAPI immunostaining showing the integrity of granule cell layer of mice in the control and ablated group.

**C**, Cell density in the granule cell layer was not affected by the ablation ($p = 0.783$, independent sample t test, two-tailed). The analysis was performed using the sections from the mice used in the experiments described in Fig. 4 and 5.

**D**, Golgi-stained granule cells of mice in the control and ablated group. The lentiviral vector was injected into the dentate gyrus in both hemisphere, and PBS or DT was systemically injected 7 days later. After another 7-day survival, the mice were euthanized for the Golgi staining. Scale bar: 50 μm.

**E**, Sholl analysis for Golgi-stained granule cells in the control and ablated group. No significant group difference was detected (two-way repeated measures ANOVA, Group: $p = 0.594$, Group x Radius interaction: $p = 0.997$, two-tailed).

**F**, Total dendritic length of the Golgi-stained granule cells. No significant, group difference was detected ($p = 0.907$, independent sample t-test, two-tailed).

**G**, The density of dendritic spines in the Golgi-stained granule cells. No significant group difference was detected ($p = 0.923$, independent sample t-test, two-tailed). Quantifications were based on 10 cells from 3 control mice and 10 cells from 2 ablated mice.

## Figure S3

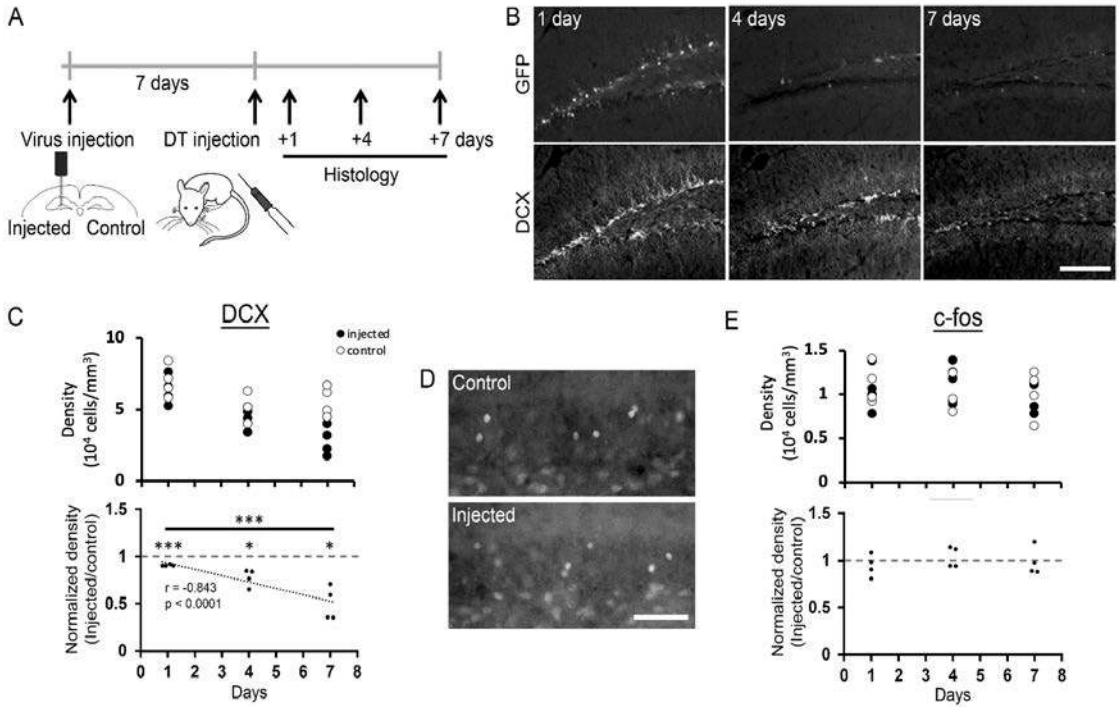



**Fig. S3. Ablation of DCX+ cells occurs over multiple days while keeping the ability of protein synthesis/activity-dependent gene expression intact in the granule cell layer**.

**A**, Experimental design. We injected the viral vector into one hemisphere of the dentate gyrus and administered DT one week later. Either at 1, 4 or 7 days after the DT injection, the mice were allowed to explore a novel, open-field environment.

**B**, Representative images showing the reduction of GFP+ cells over 7 days after DT injection.

**C**, Densities of DCX+ cells in the virus-injected hemisphere after DT injection. Normalized DCX+ cell density and days after DT injection showed a significant, negative correlation ($r = -0.843$, $p = 5.7 \times 10^{-4}$), indicating that normalized DCX+ cell density reduced over time. Further two-way (hemisphere x day) repeated measures ANOVA detected significance in the main effect of day and hemisphere and hemisphere x day interaction (Day: $p = 0.0023$, Hemisphere: $p = 2.0 \times 10^{-5}$, Hemisphere x Day: $p = 0.0023$). Post-hoc, one sample t-test against 1 indicated that the normalized density of DCX+ cells were significantly reduced in injected hemispheres than in non-injected controls at all three time points (1day: $p = 3.0 \times 10^{-4}$, 4 days: $p = 0.016$, 7 days: $p = 0.011$, two-tailed). Post-hoc independent sample t-test between days showed that the normalized density at 4 days after DT injection was not significantly lower than that at 1 day ($p = 0.062$, independent t-test, two-tailed), but reached significance at 7 days ($p = 9.6 \times 10^{-4}$). Thus, the ablation of immature DGCs gradually occurred over multiple days.

**D**, Representative images of c-fos expression in the granule cell layer of virus-injected and non-injected control hemispheres.

**E**, Densities of c-fos+ cells over 7 days after DT injection. Two-way (hemisphere x day) repeated measures ANOVA did not detect any significance in the main effects or interaction (Day: $p = 0.613$, Hemisphere: $p = 0.772$, Hemisphere x Day: $p = 0.613$), which indicates that normalized c-fos+ cell density is not affected in the injected hemisphere or over time. This conclusion was further supported by failing to detect significant correlation between the normalized c-fos+ cell density and days after DT injection ($r = 0.144$, $p = 0.654$). Scale bars: 150 µm in B and 100 µm in D. Quantifications were based on 4 mice each day after DT injection (12 mice in total, a section/hemisphere was used).

## Figure S4

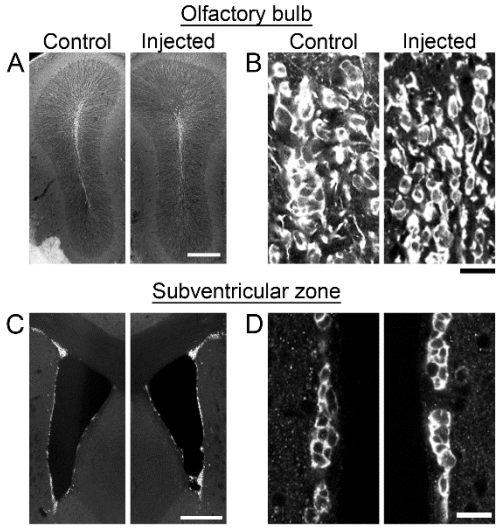



**Fig. S4. Intact olfactory bulb neurogenesis after DT-induced ablation in the dentate gyrus.**

Fluorescent images of DCX+ cells in the olfactory bulb (**A**, **B**) and subventricular zone (**C**, **D**) 1 week after DT-induced ablation in the dentate gyrus. Images from virus-injected and control hemispheres are shown. We did not observe any obvious difference between the virus-injected hemisphere and non-injected control hemisphere either in the olfactory bulb or in the subventricular zone. This observation was confirmed by quantifying the density of DCX+ cells in the subventricular zone (control hemisphere: 2.97 ± 0.02; ablated hemisphere: 3.22 ± 0.15 in $10^3$ cells/mm$^2$, n = 3, p = 0.248, paired t-test, two-tailed) and the granule cell layer of the olfactory bulb (control hemisphere: 5.35 ± 1.47; ablated hemisphere: 5.68 ± 1.14 in $10^4$ cells/mm$^3$, n = 3, p = 0.825, paired t-test, two-tailed). Scale bars: 300 µm in A and C and 15 µm in B and D.

## Figure S5

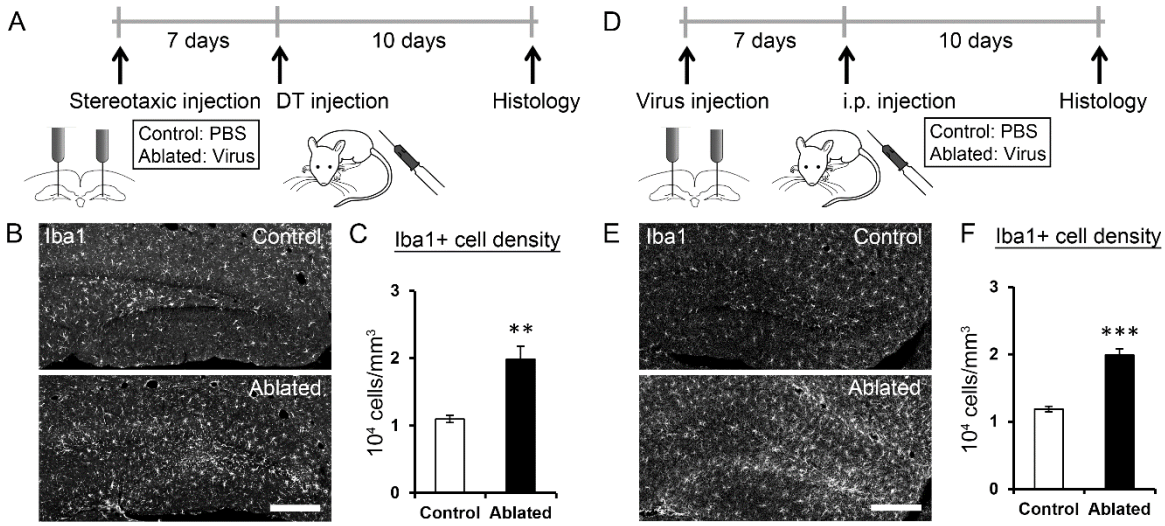



**Fig. S5. Iba1 expression in the dentate gyrus is associated with virus injection and ablation.**

**A** previous study showed that the ablation of adult-born DGCs induces moderate inflammation associated with increased Iba1+ microglia in the dentate gyrus (Arruda-Carvalho *et al.*, 2011). Therefore, we performed immunostaining against Iba1 to evaluate the extent of inflammation. For this experiment, we used brain sections from the mice used in the water maze experiment described in Fig. 3. A, Experimental design. Mice were stereotaxically injected with PBS (control group) or the viral vector (ablated group) into the dentate gyrus. After 7 days of recovery, DT was injected into both groups. Brain sections were prepared from the mice after an additional ten days of survival.

**B**, Representative images of Iba1 immunostaining of brain sections from the control and ablated groups.

**C**, Density of Iba1+ cells in the dentate gyrus. Although vehicle injection into the dentate gyrus alone appeared to increase the number of Iba1+ cells compared to the number in the non-injected hemispheres of mice from other experiments, an increase was observed after the induction of ablation by a combination of the viral vector and DT injections ($p = 0.0081$, independent sample t-test, two-tailed, $n = 5$ for each group), as was observed in the previous study (Arruda-Carvalho *et al.*, 2011).

**D**, Experimental design. Mice were stereotaxically injected with the viral vector into the dentate gyrus. After 7 days of recovery, PBS (control group) or DT (ablated group) was injected into both groups. Brain sections were prepared from the mice after an additional ten days of survival.

**E**, Representative images of Iba1 immunostaining of brain sections from the control and ablated groups.

**F**, Density of Iba1+ cells in the dentate gyrus. An increase was observed after the induction of ablation by a combination of the viral vector and DT injections ($p = 3.9 \times 10^{-5}$, independent sample t-test, two-tailed, $n = 5$ for each group), as was observed in the previous study (Arruda-Carvalho *et al.*, 2011). **: $p < 0.01$, ***: $p < 0.005$. Scale bars: 300 μm.

injections (SI Appendix, Fig. S5). The increase of Iba1+ cells by DT-mediated ablation or inhibition of protein synthesis induced by DT (Collier, 1975) may compromise the function of remaining DGCs. Nonetheless, the function of the dentate gyrus seemed to be intact as an activity-dependent gene (c-fos) expression, an index of neuronal activation (Morgan and Curran, 1991), were intact in DGCs after the DT-induced ablation (SI Appendix, Fig. S3D, E).

Next, to determine the maturational stage of ablated DGCs, we labeled different ages of adult-born DGCs by injecting a thymidine analog, 5-bromo-2'-deoxyuridine (BrdU), into three groups of mice 8-14 days (for DCX+ immature DGCs), 4 weeks (for young DGCs mostly DCX-negative) and 9 weeks (for mature DGCs) before DT injection (Fig. 2A). One week before the DT injection, the viral vector was injected into the dentate gyrus of one hemisphere of all mice. We perfused the mice 1 week after the DT injection and analyzed the density of adult born DGCs (BrdU+/NeuN+ cells) (Fig. 2B, C). The normalized densities of 8-14 day-old DGCs were significantly reduced in the granule cell layer of the virus-injected hemisphere compared with the control hemisphere [65.8 ± 5.7% reduction; two-way (hemisphere x maturation stage) repeated measures ANOVA, hemisphere: $p = 5.9 \times 10^{-4}$, maturation stage: $p = 2.2 \times 10^{-4}$, hemisphere x maturation stage: $p = 2.2 \times 10^{-4}$, two-tailed, n = 13 mice in total; post-hoc, one sample t-tests, $p = 3.3 \times 10^{-4}$, two-tailed]. On the other hand, the normalized density of 4- and 9-week-old DGCs did not show any significant reduction while 4-week-old DGCs showed a slight, non-significant decrease ($p > 0.44$ for both). The normalized densities of 8-14-day-old DGCs were significantly lower than 4-week-old ($p = 0.0028$, post-hoc, independent t-tests, two-tailed) and 9-week-old DGCs ($p = 8.7 \times 10^{-5}$). It is known that DCX expression occurs in >80% of new DGCs at <2 weeks of neuronal age, reduces to a large extent at 4 weeks and is then virtually none at neuronal maturity (Jagasia *et al.*, 2009; Snyder *et al.*, 2009). Thus, the pattern of ablation is consistent with DCX expression along neuronal maturation. Furthermore, 77.4 and 90.2% of the ablated new DGCs are estimated to be younger than 14 and 21 day old at the time of DT injection, respectively, indicating that most ablated DGCs are at the early maturational stage (SI Appendix, Fig. S6).

Figure 2

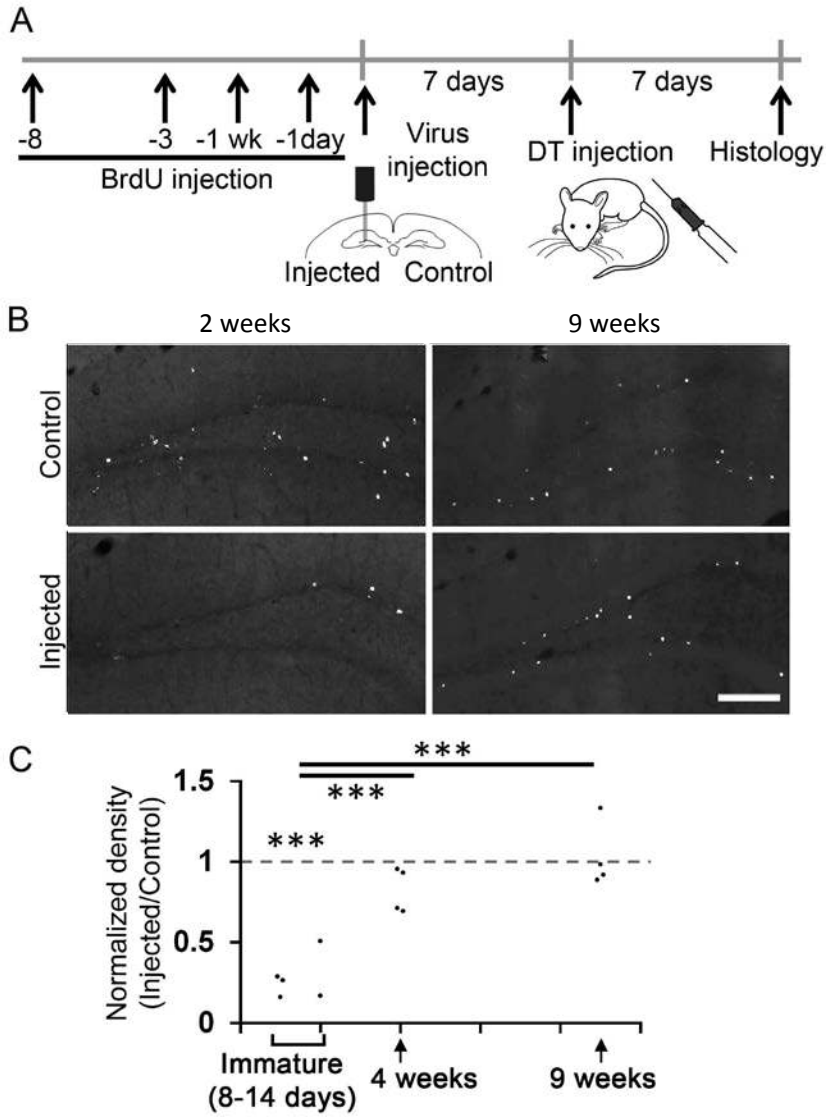



**Figure 2. DT-induced ablation of new DGCs at the early maturational stage.**

**A,** Experimental design. To label the different ages of newborn DGCs, BrdU was injected into mice at four different time points (8 days, 2, 4, and 9 weeks) before DT administration. For each mouse, one dose of BrdU (100 µg/g body weight) was injected intraperitoneally.

**B,** Representative images showing 2-week-old or 9-week-old BrdU+ cells from virus-injected and non-injected control hemispheres after DT injection. Scale bar: 150 µm.

**C,** Normalized densities of BrdU+/NeuN+ cells. The densities in the injected hemispheres were normalized by dividing by the densities in the non-injected hemispheres of the same mice. Each data point represents one mouse (8 days: n = 3 mice, 2 weeks: n = 2, 4 weeks: n = 4, 9 weeks: n = 4). A value of 1 indicates that the densities of both hemispheres are equal. Note that a significant reduction in immature (8-14-day-old) DGCs but minimal effects on 4- and 9-week-old DGCs. The densities were measured from three sections (every twelve 40-µm sections, corresponding to sections 2-4 in SI Appendix, Fig. S1B) for each mouse. These three sections cover the most part of the dorsal region of the dentate gyrus. ***: $p < 0.005$.

## Figure S6

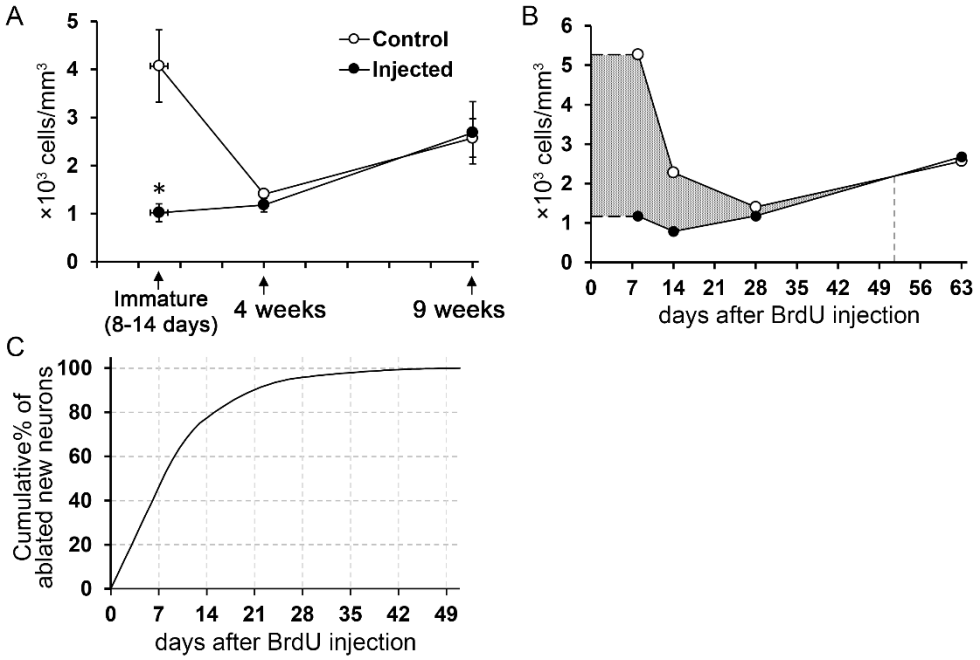



**Fig. S6. Age of new DGCs removed by DT-induced ablation.**

**A**, The density of BrdU+/NeuN+ cells in virus-injected and non-injected control hemispheres (absolute values before normalization in Fig. 2C). Two-way (hemisphere x maturation stage) repeated measures ANOVA showed that the main effects of hemisphere and hemisphere x maturation stage interaction were significant while the main effect of maturation stage was not (Hemisphere: $p = 0.0046$, Hemisphere x Maturation stage: $p = 0.0018$, Maturation stage: $p = 0.076$, two-tailed). Post-hoc, paired t-tests showed that BrdU+/NeuN+ cell density at 8-14 days was significantly reduced in the virus-injected hemispheres than in non-injected controls ($p = 0.011$, two-tailed) while those at 4 and 9 weeks were not ($p > 0.15$ for both, two-tailed).

**B**, Estimation of the numbers of ablated DGCs at different ages, using trapezoidal rule integration. Reduction in the number of new DGCs from the control to injected hemisphere reflects the number of ablated new DGCs. Therefore, we estimated the number of ablated new DGCs at different ages using the following three extrapolations. 1) We used the value on day 8 to estimate the number on days 1-7. 2) The numbers between time points were estimated by the trapezoidal rule. 3) We assumed that new DGCs up to 51-day-old were ablated. This is because, with the trapezoid rule, the density in the control hemisphere becomes lower than that in the injected hemisphere from day 52 onwards, and the numbers of ablated new DGCs become negative. Using these assumptions, the number of ablated DGCs can be estimated by calculating the area in the graph which is below the line formed by control hemisphere data and above the line formed by the injected hemisphere (gray area).

**C**, Cumulative percentage of ablated new DGCs at different ages, using trapezoidal rule integration. Percentages of ablated DGCs younger than different ages in the total ablated population were plotted. *: $p < 0.05$.

### *3.2 Impaired persistency of learned behavior in water maze probe tests after ablation of immature DGCs*

To understand the role of young DGCs in memory processes after initial acquisition, we ablated young DGCs after normal memory acquisition in a water maze task. Then we examined its effects on memory retention in a probe test (Fig. 3A). First, we pre-trained the mice over 4 days using a visible platform to familiarize them with the water maze procedures ("pre-training"). The mice were then bilaterally injected in the dentate gyrus with the viral vector. After a 7-day recovery period, the mice were trained in a hippocampus-dependent water maze task using a hidden platform in a constant position over 24 trials in one day ("training") (Trouche *et al.*, 2009). At the completion of the 24 trials, DT was systemically administered to the mice to induce the ablation of young DGCs (ablated group). Seven days after training, probe tests were performed to evaluate the retention of memory for the position where the platform was located during training. These tests were followed by "re-training" with new platform positions each day over three days. The control group were trained and tested in the same protocol except ablation was not induced (Control: PBS injection into the brain + DT injection intraperitonially, or LV injection into the brain + PBS injection intraperitonially).

We confirmed a reduction of DCX+ cells in the ablated group, while the volume of the granule cell layer was intact (SI Appendix, Fig. S7A-C). After water maze training and three additional days of survival, the extent of DCX+ cell reduction was lower than the experiment shown in Fig. 1. In pre-training and training (which were conducted before the ablation of immature DGCs), both groups learned the platform positions similarly well (Fig. 3B, SI Appendix, Fig. S7D, SI Appendix, TableS1).

For the probe tests, we initially analyzed 1) the time that the mice spent in proximity to the platform and 2) the number of entries into the area where the platform was located (Fig. 3C). The ablated group (n = 18 mice) showed significantly lower values in both parameters than the control group (n = 17 mice) (Fig. 3D, E, $p = 0.008$ and 0.019, respectively, independent sample t tests, two-tailed), whereas both control and ablated groups spent significantly longer time near platform than the chance level (Fig. 3E, Control: $p = 0.0025$, Ablated: $p = 0.042$, one-sample t-test, two tailed). These results

indicate that, while both groups remembered the platform position, the ablated group showed a deficit in memory retrieval.

To further examine the details in pattern of the deficit, we examined other parameters. Unexpectedly, latency to reach former platform area was comparable between the two groups (Fig. 3F). However, after the first entry to the platform area, the proportion of time spent near platform and the number of entries into the former platform area in ablated group were significantly lower than the control group (Fig. 3G, $p = 0.006$ and 0.019, respectively, independent sample t-test, two tailed). These results indicate that memory retrieval was initially intact in the ablated group and as accurate as the control group. The control group persisted to search platform after initial failure to find the platform, while the ablated group reduced search prematurely. In addition, we found that the duration of individual visits near platform was shorter in the ablated group (Fig. 3H, $p = 0.027$, independent sample t-test, two tailed), indicating that persistency of platform search in individual visits was impaired in the ablated group. Swimming speed was not significantly different between the groups (Fig. 3E, $p = 0.88$, independent-sample t-test, two tailed). Swimming speed was similar between the groups in the first (0 – 30 sec) and the late half (30 – 60 sec) of trial (SI Appendix, Fig. S9A, 0 – 30 sec: $p = 0.73$, 30 – 60 sec: $p = 0.42$, independent-sample t-test, two tailed), indicating that the ablation did not affect general activity in whole probe trial. In re-training, both groups learned the platform positions similarly well (SI Appendix, Fig. S7F; SI Appendix, TableS1).

Figure 3 (Continuing on p.40)

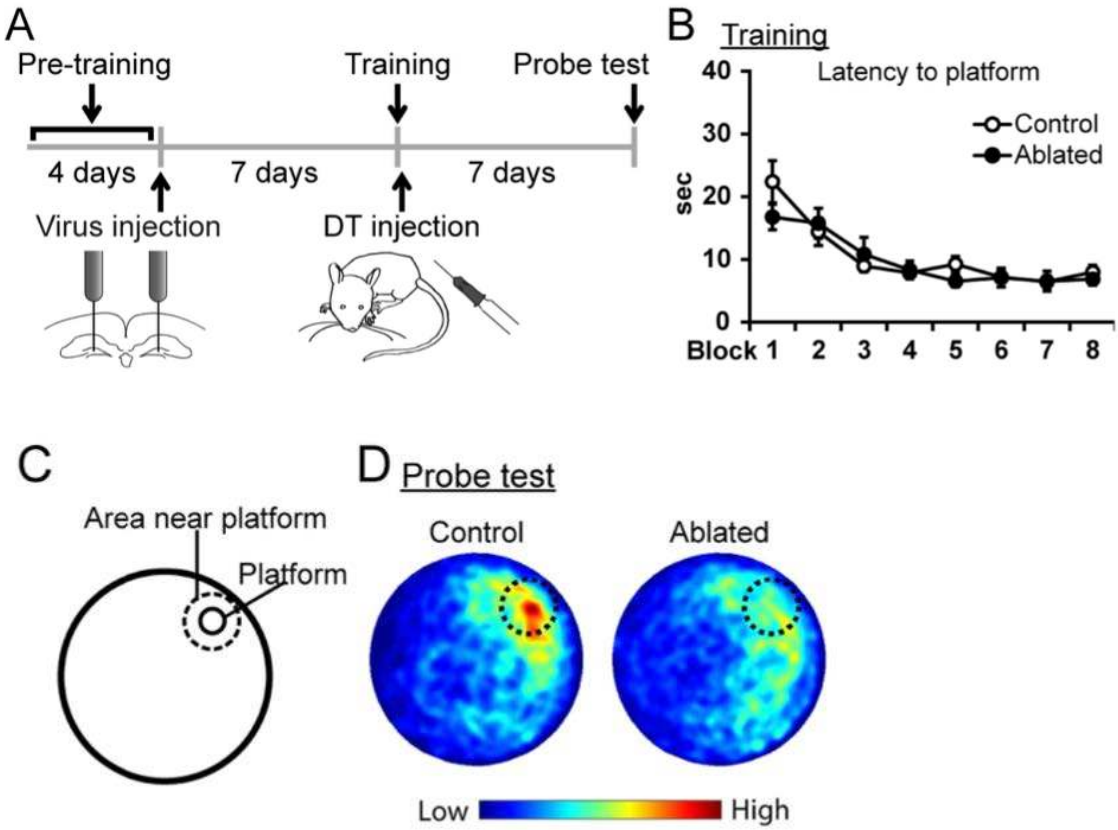



**Figure 3. Post-training ablation of DCX+ immature DGCs impaired the persistence of platform search behavior in a water maze task.**

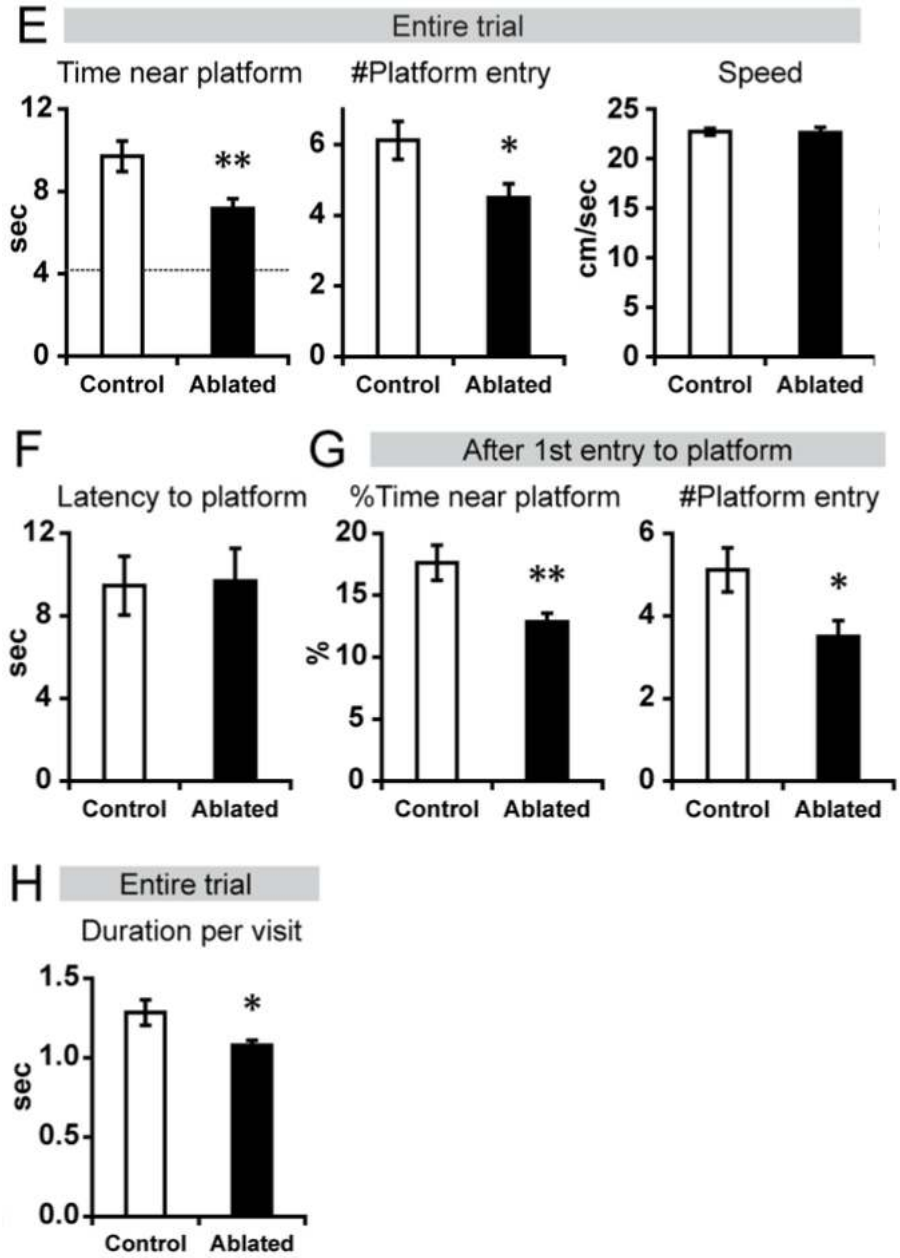
**A,** Experimental design.

**B,** Latency to reach platform during training. The two groups improved performance similarly well.

**C,** The position of the removed platform and the area near the platform in probe trials. The area near the platform is defined as the circular area within 14 cm of the center of the former platform position (~2% of whole area).

**D,** Occupancy plots showing average time that mice spent at different positions in the pool. The color bar below shows the color code for occupancy time; warmer colors indicate high occupancy, while cooler colors represent low occupancy. Dotted circles indicate the area near the platform.

Figure 3 (Continued from p.39)




**E,** Time spent in the area near the platform position, the number of platform entries and swimming speed in probe tests. A dotted line indicates the chance level in time spent near the platform position.
**F,** Latency to reach the position of the removed platform.
**G,** Percentage of time spent in the area near the platform position and the number of platform entries after the first entries into the position of the removed platform.
**H,** Duration per visit to the area near the platform. *: $p < 0.05$, **: $p < 0.01$.

## Figure S7

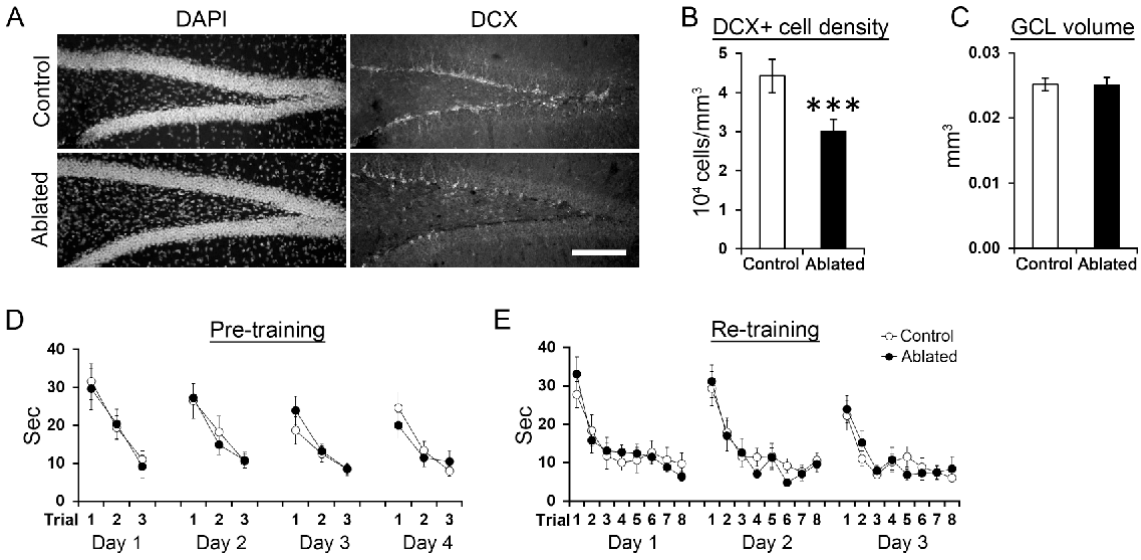



**Fig. S7. DT-induced ablation and water maze training performance in the mice for Fig. 3.**

**A**, Representative images of DAPI staining and DCX+ cells in the dentate gyrus of mice in the ablated and control groups. Scale bar: 150 μm.

**B**, The densities of DCX+ cells. The density was significantly lower in the ablated group than in the control (n = 18 mice for each group, $p = 4.6 \times 10^{-4}$, independent sample t-test, two-tailed).

**C**, The volume of the granule cell layer. The volume was not significantly different between the two groups (n = 18 mice for each group, p = 0.647, independent sample t-test, two-tailed)

**D**, **E**, Latency to locate the platform during pre-training (D), and re-training (E). For pre-training and re-training, repeated-measures ANOVAs (n = 18 mice for each group, SI Appendix, Table S1) detected significance in the main effects of trial/block ($p < 0.001$ for all) but not the main effects of group or any interactions involving group ($p > 0.05$ for all). Post hoc Fisher's LSD tests showed a significant reduction from the first trials/blocks to the last trials/blocks in pre-training and re-training ($p < 0.001$ for all). Thus, both groups learned the platform positions similarly well in pre-training and re-training. For pre-training, no group difference was expected because these tests were performed before the ablation of immature DGCs. ***: $p < 0.005$.

### *3.3 Impaired persistency of learned tone-induced freezing behavior after ablation of immature DGCs*

To test whether the post-learning role of immature DGCs is generalized to another form of hippocampus-dependent memory, we examined the effect of immature neuron ablation on a trace fear conditioning task. Similarly to the water maze experiment, 1 week after virus injection, mice received training for trace fear conditioning (Fig. 4A). The mice were exposed to five pairings of a tone (a conditioned stimulus) and an electrical shock (an unconditioned stimulus) with a 20-sec interval in context A (Fig. 4B). After the completion of training, mice in the control and ablated groups (n = 13 and 12 mice, respectively) received systemic injection of PBS and DT, respectively. The two groups showed similar freezing level during training (which was conducted before the induction of immature neuron ablation) both before and after the first tone was given (Fig. 4C, p = 0.47 for control and 0.64 for ablated, independent sample t tests, two-tailed). We confirmed a reduction of DCX+ cells in the ablated group while the volume of the granule cell layer was intact (SI Appendix, Fig. S8A-C).

1 week after training, mice underwent a tone test in context B, in which mice were exposed to five tones without a foot shock (Fig. 4D, E). We analyzed percentage time in freezing during 180-sec baseline period (before 1$^{st}$ tone exposure) and five consecutive 20-sec periods (Tone, Post-Tone 1, 2, 3, and 4) averaged over 5 tone exposures (Fig. 4D, F). Changes in freezing behavior over periods in ablated group are different from control group (Fig. 4F, Two-way (Period x Group) repeated measures ANOVA, Period x Group: p = 0.002). While the two groups showed comparable level of freezing during the baseline period and similarly increased freezing level in tone period (Fig. 4F , p = 0.25 and 0.93, respectively, independent sample t tests, two-tailed), from post-tone 1 onwards, freezing level of the ablated group became lower than the control group, which became significant during post-tone 3 and 4 periods (Fig. 4F, post-tone 1: p = 0.055, post-tone 2: p = 0.081, post-tone3: p = 0.001; post-tone 4: p = 0.017, independent sample t-tests, two-tailed). When we focused on the response to the first tone exposure only (instead of average over 5 exposures), the ablated group showed similar differences from the control group (Fig. 4G). Thus, this suggests that tone-conditioned freezing response of the ablated group was initially increased as control, but its increase did not persist as long as

control group. We found similar values in exploration speed before tone generation between control and ablated groups (Fig. S9B), suggesting that the ablation did not affect general activity.

To further look into the impaired persistency, we have also analyzed the frequency and duration of individual freezing episodes. The frequency of freezing episodes was not significantly different between the two groups and similarly increased after the first tone exposure (Fig. 4H, Period: $p = 3.2 \times 10^{-7}$, Group: $p = 0.743$, Period x Group: 0.097, two-way repeated measures ANOVA, two-tailed). While the duration of freezing episodes in both groups increased after the first tone exposure (Fig. 4I, Control: $p = 5.5 \times 10^{-5}$, Ablated: $p = 8.1 \times 10^{-5}$, paired t-test, two-tailed), the duration was shorter in ablated group than control group; (Period: $p = 4.5 \times 10^{-8}$, Group: $p = 0.029$, Period x Group: $p = 0.017$, two-way repeated measures ANOVA). During the baseline period the duration of freezing episodes was comparable between the groups (Fig. 4I, $p = 0.356$, independent t-test, two tailed), but after the onset of 1$^{st}$ tone, the duration was significantly shorter in the ablated group than in the control group (Fig. 4I, $p = 0.019$, independent t-test, two tailed). These analyses suggest that the reduced overall persistency of tone-conditioned freezing behavior after the initial retrieval in the ablated group was due to the reduced duration of individual episode, but not the frequency.

Figure 4 (Continuing on p.45)

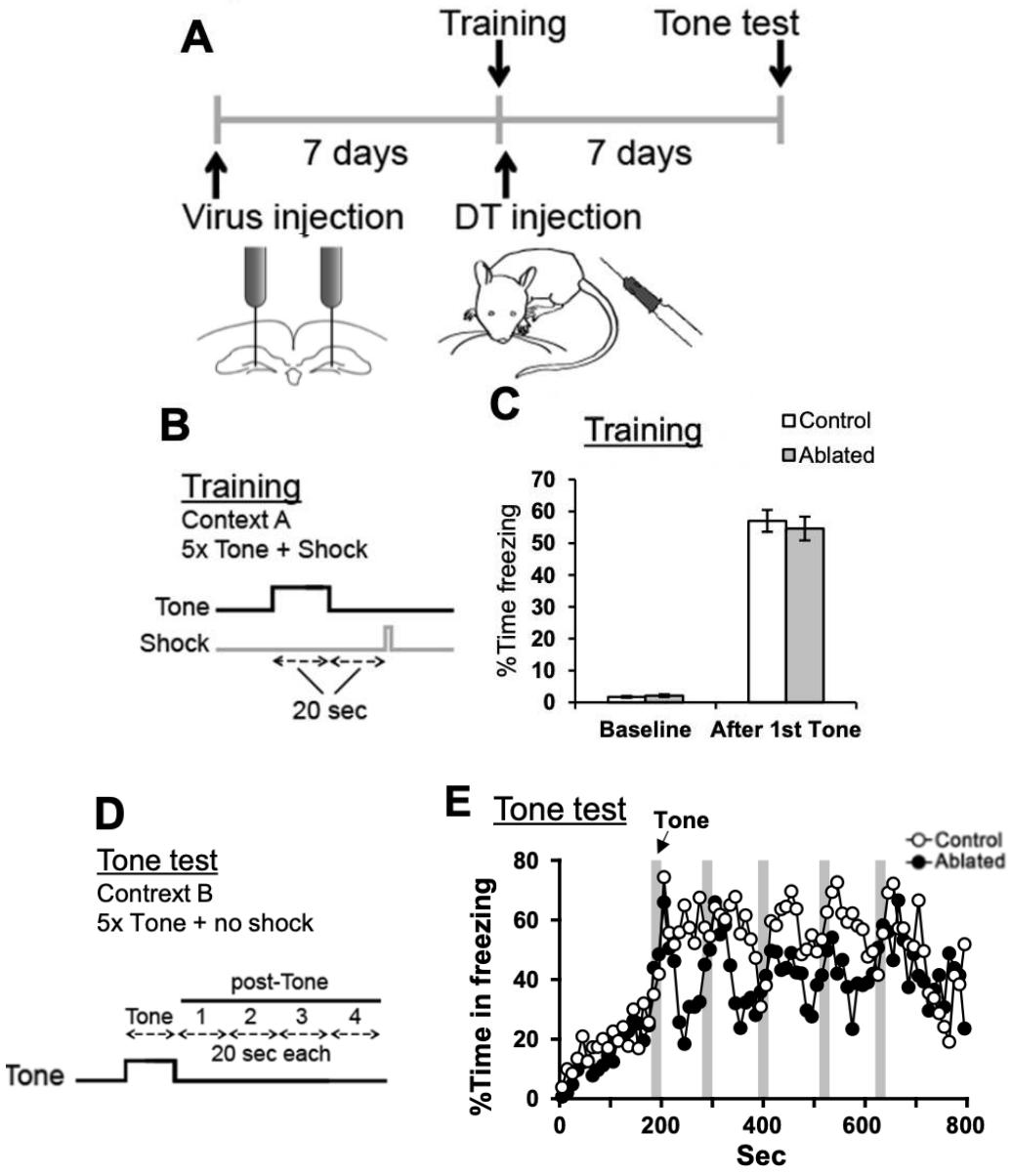



**Figure 4. Post-training ablation of DCX+ immature DGCs impaired the persistence of tone-induced freezing in a tone trace fear conditioning task.**

**A,** Experimental design.

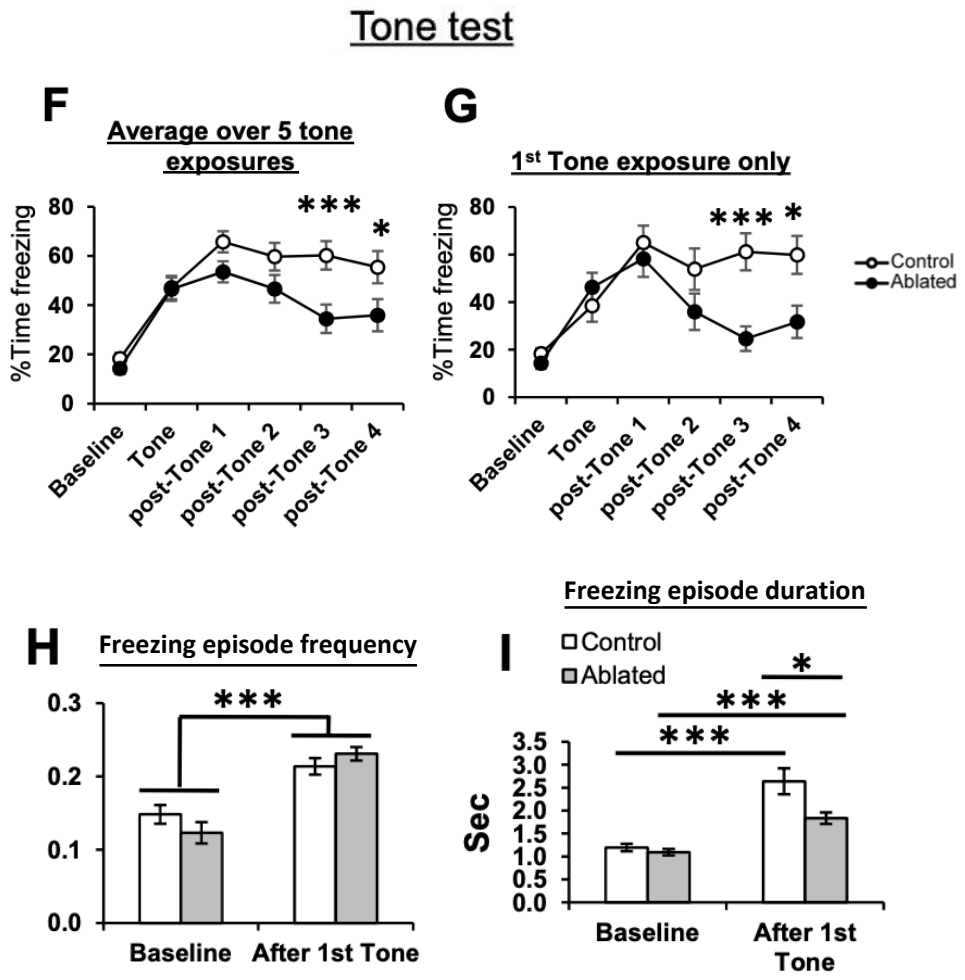
**B,** Timings of a tone (conditional stimulus) and an electrical shock (unconditioned stimulus) during training.

**C,** Percentage time in freezing during the baseline period and after the onset of 1st tone. No significant different was detected between the groups.

**D,** The definition of the tone and post-tone period in the tone test.

**E,** Percentage time in freezing for every 10 sec during the tone test. Gray areas indicate the timing of tone deliveries.

Figure 4 (Continued from p.44)




**F, G,** Percentage time in freezing during baseline, tone, post-Tone 1, 2, 3, and 4 periods, averaged over 5 tones (F) or for 1st tone only (G).
**H, I,** Frequency (H) and duration (I) of freezing episodes during the baseline period and after the onset of 1st tone in the tone test. *: $p < 0.05$, ***: $p < 0.005$.

## Figure S8

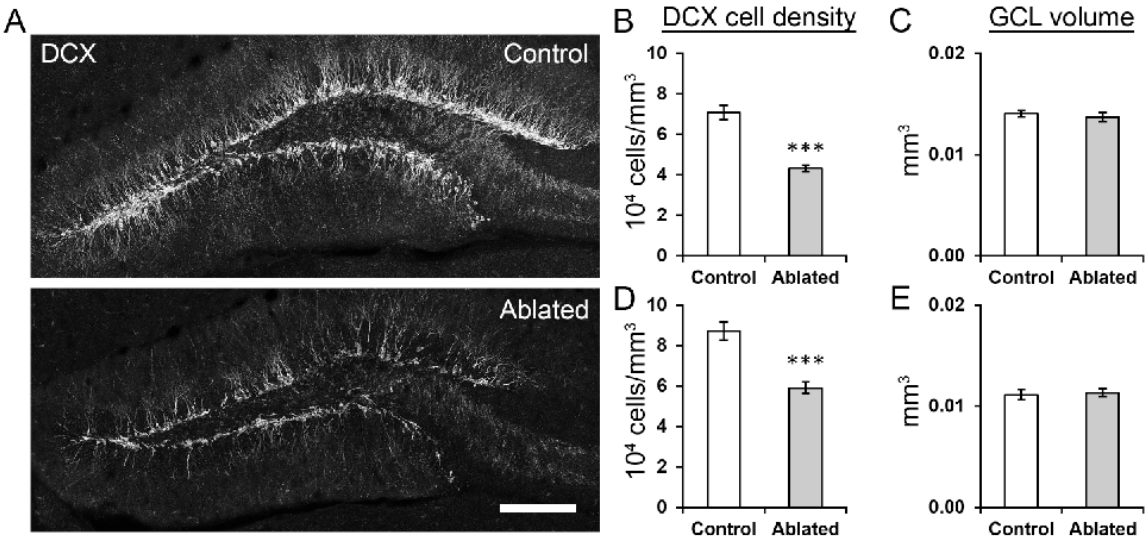



**Fig. S8. DT-induced ablation of DCX+ DGCs in the mice for Figs. 4 and 5.**

**A**, Representative images of DCX+ cells in the dentate gyrus of mice in the ablated and control groups. Scale bar: 200 μm.

**B**, The densities of DCX+ cells in the mice for Fig. 4. The density was significantly lower in the ablated group than in the control ($p = 3.0 \times 10^{-6}$, control: n = 13 mice, ablated: n = 12, independent sample t-test, two-tailed).

**C**, The volume of the granule cell layer in the mice for Fig. 4. The volume was not significantly different between the two groups ($p = 0.556$, control: n = 13 mice, ablated: n = 12, independent sample t-test, two-tailed).

**D**, The densities of DCX+ cells in the mice for Fig. 5. The density was significantly lower in the ablated group than in the control ($p = 7.0 \times 10^{-6}$, control: n = 20 mice, ablated: n = 18, independent sample t-test, two-tailed).

**E**, The volume of the granule cell layer in the mice for Fig. 5. The volume was not significantly different between the two groups ($p = 0.768$, control: n = 20 mice, ablated: n = 18, independent sample t-test, two-tailed). ***: $p < 0.005$.

## Figure S9

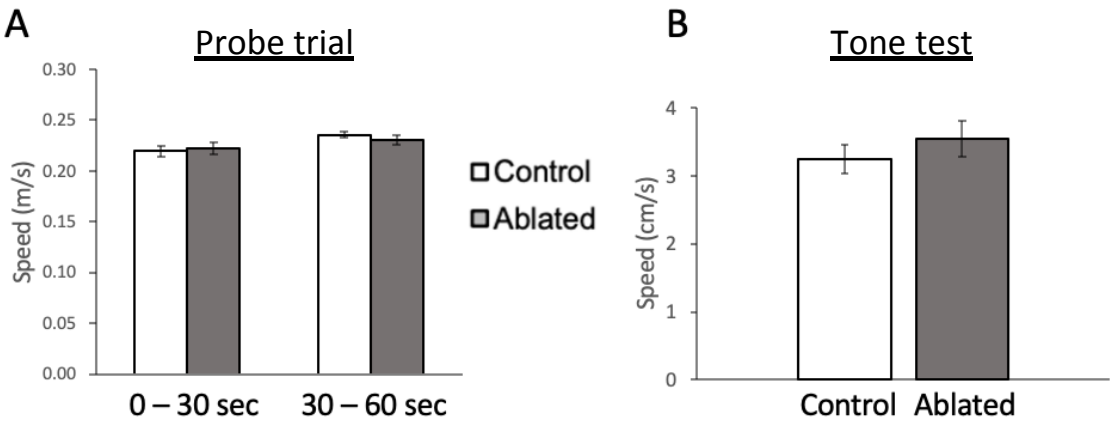



**Fig. S9. General activity during retrieval tests.**

**A**, The swimming speed the first half (0 – 30 sec) and the late half (30 -60 sec) of probe trial. The swimming speed is similar between control and ablated groups (0 -30 sec: $p = 0.73$, 30 – 60 sec: $p = 0.42$, independent samples t-test, two tailed)

**B**, The exploration speed before tone generation in tone test. The exploration speed is similar between control and ablated groups ($p = 0.39$, independent samples t-test, two tailed).

### *3.4 Impaired persistency of learned context-induced freezing behavior after ablation of immature DGCs*

Next, we examined whether immature neuron ablation affects contextual fear conditioning, another form of hippocampus-dependent fear conditioning paradigm (Fig. 5A). 7 days after viral vector injection, the mice underwent training for contextual fear conditioning. The mice were moved to context A, and an electrical shock (2 sec) was given after 180 sec (training). Next day, a shorter shock (0.2sec) was given as a reminder in the same context ("reminder" training), and then DT was systemically injected (ablated group, n = 18 mice). After 7 days, context test was conducted. The control group (n = 20 mice) underwent the same protocol except that they were injected with PBS instead of viral vector or DT. We confirmed a reduction of DCX+ cells in the ablated group, while the volume of the granule cell layer was intact (SI Appendix, Fig. S8D, E). In training and reminder (which were conducted before the induction of immature neuron ablation), freezing level is similar between the two groups before or after foot shocks (Fig. 5B, C; $p > 0.46$ for all, independent t-tests, two-tailed), suggesting that both groups conditioned similarly.

In the context test, overall freezing level was significantly lower in the ablated group than control (Fig. 5D, E, $p = 0.046$, independent t-test, two-tailed), indicating that ablation of young DGCs impaired fear memory retention or retrieval. Motivated from the previous two experiments, as the indexes of memory retrieval performance at early time points in the test, we examined 1) latency for cumulative freezing duration to reach 20 sec and 2) percentage of time in freezing before reaching cumulative freezing duration of 20 sec, and as the index of performance at late time points, we examined 3) the same percentage after reaching cumulative freezing of 20 sec. Cumulative duration of 20 sec is an arbitrary value which corresponds to ~20% of mean total freezing time. There was no significant group difference in the first two (Fig. 5F, G; $p = 0.62$ and 0.32, independent t tests, two-tailed), suggesting that newborn ablation did not affect initial retrieval. In contrast, there was a significant reduction in the third index (Fig. 5H, $p = 0.033$, independent t-test, two-tailed), suggesting that the persistency of learned fear response to the context was impaired by post-training ablation of young DGCs. Consistent with tone fear conditioning, the duration, but not the number, of freezing episodes was significantly

shorter in the ablated group than control (Fig. 5I, duration: p = 0.028; number: p = 0.29, independent- sample t-test, two-tailed).

Figure 5 (Continuing on p.50)

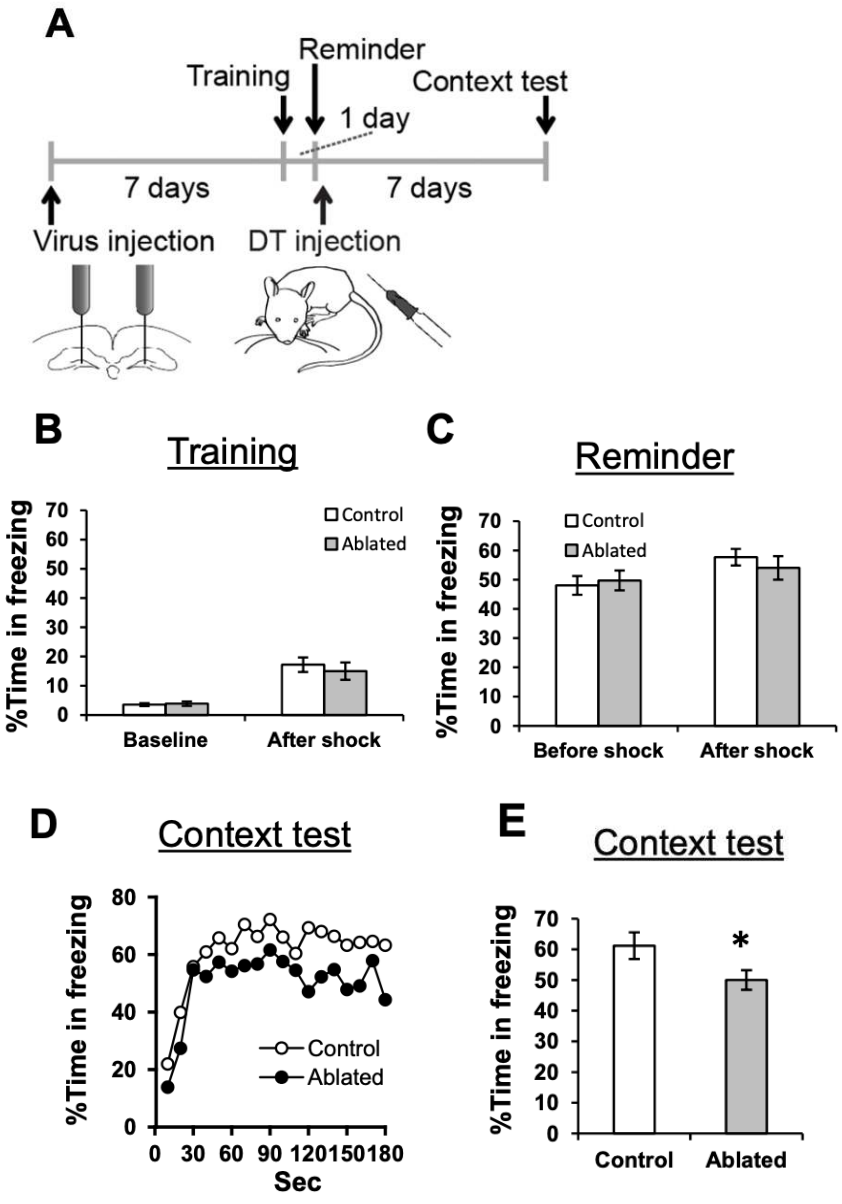
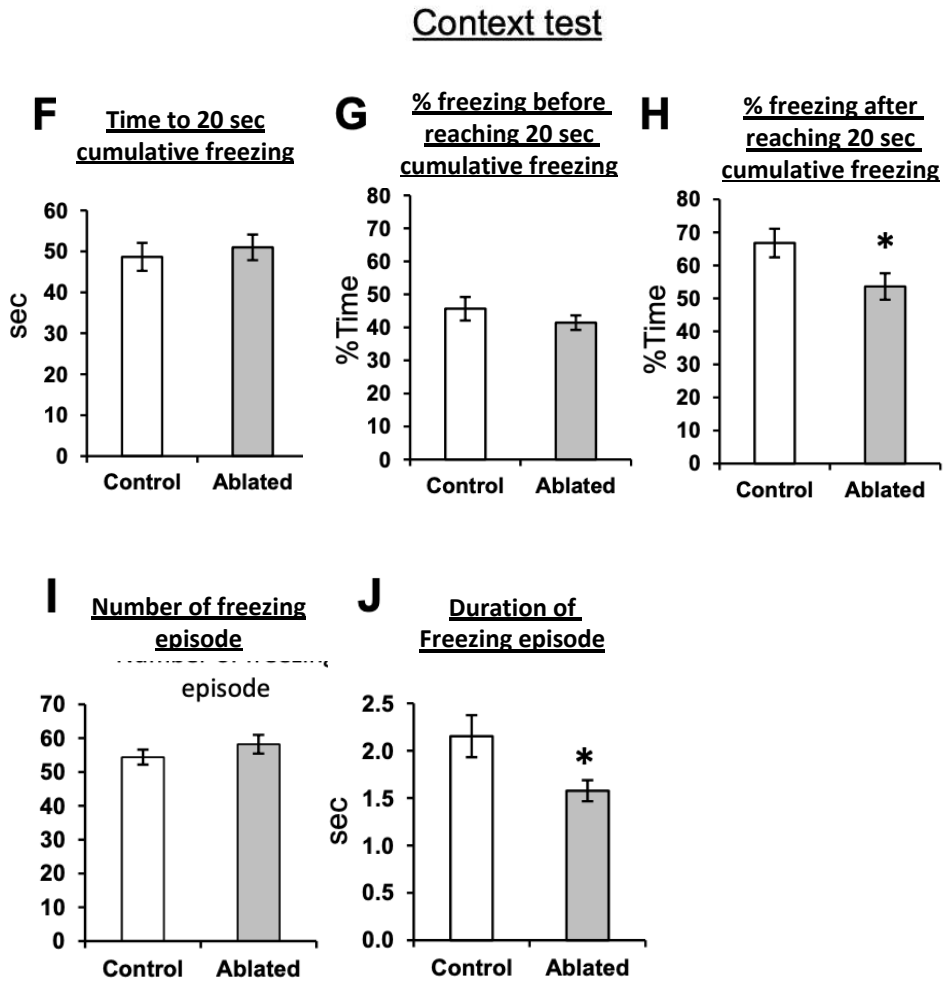



**Figure 5. Post-training ablation of DCX+ immature DGCs impaired the persistence of context-induced freezing in a contextual fear conditioning task.**
**A,** Experimental design. **B, C,** Percentage time in freezing during the baseline period and after the shock in training (B) and remainder training (C). No significant different was detected between the groups. **D,** Percentage time in freezing for every 10 sec during the context test. **E,** Percentage time in freezing in the context test.

Figure 5 (Continued from p.49)




**F,** Latency to reach 20-sec cumulative freezing in the context test. **G, H,** Percentage time in freezing before (G) or after (H) reaching cumulative freezing of 20 sec. **I,** The number and duration of freezing episodes. *: $p < 0.05$.

# 4. Discussion

### *4.1 Methodological consideration for investigating post-acquisition role of immature DGCs*

In previous studies, pre-acquisition ablation of immature DGCs caused deficits in memory retention tests (Deng *et al.*, 2009; Seo *et al.*, 2015). Since they manipulated them before acquisition, there is a possibility that the ablation firstly disturbed memory process during acquisition despite seemingly intact acquisition of learned behavior, so that the effect of ablation on retention was secondary. Under some conditions, such impairment in acquisition has been clearly detected after pre-acquisition ablation of adult-born DGCs (Dupret *et al.*, 2008; Zhang *et al.*, 2008; Garthe, Behr and Kempermann, 2009; Vukovic *et al.*, 2013). Thus, post-acquisition role of immature DGCs remained unclear. To understand the post-acquisition role, manipulation after acquisition was required.

To manipulate immature DGCs, we established a novel ablation method targeting to DCX+ immature DGCs. In this method, we injected lentivirus which induces DTR expression under the control of DCX promoter. 1 week after the injection, we injected DT to ablate DGCs expressing DTR. We confirmed that the reduction of DCX+ immature DGCs 1 week after DT injection (Fig. S3C, S7B, S8B and S8D). We found that up to around 50% of cells expressing GFP did not express DCX 1 week after lentivirus injection (Fig. S1E), raising the concern that our method ablated more mature DGCs in addition to immature DGCs. However, our estimation showed that more than 90% of ablated DGCs are younger than 3 weeks old at the timing of DT injection (Fig. S6), suggesting that this method mainly targets the early maturation stage of newborn DGCs. As observed before (Arruda-Carvalho *et al.*, 2011), an increase in the number of Iba1+ cells was detected (Fig. S5). Despite the increase in microglia, this method did not affect the activity and the morphology of DGCs as we found the intact number of DGCs expressing activity marker (c-fos) and the intact volume of the granule cell layer, cell density, dendritic length and spine density of granule cells (Fig. S2, S3D and E). In addition, this method did not affect neurogenesis in olfactory bulb and the subventricular zone (Fig. S4), giving an advantage over existing transgenic mouse lines which cannot

manipulate neurogenesis specifically in the dentate gyrus (Imayoshi *et al.*, 2008; Deng *et al.*, 2009; Vukovic *et al.*, 2013; Seo *et al.*, 2015).

We observed some GFP+DCX- cells which appeared to include non-neuronal and granule cells based on their morphology. Without an active degradation mechanism, GFP protein is highly stable even after the transcription activity of the promoter is shut off (Andersen *et al.*, 1998). Therefore, GFP protein is expected to be maintained in new DGCs that expressed DCX at the time of virus injection but thereafter lost the expression of DCX (and presumably DTR) during the survival time. Thus, some of GFP+DCX- cells were likely to be new DGCs that had just lost DCX expression. In consistent with this possibility, the proportion expressing DCX was higher in high GFP-expressing cells (>50%) than that of low-expressing cells (<20%) (Fig. S1C-E). Nonetheless, we cannot completely exclude the possibility that some of GFP+ cells were mature DGCs. Our estimation revealed that the proportion of GFP+/DCX- cells among the total granule cell population in the dentate gyrus would be small (0.6%, section 2.8, p.21). This is consistent with our observation of no ablation of mature granule cells (9 weeks old), shown in Fig. 2.

### *4.2 Post-acquisition role of immature DGCs in memory*

Using this method, we ablated immature DGCs after training of memory tasks was completed and then examined its effect on retrieval. We found impairment in performance reflecting memory retrieval, measured as time spent near platform and the number of entries to platform zone in water maze and %time in freezing behavior in tone and context fear conditioning (Fig. 3E, 4F and 5E). The reduction in the number of entries to platform zone could be due to the reduction in the general activity in probe trial. If the general activity is lower, it then reduces the frequency of crossing a certain area of the pool, decreasing the number of entries to platform zone. If the general activity is higher in fear conditioning test, it may reduce the frequency of the detection of freezing behavior. However, this concern is excluded because control and ablated groups have similar values in the swimming speed during probe trial (Fig. 3E and S9A). In addition, both groups have similar values in exploration speed before tone generation (Fig. S9B),

suggesting that general activity in fear conditioning test was also not affected with the ablation. Previous studies failed to find impairment in memory retrieval with post-acquisition manipulation of immature DGCs (Gu *et al.*, 2012; Vukovic *et al.*, 2013). Thus, our study, for the first time, demonstrates that immature DGCs have an important role in memory after acquisition process.

Discrepancy between a previous finding and ours suggests at which timing immature DGCs are involved in memory. The previous study also investigated post-acquisition role of immature DGCs in memory retention tests (Gu *et al.*, 2012). Nevertheless, they did not detect deficits in those tests after post-acquisition manipulation of 2-3-week-old immature DGCs. The discrepancy with Gu et al. may be due to the difference in the timing of manipulation. They did optogenetic inhibition to immature DGCs only during retention tests of Morris water maze (MWM) or fear conditioning, while new DGCs were lacked during all the time after training in our experiments, including period of retention and retrieval. Thus, the discrepancy in the results may suggest that immature DGCs are involved in some memory process after acquisition and before retrieval, but not during retrieval.

Our study with Gu et al. further suggests distinct roles of adult-born DGCs depending on their maturation stages. Gu et al. found that optogenetic inhibition of more matured DGCs (4-5 weeks old), but not immature DGCs (2-3 weeks old), during retention tests lowered performance reflecting memory retrieval in retention tests, indicating that 4-5-week-old DGCs have a role in memory during retrieval. Thus, our study along with their study suggests that retention/retrieval are differently controlled by DGCs depending on their maturational stages; immature DGCs are involved in memory processes before retrieval, and older DGCs are involved in memory processes during retrieval although their involvement before retrieval has not been investigated.

### *4.3 Role of immature DGCs in persistency of learned behavior*

Then, we looked further into the performance in retrieval. We examined the performance related to initial retrieval (how fast/well memory is retrieved) and

performance after initial retrieval of those retention tests. We examined the latency to the platform zone in probe trial which is an indication of how fast/well memory is retrieved. The increase in freezing behavior immediately after tone exposure in tone test, and %time in freezing behavior until reaching total 20 sec freezing behavior in context test were examined as indications of how well/fast initial retrieval occurred. Control and ablated groups showed similar performance in the latency to reach the platform in probe trial (Fig. 3E), indicating that initial retrieval of spatial memory was intact. Control and ablated groups showed similar extent of increase in freezing behavior immediately after tone exposure in tone test (Fig. 4F and 4G), latency to reach 20 sec cumulative freezing behavior and %time in freezing behavior until reaching total 20 sec freezing behavior in context test (Fig. 5G), suggesting intact initial retrieval of fear memory too.

In contrast, the performances were lowered in the later phase of trial with ablation of immature DGCs as we found decrease in %time spent near platform and the number of entries to the platform zone after the first entry to the platform zone (Fig. 3G). In addition, we found the decrease in the freezing behavior during the later part of post-tone period in tone test (Fig. 4F and 4G), and in the freezing behavior after reaching total 20 sec-freezing behavior in context test (Fig. 5H). These observations indicate that, after recalling spatial memory or fear memory normally, the expression of learned behavior was not maintained in ablated group. In other words, the persistency of learned behavior was impaired in ablated group. Thus, these further analyses suggest that immature DGCs are involved in persistency of leaned behavior. In addition, we found reduction in the duration of each episode (each entry to platform zone or each freezing episode) in ablated group (Fig. 3H, 4I and 5I), suggesting that immature DGCs support the persistency in a shorter time scale too.

The persistency of learned behavior was not analyzed in previous studies which did not find deficits in retention tests with post-acquisition ablation/inhibition of immature DGCs (Gu *et al.*, 2012; Vukovic *et al.*, 2013). If they focused on it, they may have detected an impairment in persistency of learned behavior. Their data showed non-significant reduction in performance in retention tests (Gu *et al.*, 2012; Vukovic *et al.*,

2013), possibly implying that there was impairment in the persistency in their retention tests.

The difference in the timing of manipulation raised above (in section 4.1) cannot explain why Vukovic *et al*. did not find impairment in retention tests because both their and our studies did manipulations at the similar timing, after the training before retention tests. Instead, the discrepancy could be explained by absence/presence of reinforcement during retention tests. Vukovic *et al*. used an active place avoidance test. In this task, mice remember a shock zone relative to room configuration in a rotated arena. In their memory retention test, as well as training, a foot-shock was given to mice. This foot-shock works as a punisher for mice to keep learned behavior, escaping from the shock zone. In contrast, our retention tests did not have a punisher/reinforcer such as a foot-shock and a platform which maintained the expression of learned behavior such as freezing behavior and searching/staying at platform zone. Due to the lack of a punisher/reinforcer, the learned behaviors in our retention tests rely more heavily on mouse's own ability of persisting learned behavior which immature DGCs may support, compared with the learned behaviors in the retention test in Vukovic et al.

Here I discuss possible brain functions which immature DGCs are involved in to support the persistency of learned behavior.

(1) Memory strength

We could think in terms of memory strength. One may think that immature DGCs support memory strength, so that their ablation induced the impairment in memory retention tests. However, our detailed analysis revealed intact initial retrieval and impaired persistency of learned behavior. Thus, there may be at least two distinct components composing memory strength which contribute to initial retrieval or the persistency of learned behavior separately, and ablation of immature DGCs impaired a component for the persistency of learned behavior while sparing another component for initial retrieval. Here I relate these two types of memory strength to "retrieval strength" and "storage strength" defined by Bjork (Bjork and Bjork, 1992). Retrieval strength refers to how easily the learned information is retrieved, while storage strength represents how

well/detailed learned information is stored. For example, previous studies showed that amnesia induced by protein-synthesis inhibitor or in mouse models of Alzheimer's disease is primarily due to compromised memory retrieval but not storage (Roy *et al.*, 2016; Roy, Muralidhar, *et al.*, 2017), implying that only retrieval strength was lowered. In contrast, in our study, possibly immature DGCs are not involved in retrieval strength as their ablation did not affect initial retrieval, while immature DGCs may increase storage strength possibly supporting persistency of learned behavior.

(2) Certainty, clarity, and detailedness of memory

When subjects are certain about a memory, they would continue showing behavior reflecting the memory. A study examined learned behavior of human subjects as a function of certainty about memory. They showed that subjects searched for an object persistently at the location where they previously learned that the object was located. When they mistakenly searched a wrong location, they searched less persistently at the wrong location. They claimed that the persistency of learned behavior was taken when subjects were certain about the location of the object (DeLoache and Brown, 1984). The certainty could be decided with clarity of memory; clear memory likely increases the certainty while non-clear memory likely decreases certainty. One of ways describing clarity of memory is whether memory contains details of information. Possibly, immature DGCs are required for retaining a fine memory with details of information, and their ablation may have made a less detailed memory which is considered as a weak memory, causing the memory more fragile and vulnerable to extinction. Previous studies using mice suggested that, with hippocampal lesions, memory lost context specificity (context information), which was observed as impaired context discrimination (Wang *et al.*, 2009). Thus, the hippocampus may be required for retaining fine memories with details of information, and immature DGCs may be involved in the function of the hippocampus. For examples, in probe trial, they remember the patterns of visual cues in more detail or simply remember the position more precisely. In fear conditioning test, they remember the detail in how painful/fearful the shock was during training. Recalling these kinds of more detailed information may be delayed from initial memory recall with less detailed information. Along with this speculation, in human recognition memory, recollection which contains

detail of information succeeds familiarity which lacks detail of information (Nicholas Dias, Yung Peng, 2019). Thus, immature DGCs may support to hold these kinds of detailed information, which makes memory clearer, and then increases certainty about memory. This may be why they are required for expression of learned behavior in later phases of memory tests.

(3) Cognitive rigidity

The other process which could promote the persistency is cognitive rigidity. Cognitive rigidity is often defined as “the tendency to develop and perseverate in particular cognitive or behavioral patterns, and such patterns being continuously employed in situations where the pattern is no longer effective” (Morris and Mansell, 2018). Along with this line, the persistency of learned behavior in control group can be interpreted to reflect high rigidity, while the impaired persistency in ablation group reflects lowered rigidity. High rigidity is thought to be worth in some situations where continuation of memory use brings merit to subjects. For example, in the situation where mice fail to reach platform even though they are close to it, cognitive rigidity would help mice continue to search around the platform zone and finally find it. In our daily life, examples of behaviors supported by cognitive rigidity are to continuously search something according to memory (e.g. you may keep looking for tomatoes in the vegetable section in a supermarket as you have learned that tomatoes are in the vegetable sections before even though you do not find tomatoes immediately in that section) or continuously avoid something according to memory (e.g. you may keep avoiding a scary teacher who has scold you badly even though he/she does not scold any more). Possibly, immature DGCs are involved in cognitive rigidity.

Another interpretation of ablation-induced behavioral effect is improved learning in general with less immature DGCs. It may be interpreted that ablated group learned new things faster during memory tests (e.g. learn no platform at the original place), so that less learned behavior after initial retrieval was observed. However, this interpretation is less likely because ablated group did not show better learning during re-training (Fig. S7E).

### *4.4 Consideration of persistency of learned behavior with proposed roles of the dentate gyrus*

Would the persistency of learned behavior be linked to proposed roles of the dentate gyrus or the hippocampus in memory (see section 1.5 too)? One of proposed role of the dentate gyrus is pattern separation. Pattern separation may segregate different memories well (e.g. pattern separation may segregate following memories that may overlap neural representation during water maze task: memory that mice reach a platform position during training using visual cues and memory that mice reach a different platform position during pre-training using the same visual cues), which may facilitate certainty, clarity, and detailedness of memory, thereby facilitating persistency of learned behavior. If mice fail to differentiate similar contexts after some manipulation of the brain, it will be interpreted that the manipulation disturbs pattern separation (e.g. McHugh *et al.*, 2007). Our tone test before tone generation may be suitable for investigating pattern separation because the test used a different context which might be similar with training context (the same size as training chamber, but different from training context in scent, wall pattern and floor material). Ablated group showed similar freezing level with control group in tone test before tone generation (baseline in Fig. 4E-G), possibly suggesting that pattern separation function of the dentate gyrus was spared after ablation. Similarly, the similar freezing level at the different contexts may also suggest that pattern completion was not impaired.

Novelty detection as a role of the dentate gyrus might also affect the behavioral phenomena in retrieval tests. No platform anymore in the pool in probe trial or no foot-shock in tone and context test may be detected as novelty. Possibly ablation improved novelty detection, which may facilitate new learning observed as less learned behavior in retrieval tests. If it facilitates new learning in general, ablated mice may have showed better performance in re-training, but did not (Fig. S7E).

Working memory as a role of the dentate gyrus seems to well support the persistency of learned behavior after initial retrieval. With working memory, subjects retain the information after recalling. For example, in our retrieval tests, the information is the

memory of platform position in probe trials or the association between foot-shock and tone or context in tone and context test. Retaining the information by the dentate gyrus may promote the expression of learned behavior. However, it is not clear that our ablation impaired working memory.

### *4.5 The neural mechanism underlying persistency of learned behavior*

How is the persistency of learned behavior achieved by the brain? In the brain, information is thought to be represented by the activation of a subset of population of neurons. The information is thought to be stored in a subset of population of neurons during training, and then thought to be recalled by the activation of the same subset of population of neurons during memory recall. This subset of population of neurons is considered to be engram cells, which are defined as "populations of cells that constitute critical cellular components of a given engram" (Josselyn and Tonegawa, 2020). The term, "engram" is originally proposed by Semon over 100 years ago. He defined engram as "the enduring through primarily latent modifications in the irritable substances produced by a stimulus" (Semon, 1921). Josselyn and Tonegawa described relationship between engram and engram cells in a way that "an experience activates a population of neurons that undergo persistent chemical and/or physical changes. Subsequent reactivation of the engram by cues available at the time of the experience induces memory retrieval" (Josselyn and Tonegawa, 2020).

Since the activation of a neuronal subset may induce memory recall and make the subjects show learned behavior, one possible brain mechanism behind persistency of learned behavior is whether stably the same subset of population of neurons is activated during memory recall. Previously, Liu et al. showed that the activation of the engram cells labeled during fear conditioning induced freezing behavior (= learned behavior) in a test with a context different from the one used for the conditioning (Liu *et al.*, 2012). They divided the test into some epochs in which engram was activated and not activated, and then found that freezing behavior was induced in only epochs where the engram was activated, but not persisted into epochs where the engram cells were not activated. This

suggests that the learned behavior is induced when the subset of population of neurons encoding memory is activated and persists only while the neuronal subset is activated. Thus, the persistency of learned behavior may be supported by the continuous activation of the same subset of neuronal population representing memory. As our studies suggest that immature DGCs support the persistency of learned behavior, immature DGCs may work to stably activate the same subset of neuronal population encoding memory to support the persistency of learned behavior. The continuous activation of neuronal subset which immature DGCs may support may increase memory strength, certainty or cognitive rigidity. The persistency of learned behavior may be enhanced through their increase.

### *4.6 The neural mechanism underlying persistency of learned behavior which immature DGCs support*

Immature DGCs up to 3 weeks old are still under the process of extending neurites, which are going to be integrated into neural circuits during this age and later age; immature DGCs have lower numbers of dendritic spines and axons reaching CA3 and smaller size of dendritic spines and synaptic boutons than mature DGCs (Hastings and Gould, 1999; Espósito *et al.*, 2005; Zhao *et al.*, 2006; Toni *et al.*, 2008), having less functional inputs and outputs (Espósito *et al.*, 2005; Gu *et al.*, 2012; Groisman, Yang and Schinder, 2020). The decision of cell death/survival, which occurs during the 1$^{st}$ week to the 3$^{rd}$ week of age of DGCs, is known to be dependent on learning opportunities including exposure to enrich environments and memory tasks (Kempermann, Kuhn and Gage, 1997; Gould *et al.*, 1999; van Praag, Kempermann and Fred H. Gage, 1999; Tashiro, Makino and Gage, 2007; Aasebø *et al.*, 2018). The learning opportunities promote maturation of dendrites, dendritic spines and functional excitatory and inhibitory inputs (Tronel *et al.*, 2010; Lemaire *et al.*, 2012; Alvarez *et al.*, 2016; Gonçalves *et al.*, 2016). Thus, the integration of immature DGCs into neural circuits may be affected by learning opportunities. In other words, neural inputs from learning may affect pathways built by immature DGCs. The newly built pathways shaped with learning opportunities

may represent memory in addition to existing circuits during memory recall. The increase in pathways representing memory may make memory representation more stable, supporting the persistency of learned behavior.

The learning-dependent circuit formation may occur in a period after acquisition which includes retention period if the period is long enough for new circuits to be formed. We found that ablation of immature DGCs after acquisition process impaired persistency of learned behavior in retention test 1 week after acquisition, while Gu et al. did not find impairment in retention test with optogenetic inhibition of 2-3-week-old immature DGCs during retention test (Gu *et al.*, 2012). The discrepancy between Gu et al. and our finding indicates that immature DGCs are involved in some memory process in a period after acquisition and before retrieval, which corresponds to a retention period. The retention period lasts for a week in our tests, during which presumably new circuits responsible for memory representation were formed. Consistent with the idea, a recent study showed that the activities of adult-born DGCs during retention period are important for retrieval later. They found that the activities of DGCs up to 4 week-old during REM sleep in retention period after contextual fear conditioning contribute to retrieval of fear memory in context test later (Kumar *et al.*, 2020).

This brings up an idea of relevance of immature DGCs to systems consolidation. System consolidation is the time-varying process where memory relies on the hippocampus less and on the cortex more over time to develop a long-lasting memory (Roesler *et al.*, 2016). The idea is supported by a systematic experimental study; it was shown that the reactivation of engram cells at the dentate gyrus occurs in retrieval test which was performed 1 day, but not around 2 weeks, after training with contextual fear conditioning (Kitamura *et al.*, 2017). In contrast, the reactivation of engram cells at the medial prefrontal cortex (mPFC) occurs in retrieval test which was performed around 2 weeks, but not 1 day, after the training (Kitamura *et al.*, 2017). This suggests that memory retrieval initially relies on the hippocampus, but this reliance is taken over by the mPFC over time. Our memory retrieval tests (1 week after the trainings) may also rely on the mPFC to some extent. Thus, immature DGCs may help consolidation processes to shift the place of memory storage from the hippocampus to the mPFC. Immature DGCs may

be involved in systems consolidation which changes the way of memory representation over time, and thereby support the persistency of learned behavior. Thus, when immature DGCs are ablated, the process of consolidation may be incomplete, resulting in fragile memory lacking some information stored in the mPFC. This could disturb keeping the memory by the mPFC, resulting in impaired persistency of learned behavior as we observed.

### *4.7 Future directions*

To fully understand the role of adult neurogenesis in memory, following questions need to be addressed.

(1) The first question is how different ages of new neurons contribute to different memory-related processes. Previous behavioral, electrophysiological and anatomical studies suggest that DGCs have different functions depending on their ages (Hastings and Gould, 1999; Schmidt-Hieber, Jones and Bischofberger, 2004; Espósito *et al.*, 2005; Zhao *et al.*, 2006; Ge *et al.*, 2007; Pathak *et al.*, 2007; Toni *et al.*, 2008; Gu *et al.*, 2012; Heigele *et al.*, 2016; Groisman, Yang and Schinder, 2020). Previously, it has been suggested that immature DGCs are involved in memory acquisition (Vukovic *et al.*, 2013). Pre-acquisition manipulation studies suggest that adult born DGCs at both early and late maturational stage are involved in memory (Deng *et al.*, 2009; Denny *et al.*, 2012; Seo *et al.*, 2015). Post-acquisition manipulation study suggests that DGCs at a late maturational stage have a post-acquisition role in memory (Gu *et al.*, 2012). Our study newly suggests that immature DGCs are involved in the persistency of learned behavior after acquisition process. To fully understand the role of adult neurogenesis in memory, we need investigate roles of adult-born DGCs at different maturational stages in different memory processes such as the persistency of learned behavior, memory extinction and forgetting.

The persistency has never been studied as a role of DGCs at the late maturation stage or matured DGCs previously. This could be examined by manipulating those older ages of DGCs with ablation, optogenetics or chemogenetics, and examining the effect of

manipulation during retention tests. Possibly, older cohorts of DGCs are involved in initial memory retrieval, but not in persistency of learned behavior, or vice versa.

Previously, it has been suggested that adult-born DGCs are involved in memory extinction, forgetting and cognitive flexibility (Deng *et al.*, 2009; Burghardt *et al.*, 2012; Akers *et al.*, 2014; Epp *et al.*, 2016; Gao *et al.*, 2018). Those functions seem to be at an opposite pole in relation to the possible brain functions underlying persistency of learned behavior discussed in section 4.3. The possible brain functions may increase the stability of memory, while extinction, forgetting and cognitive flexibility may decrease the stability of memory. To understand the opposite roles of adult-born DGCs it may be necessary to consider that they may be involved in different memory processes depending on their age. Burghardt et al. suggested up to around 12-week-old DGCs are involved in cognitive flexibility (Burghardt *et al.*, 2012). Deng et al., suggested that 2-4-week-old, but not 5-7-week-old, DGCs are involved in memory extinction (Deng *et al.*, 2009). Their findings are based on pre-acquisition ablation of adult-born DGCs, so that there is room for investigation of post-acquisition role in extinction and cognitive flexibility. In addition, since Burghardt et al., manipulated DGCs at a wide range of age, it is required to investigate which ages of DGCs in a narrower range are involved in cognitive flexibility. Akers et al., Epp et al. and Gao et al. showed that DGCs born after memory acquisition are responsible for forgetting (Akers *et al.*, 2014; Epp *et al.*, 2016). Since increased neurogenesis during the interval of 4 weeks between acquisition and retrieval resulted in forgetting, DGCs responsible for forgetting are younger than 4 weeks old. However, from these studies, the exact age of DGCs involving in forgetting remains unclear. To elucidate it, it is required to manipulate different ages of DGCs and then examine the effect of manipulation on forgetting.

(2) The second question is whether experience-dependent plasticity through adult neurogenesis underlies memory. So far it has been shown that learning and memory modify adult neurogenesis. Learning opportunities increase the survival of newborn DGCs (Kempermann, Kuhn and Gage, 1997; Gould *et al.*, 1999; van Praag, Kempermann and Fred H. Gage, 1999; Tashiro, Makino and Gage, 2007; Aasebø *et al.*, 2018). In addition, spatial learning or exposure to enrich environment increases dendritic

length and complexity and the number and size of dendritic spines of newborn DGCs (Tronel *et al.*, 2010; Lemaire *et al.*, 2012; Alvarez *et al.*, 2016; Gonçalves *et al.*, 2016), suggesting that learning and memory promote circuit formation of newborn DGCs. Furthermore, enrich environment increases the number of excitatory inputs, presumably from the entorhinal cortex, and inhibitory inputs from interneurons in CA1, CA3 and the dentate gyrus to newborn DGCs (Bergami *et al.*, 2015; Alvarez *et al.*, 2016). However, whether such experience-dependent plasticity underlies memory remains unclear. To understand it, I suggest to experimentally manipulate experience-dependent modification of dendrites or spines specifically in vivo during memory-related events, and then examine an effect on behavior.

(3) It remains unclear what role is played by integrated mature DGCs which have been involved in memory previously during their maturation. They may or may not contribute to memory after maturation. For this question, it is required to manipulate mature DGCs which have been involved in memory during their maturation. We need to label immature DGCs which are active during memory acquisition (e.g. activity-dependent ChR2 expression in immature DGCs, which persists even after they mature) for manipulation after they mature (e.g. activation of mature DGCs expressing ChR2 with blue light) to examine whether adult-born DGCs become a part of memory trace. In this way, we could investigate whether or not DGCs which are involved in a memory during their maturation continue to contribute to the same memory after their maturation. In addition, we should investigate in which ages of mature DGCs contribute to memory by manipulating them at different time points.

(4) Another question is what kind of neuronal activities of newborn DGCs contribute to memory. It is required to monitor neuronal activities of newborn DGCs during memory-related events using in vivo electrophysiological recording or optical imaging. Then we can inhibit the activity to examine whether the activity is involved in memory. Since newborn DGCs form neural circuits with local interneurons and mature DGCs (Hastings and Gould, 1999; Schmidt-Hieber, Jones and Bischofberger, 2004; Espósito *et al.*, 2005; Zhao *et al.*, 2006; Ge *et al.*, 2007; Pathak *et al.*, 2007; Toni *et al.*, 2008; Heigele *et al.*, 2016; Luna *et al.*, 2019; Groisman, Yang and Schinder, 2020), it is required to

understand how the circuits between newborn DGCs and these neuronal population in the dentate gyrus contribute to memory. To elucidate it, we need to manipulate the activity of a specific neural circuit during memory-related events, and then examine the effect of manipulation on memory.

(5) In addition, it remains unclear how newborn DGCs contribute to memory processes with other brain regions. For example, the medial prefrontal cortex (mPFC) should be considered to be a brain region that newborn DGCs may interact with since memory consolidation, which may regulate persistency of learned behavior, relies on the mPFC (Wimber *et al.*, 2015; Kitamura *et al.*, 2017; Bekinschtein *et al.*, 2018). Previously, it has been reported that there are direct projections from the hippocampus to the mPFC (Hoover and Vertes, 2007; Padilla-Coreano *et al.*, 2016; Ye *et al.*, 2017). However, it remains unknown how neurogenesis in the dentate gyrus cooperates with other cell types in the hippocampus to engage those memory processes in which mPFC is involved. To understand how neural circuits between newborn DGCs and other brain regions contribute to memory processes, we could record activity from both the hippocampus and the other regions simultaneously during memory-related events, and then examine the effect of manipulation of activity of newborn DGCs on the activities of neurons in the other regions.

# 5. Conclusion

Overall, our studies reshape understanding the role of immature DGCs on learning and memory. With the LV- and DT-mediated ablation method, we ablated immature DGCs after acquisition process of the three hippocampus-dependent memory tasks, water maze and trace and contextual fear conditioning. We found the impairment in those retention test, suggesting the post-acquisition role of immature DGCs in spatial and fear memory. More specifically, our results showed that immature DGCs are not involved in how fast/well initial retrieval occurs, but are involved in the performance of the later phase, suggesting that immature DGCs work for persistency of learned behavior within single retention tests. This finding unravelling the post-acquisition role of immature DGCs in persistency of learned behavior contributes to understanding of the role of neurogenesis in memory.

Table S1. Statistical results for Fig. 3 and SI Appendix, Fig. S7

**Pre-training (three-way, group x trial x day, two-tailed, control, n = 18 mice; ablated, n = 18 mice)**

| | Latency to platform | | | Speed | | |
|---|---|---|---|---|---|---|
| | p | df | F | p | Df | F |
| Day | 0.016 | 3, 102 | 3.61 | $6.5 \times 10^{-7}$ | 2.43, 82.8 | 14.7 |
| Day*Group | 0.858 | 3, 102 | 0.254 | 0.630 | 2.43, 82.8 | 0.524 |
| Trial | $1.4 \times 10^{-11}$ | 1.53, 52.0 | 52.0 | 0.006 | 2, 68 | 5.49 |
| Trial*Group | 0.885 | 1.53, 52.0 | 0.072 | 0.612 | 2, 68 | 0.495 |
| Day*Trial | 0.459 | 4.99, 170 | 0.936 | 0.007 | 6, 204 | 3.02 |
| Day*Trial*Group | 0.713 | 4.99, 170 | 0.583 | 0.226 | 6, 204 | 1.38 |
| Group | 0.870 | 1, 34 | 0.027 | 0.157 | 1, 34 | 2.07 |

**Training (two-way, block x group, control, two-tailed, n = 18 mice; ablated, n = 18 mice)**

| | Latency to platform | | | Speed | | |
|---|---|---|---|---|---|---|
| | p | df | F | p | df | F |
| Block | $2.6 \times 10^{-12}$ | 4.31, 147 | 17.43 | $7.8 \times 10^{-5}$ | 5.29, 180 | 5.44 |
| Block*Group | 0.321 | 4.31, 147 | 1.18 | 0.474 | 5.29, 180 | 0.919 |
| Group | 0.566 | 1, 34 | 0.336 | 0.533 | 1, 34 | 0.398 |

**Re-training (three-way, group x trial x day, two-tailed, control, n = 18 mice; ablated, n = 18 mice)**

| | Latency to platform | | | Speed | | |
|---|---|---|---|---|---|---|
| | p | df | F | p | df | F |
| Day | 0.034 | 2, 68 | 3.57 | 0.065 | 1.68, 57.2 | 3.02 |
| Day*Group | 0.815 | 2, 68 | 0.205 | 0.826 | 1.68, 57.2 | 0.149 |
| Trial | $1.7 \times 10^{-30}$ | 5.85, 199 | 38.7 | 0.0002 | 7, 238 | 4.20 |
| Trial*Group | 0.756 | 5.86, 199 | 0.562 | 0.494 | 7, 238 | 0.917 |
| Day*Trial | 0.478 | 9.89, 336 | 0.960 | 0.127 | 12.3, 418 | 1.48 |
| Day*Trial*Group | 0.871 | 9.89, 336 | 0.524 | 0.627 | 12.3, 418 | 0.826 |
| Group | 0.920 | 1, 34 | 0.010 | 0.265 | 1, 34 | 1.28 |

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