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ABSTRACT

The hippocampus encodes sensory information into memories. The dentate gyrus (DG) region is viewed as the entry point into the hippocampus, receiving sensory and spatial signals from perforant path axons of the entorhinal cortex (EC). Ever since researchers discovered a neurogenic niche in the subgranular zone (SGZ) of the DG in adult animals and humans, many studies have been aimed at discovering how these continually proliferating granule cells (GCs) contribute to the network. Immature GCs have dendrites that project densely into the molecular layer alongside mature GCs and there is morphological evidence that immature excitatory spines preferentially synapse onto existing boutons. Therefore, it is likely that the addition of new GCs alters preexisting connections. Here we give an introduction to the possible functions of the DG and its relevance to human memory, then briefly review what is known about cell death, plasticity and synapse competition in the DG as well as other CNS regions. We then present a body of work that shows evidence for functional synaptic competition between immature and mature GCs. We also show that eliminating the pro-apoptotic Bax gene in mature GCs prevents competition-induced rewiring of the circuit. Finally, we discuss the implications of this work and present further evidence that manipulating neurogenesis affects the DG network.

Keywords: Dentate gyrus, neurogenesis, synaptic competition, Bax

DEDICATION

This culmination of my Ph.D. work is dedicated to my father, Jerry Henry, whose memory inspires my current and future research.

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INTRODUCTION

From an evolutionary standpoint, memory is essential to survival – it is needed to locate food sources, avoid threats and interact with mates and offspring. For humans, memories also hold immense social and emotional value. They form the basis of knowledge, inform daily decisions and ultimately define who we are, making the loss of memory one of the most debilitating symptoms of neurological disease. Thus, understanding the biological processes responsible for memory formation and recall is one of the aspirations of neuroscience. Early temporal lobe resections in patients provided the first evidence that this region of the brain is necessary for the retention of long-term memories[1], and subsequent animal studies have confirmed the involvement of the entorhinal-hippocampal circuit in the conversion of sensory information into episodic memories.

Evolution of the dentate gyrus and evidence for neurogenesis in humans

The hippocampus itself consists of the dentate gyrus (DG), CA1, CA2 and CA3. Collectively, they generate memories based on sensory and spatial information received from the entorhinal cortex (EC) (see Figure1). The DG in particular has been an area of intense study because it continues to generate new granule cells (GCs) throughout

adulthood in mice, rats, primates and humans. Its role in the greater scheme of memory encoding is not clear, for while some information from the EC passes through the DG before innervating CA3, the EC also directly connects to both CA3 and CA1. In the animal kingdom, while some non-mammalian species possess analogous structures with regenerative capacity, the modern DG seemed to evolve along with the neocortex and corpus callosum in mammals[2]. Furthermore, not all mammals undergo DG adult neurogenesis, including some species of bats and cetaceans (dolphins and orcas), but are still able to perform complex spatial navigation tasks. Nevertheless, the existence of the DG and adult neurogenesis is likely to play a crucial role in human memory formation. The size of all hippocampal regions including the DG are larger in humans than in more primitive primates of comparable body weight (the DG by a factor of 2.6, CA1 by 6.6). In rodents, new GCs constitute only ~5% of the overall population, with little or no replacement of existing mature GCs[3], and proliferation is dramatically reduced as animals age. However, by comparing levels of ^{14}C in neurons from postmortem human hippocampi to changing atmospheric levels of ^{14}C from the era of above-ground atomic bomb testing, researchers showed that proliferation and turnover of hippocampal cells occur to a much larger degree in humans than in rodents, with a minimal decline in neurogenesis during aging[4].

The EC-hippocampal circuit

Signals from multiple cortical sensory modalities converge in the EC, which projects to all regions of the hippocampal tri-synaptic circuit. The EC includes the medial entorhinal cortex (MEC), which processes spatial information, and lateral entorhinal cortex (LEC), which processes object and sensory information.

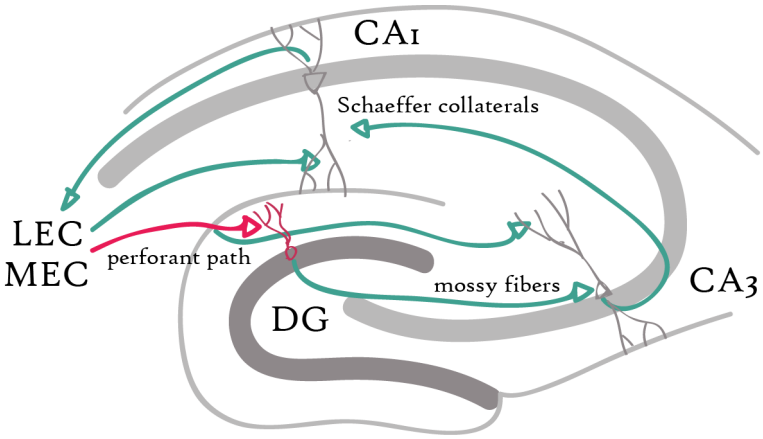

Figure 1. The simplified EC-hippocampal circuit. The EC projects axons to the GC dendrites in the molecular layer of the DG. The GCs project onto CA3 pyramidal cells through mossy fibers, and CA3 pyramidal cells then project onto CA1 through the Schaeffer collaterals. Note that the EC also projects directly to both CA3 and CA1, and CA1 projects back onto the EC, forming several excitation loops. (Illustration based on Petrantonakis 2014)

Large-scale in vivo population recordings in the MEC of rodents have revealed distinct cell types tuned to different navigational parameters, such as head direction, boundaries or a grid-like spatial map of the animal's environment (grid cells), as well as some cells that are not tuned to spatial changes[5]. Firing in the MEC activates perforant path axons

which project to the molecular layer of the DG (Fig. 1). A defining characteristic of the DG is sparse activity, with only 2-5% of GCs firing in response to perforant path stimulation both *in vitro* and *in vivo*[6,7]. This is due to both intrinsic properties of the cells (hyperpolarized resting membrane potential, decreased threshold to fire) and abundant GABAergic tonic, feed-forward, and feed-back inhibition from molecular layer interneurons[8]. Despite this sparse activation, a single GC mossy fiber axon can reliably activate a CA3 pyramidal cell because of large, perisomatic boutons known as detonator synapses. The CA3 neurons then project onto CA1 pyramidal cells through the Schaeffer Collaterals.

Sparse activity serves the function of the DG as a pattern separator and an excitation gate

It has been proposed that, while not necessary for the formation or retrieval of all memories, the DG serves to disambiguate potentially overlapping memories by separating patterns from similar environmental stimuli[9]. Both early and recent computational models predict the dense cell number and low probability of activation would contribute to the DGs function as an effective pattern separator[10,11]. *In vivo* population recordings confirmed that for largely different environments, distinct cell populations were activated in CA3, with no change in DG population firing, suggesting direct signaling from EC to CA3. Small environmental differences resulted in rate changes to different firing fields in DG with no change in CA3 place fields, indicating that it is the distinction of similar stimuli that requires filtering through the DG[5]. Many

behavioral studies have supported the role of the DG in spatial and contextual pattern separation[12]. More recently the paradigm in the neurogenesis field has shifted to the belief that young rather than mature GCs are responsible for pattern separation. Ablating newborn GCs negatively affects pattern separation ability[13,14], and increasing the population of immature GCs by preventing the death of progenitor cells improved performance on a context discrimination task[15]. However, genetically blocking the output of mature GCs was sufficient to improve performance on a similar context discrimination task[14].

Sparse firing in the DG may also help prevent the formation of epileptic activity. The hippocampus is a concentrated region of increased plasticity, making it highly adaptable to changing environments. The trade-off is an increased susceptibility to overexcitation. The excitatory loops among regions in the circuit also present a risk for recurrent epileptiform activity[16]. Because of the low excitability of GCs, the DG counteracts this hazard by acting as a dampening filter, or 'gate', for the barrage of incoming excitatory signals from the EC. In fact, disrupted inhibition to the DG is a hallmark of temporal lobe epilepsy (TLE) in animal models[17].

Development and integration of adult-born GCs

Learning how newly-generated GCs integrate into an adult network is a priority in the neurogenesis field. The various stages of GC development have been well-characterized, although how the each of these cell phases contributes functionally to the

DG is uncertain. A population of subgranular zone (SGZ) neural stem cells gives rise to dividing Type II progenitors, which, upon exiting the cell cycle, differentiate into newborn GC neurons[18]. The developing granule cell layer (GCL) is assembled according to an outside-in arrangement, with embryonically-generated GCs migrating closest to the molecular layer and adult-generated GCs mostly remaining near the hilus (Fig. 2).

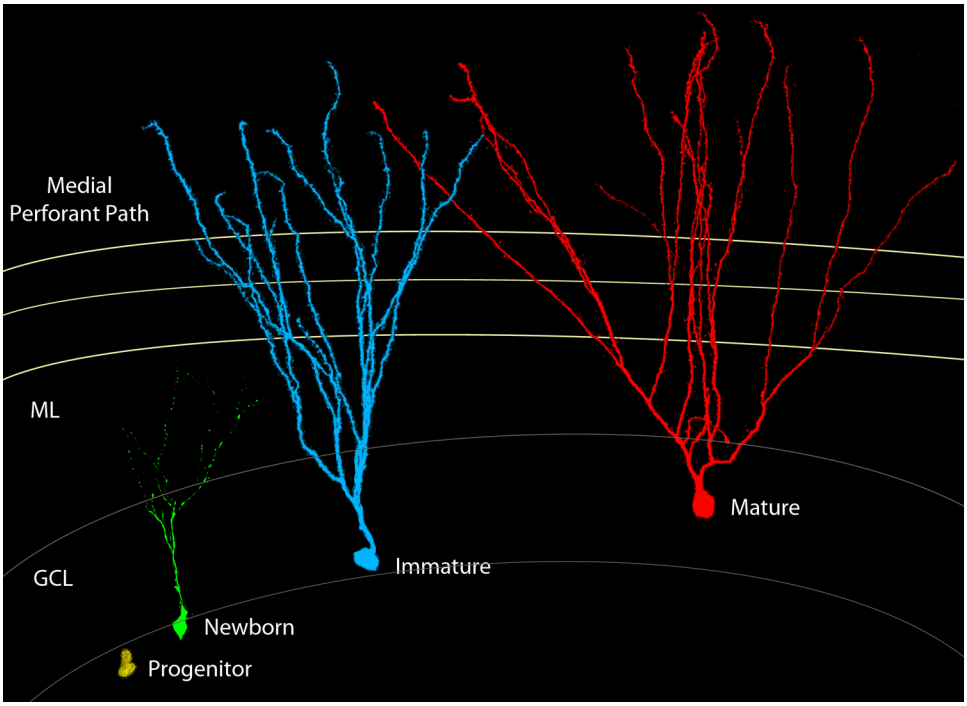

Figure 2. Developmental stages of GCs. GCs slowly pass through several stages of development as they build up the granule cell layer in an outside-in pattern. The progenitor cell was immuno-labeled with Ki67 antibody, newborn was genetically labeled by the POMC-eGFP gene, and immature and mature GCs were biocytin filled and tagged with fluorescent antibody. ML=molecular layer, GCL=granule cell layer. Image not to scale.

Progenitor cells express markers Nestin, ki67 and MCM2, and their proliferation can be enhanced in the mouse DG in many ways, including running[19] or blocking Bax-mediated

cell death[15]. Early postmitotic GCs have short dendritic projections and are labeled by DCX, PSA-nCAM, NeuroD, Prox1 and POMC-eGFP. Despite having glutamate receptors, they receive only GABAergic signaling from direct connections to interneurons, which at the time is depolarizing due to a higher expression of the NKCC1 transporter[8,20,21]. GCs in this developmental stage are thought to be in a "critical period" because of heightened excitatory synaptogenesis, susceptibility to apoptotic signals and recruitment to memory tasks[22,23]. New GCs develop gradually over 7-8 weeks and eventually become indistinguishable from mature GCs in morphology, intrinsic membrane properties, excitatory and inhibitory transmission and recruitment to memory tasks, regardless of whether they were generated embryonically, postnatally or in adulthood[18,24,25]. By the time the cells are 2 weeks post-mitosis, they have developed glutamatergic dendritic spines and receive excitatory inputs from contralateral hilar mossy cells[26] as well as the EC via perforant path axons[27,28]. By 4 weeks post-mitosis, they project mossy fiber axons onto CA3 pyramidal cells[29,30]. GCs that are functionally connected but not yet mature possess unique physiological properties, such as higher input resistance, higher intrinsic excitability and lower threshold for LTP induction[31,32].

How does adding new GCs affect the sparseness of activation?

With the importance of inhibition in the DG, adding young GCs to the network presents a conundrum, since immature GCs are regarded as hyperexcitable. Immature GCs also receive less direct GABAergic inhibition[31] and receive and induce less feedback

inhibition than mature GCs[30]. Adding easily-activated, broadly-tuned units to the system would counteract the proposed functions of the DG, so the new cells must have another way of maintaining low activity or dampening existing activity. In fact, increasing neurogenesis has been shown to decrease overall excitability in the DG[33]. This may be due to the fact that immature GCs receive low excitatory drive from the EC, making them less likely to fire under *in vivo* conditions[32]. On the other hand, the firing properties of the relatively small number of new GCs may not account for widespread changes to the network. Therefore, it is also possible that adding immature GCs drives down the activity of mature GCs.

The Bax gene and programmed cell death in the DG

Normally-occurring programmed cell death has been well established as a necessary process throughout the central nervous system (CNS), especially in the developing brain[34-36]. The reason the process of overproduction and scaling back of neurons occurs in almost every brain region is not completely understood, but some possibilities are that it limits the pool of proliferating neurons, corrects errors (i.e. in migration) and fine tunes networks by allowing only cells with trophic support from their targets to survive[36]. The pro-apoptotic gene, Bax, controls apoptosis in progenitor cells and immature neurons, including in adult-generated GCs of the dentate[37]. Bax is a member of the BCL-2 family of proteins and functions midstream in the intrinsic apoptotic pathway. Activation of Bax permeabalizes the mitochondrial membrane, which

triggers the release of soluble proteins (such as cytochrome c) into the cytosol, leading to the formation of an apoptosome by APAF1 and eventually the activations of caspase-9 and caspase-3 which cleaves DNA, causing cell death[38] (Fig. 3).

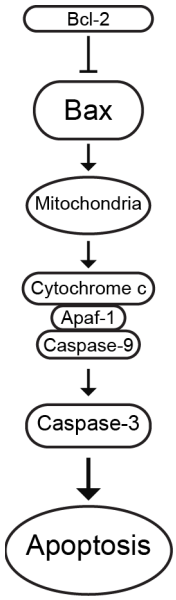

Figure 3. The intrinsic apoptotic pathway. Activation of the pro-apoptotic protein, Bax, sets off a cascade of factors that activates the "executioner" protease, caspase-3, leading to apoptosis of the cell.

Normally, less than 20% of GCs generated in the SGZ will survive to maturity due to periods of massive cell death in both the progenitor and postmitotic stages[39]. However, once GCs survive past these critical periods, they remain in the granule cell layer for the lifetime of the animal[3]. Knocking out Bax does not affect the proliferation rate of GCs, but facilitates survival since Bax KO mice have increased numbers of both mature and newborn GCs[37]. Because the peak GC death rate does not occur until around 1 month of age[39,40], the accumulation of GCs in Bax KOs is less apparent in younger

animals and becomes more pronounced with age. Importantly, the gross morphology of the EC and other hippocampal regions is similar to controls. The volume of the molecular layer was unchanged and electron microscopy showed a similar density and morphology of perforant path synapses, indicating that the number of presynaptic EC terminals is the same despite the increase in GC number. The increasing density of GCs innervating the static population of CA3 pyramidal neurons causes a decreased ratio of CA3 dendritic spines to mossy fiber boutons[40]. GCs in older Bax KO mice no longer express the calcium-binding protein, calbindin, suggesting that some excess Bax KO GCs may not develop into a mature phenotype[37]. This may be due to limited availability of neurotrophic factors. Although they appear outwardly normal and have typical life spans, older Bax KOs experience severe learning deficits[40,41], aberrant cell migration[37,42] and altered social behavior[43]. Conditional elimination of Bax in progenitor cells is an effective way of inflating the newborn GC population in adult animals[15].

The fact that so many cells are pruned before joining with the network raises the question of how GCs are selected for either integration or elimination. Some studies suggest that the presence of presynaptic innervation prevents cell death. Scenarios that cause activation of the DG enhance survival of newborn GCs, such as environmental enrichment or seizures[22,23,44]. Knocking out the NMDAR-NR1 subunit from some adult-born GCs, which would decrease glutamatergic signaling, caused significantly more cell death in those cells than in neighboring cells of the same age with NMDARs intact, showing that altering activity can affect survival at a cell-specific level[45]. However, injection of an NMDAR antagonist for a week, uniformly decreasing activity across all GCs, rescued some of the cell death in the NR1KO cells. This suggests that lack of

activity alone does not lead to pruning, but rather the relative difference in activity levels between cells that prompts selective survival of one over the other. This phenomenon occurs at the synapse level and has been demonstrated many times in sensory systems of the developing brain. Imbalanced activity levels between afferents will cause the pruning of the less active inputs, while a global decrease in activity does not alter projection patterns[46].

Network refinement in the developing and adult brain

The mammalian brain can alter its connectivity to adapt to changing environmental factors. Long-lasting changes in plasticity are thought to occur through long-term potentiation (LTP), which correlates to the formation/strengthening of synapses through the addition of AMPA receptors, and long-term depression (LTD), which correlates to the weakening/elimination of synapses through the endocytosis of AMPA receptors. These forms of plasticity are especially important in the hippocampus, where activity-dependent plasticity takes place regularly, and LTP and LTD are proposed cellular mechanisms of learning. Neuronal plasticity is higher throughout the brain during development, but the level in adulthood varies between brain regions. Plasticity in the visual cortex is restricted to a "critical period" during development, when changes in visual input can result in experience-dependent rewiring. This has been described largely in terms of axonal retraction, as when afferents from both eyes initially converge on the same downstream visual region, but are pruned to a single input by maturity[46]. In

contrast, the adult sensory cortex and hippocampus remain highly plastic throughout the lifetime of an animal. For instance, the part of the adult mouse sensory cortex corresponding to whisker sensation, the barrel cortex, will alter connectivity if whiskers are stimulated or trimmed. When changes occur in a mature network, the afferent connections are more concrete, and plasticity takes the form of activity-dependent formation and elimination of post-synaptic terminals. Researchers imaged the barrel cortex of whisker-trimmed mice over a 1-month period and found that during the addition of new contacts, dendritic spine formation preceded synapse formation. Ultrastructural analysis also showed that new spines preferentially synapsed onto preexisting axonal boutons, sometimes forming multi-synaptic boutons[47].

The adult hippocampus is considered a region of heightened plasticity due to its ability to change synaptic strength in response to changing levels of input[48]. However, the capacity for hippocampal spines to remain motile is still decreased after development[49]. The pool of completely novel synapses offered by continuous DG neurogenesis represents a significant contribution to circuit plasticity[50]. Integration of synapses from new GCs into the mature network seems to follow the same process as addition of synapses in the adult sensory cortex. Rather than inducing the generation of *de novo* boutons, 4-week-old GCs form contacts with boutons that are already connected with a mature GC spine[51]. Since at later time points, the adult-generated spines tend to occupy single-synapse boutons, it is conjectured that the more excitable immature GCs will undergo Hebbian strengthening while the mature spine weakens and is eventually eliminated. In this scenario, the new GC effectively "steals" the existing connection from

the mature GC[52]. However, the elimination of mature synapses as a result of competition for presynaptic input has never been functionally shown.

The role of Bax in activity-dependent competition

In addition to its established role in programmed cell death, Bax has become recognized as a key player in synaptic plasticity. Bax and downstream caspases are locally contained in axons, dendrites and pre- and post-synaptic terminals[53]. Eliminating BAX impairs the developmental pruning of sensory axons in mice and dendrites in *Drosophila*[54]. Furthermore, caspase-3 and Bax were shown to be necessary for AMPAR internalization and NMDA-dependent LTD in hippocampal culture and mouse CA1[55,56]. These data together support a localized function of the apoptotic pathway that is independent of cell death. Activating caspase-3 by photostimulation in localized dendritic compartments induced spine elimination without causing cell death, and hippocampal neurons lacking caspase-3 resist spine shrinkage after NMDA excitotoxicity[57]. Functional caspase-3 is also implicated in attention control (alertness, reorientation and executive control), which may be related to proper synaptic connections in the DG. In the same study, exploration of a novel environment induced significantly more activation of the immediate early gene, c-fos, in GCs of a caspase-3 KO mouse[58]. Intriguingly, expression of Bax mRNA remains high in mature cells of the adult hippocampus[59,60] even though cell death is rare, implying an ongoing need for the protein -- possibly in synapse elimination related to mature circuit refinement.

HYPOTHESIS AND EXPERIMENTAL GOALS

We know that adult-born GCs receive functional input from the perforant path. There is also evidence that newly-forming dendritic spines on immature GCs can share presynaptic terminals with established mature spines. However, it is unclear what the fate of the mature spines is after these multisynaptic boutons are formed. Knowing that the number of synapses in the molecular layer remains constant even when GC efferents are increased, we predict that existing and new synapses must compete for a finite number of inputs. Therefore, we propose to test how the addition of new synapses alters the DG circuit by measuring how excitatory synaptic transmission is affected in the mature population after enhancing or suppressing neurogenesis. Eliminating Bax conditionally from proliferating GCs is an effective way of enhancing adult neurogenesis without genetically altering developmentally-generated cells. However, recent evidence has shown that Bax and its downstream effector caspases are essential to synaptic pruning. Therefore we also aim to show that cells lacking Bax have a competitive advantage and will retain active synapses, even in the face of competition. Overall, the results of this study will demonstrate that changes to the immature GC population will affect connections to all GCs, and thus cannot be isolated in their contribution to DG function.

ADULT-BORN NEURONS REDUCE SYNAPTIC CONNECTIVITY OF EXISTING NEURONS BY BAX-DEPENDENT SYNAPTIC COMPETITION

by

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Adult-Born Neurons Reduce Synaptic Connectivity Of Existing Neurons By Bax-Dependent Synaptic Competition

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Summary

Continual neurogenesis in the adult hippocampus enables circuit refinement through the addition of new neurons, the dentate gyrus (DG) granule cells (GCs). Adult-born GCs transiently display distinct functionality that is thought to add unique processing capabilities to the network, but synaptic integration of new GCs also has the potential to disrupt preexisting circuits. Here we show that increasing neurogenesis by conditional deletion of the pro-apoptotic gene, Bax, in neural stem cells decreases excitatory transmission to unmodified mature GCs. Consistent with its non-apoptotic function in synapse pruning, deletion of Bax in postmitotic GCs increases synaptic strength and prevents neurogenesis-induced synapse loss. Furthermore, conditional ablation of immature GCs enhances synaptic transmission to mature GCs, potentially suggesting how deletion of excitable immature GCs can counter-intuitively enhance afferent-induced excitation. Together these results show that synaptic competition from adult born neurons leads to a Bax-dependent redistribution of synaptic connectivity from the entorhinal cortex.

Highlights

- Increasing neurogenesis reduces synaptic strength of mature neurons
- Neurogenesis-induced loss of synaptic strength requires intact Bax signaling
- Neurons with Bax deletion have increased synapse strength and spine density
- Ablation of immature neurons increases synaptic strength of mature neurons

Introduction

Continual neurogenesis in the adult dentate gyrus produces new GCs that integrate into the hippocampal circuit by establishing synapses with existing neurons[1-4]. During a transient period of maturation, new GCs exhibit intrinsic and synaptic properties that are thought to endow distinct network functions compared to mature GCs[5,6] and potentially underlie the contribution of neurogenesis to DG-specific behaviors including context discrimination[7]. Yet computational models also predict that remodeling of pre-existing circuits by continual neurogenesis can degrade established memories[8], a possibility that has recently gained experimental support by the observation that neurogenesis facilitates "forgetting"[9]. The idea that synaptic competition from newly generated GCs alters the pre-existing circuit is supported by the anatomical finding that immature dendritic spines transiently receive synapses from multiple-synapse boutons[10]. A competition model implies that existing EC synaptic terminals are redistributed to newly integrating GCs, and thus up-regulation of neurogenesis is accompanied by a loss of synaptic strength to mature GCs.

One effective method to increase the number of adult born neurons is by manipulating cell survival, since the majority of proliferating DG progenitors undergo apoptosis during the first days after cell division[11]. Cell death is controlled by the pro-apoptotic protein Bax, a member of the BCL-2 family of proteins in the intrinsic apoptotic pathway[12]. Both germline and conditional Bax deletion block cell death of adult-generated GCs without altering proliferation or the gross structural integrity of the DG[12-14]. Interestingly, Bax and downstream effector caspases also contribute to synaptic plasticity in surviving neurons. In the hippocampus, the Bax-caspase signaling pathway

contributes to AMPA receptor internalization, NMDA-dependent LTD and activity-dependent spine elimination[15-17]. Thus, the high expression of Bax mRNA in mature GCs[18,19] raised the possibility that this pathway is involved in neurogenesis-induced synaptic refinement of the mature circuit. Here we directly test how manipulating the number of immature GCs affects the strength of synaptic transmission to mature GCs. We compared synaptic transmission to mature GCs after inducible Bax deletion in DG progenitors (to enhance neurogenesis) with conditional Bax deletion in early postmitotic neurons (to enhance neurogenesis and disrupt Bax in mature GCs).

Results

Adult-born GCs functionally integrate into the hippocampal circuit by receiving synapses from the major afferent pathway from the entorhinal cortex (EC). Synaptic integration can potentially modify the EC-DG circuit either by increasing the number of synapses from EC or by competition for existing EC synapses. If developing GCs establish new synapses, enhancing neurogenesis will increase the total number of synapses but have no effect on synapse number per mature GC **(Fig. 1A)**, whereas a competitive process would maintain the total pool of synapses while requiring mature GCs to relinquish existing synapses **(Fig. 1B)**. Since the Bax signaling pathway in hippocampal neurons is required for synaptic depression[15] and activation of a downstream effector, caspase-3, locally prunes spines[17], we predicted that Bax is necessary for mature GCs to relinquish synapses during synaptic competition with adult-born GCs **(Fig. 1C)**.

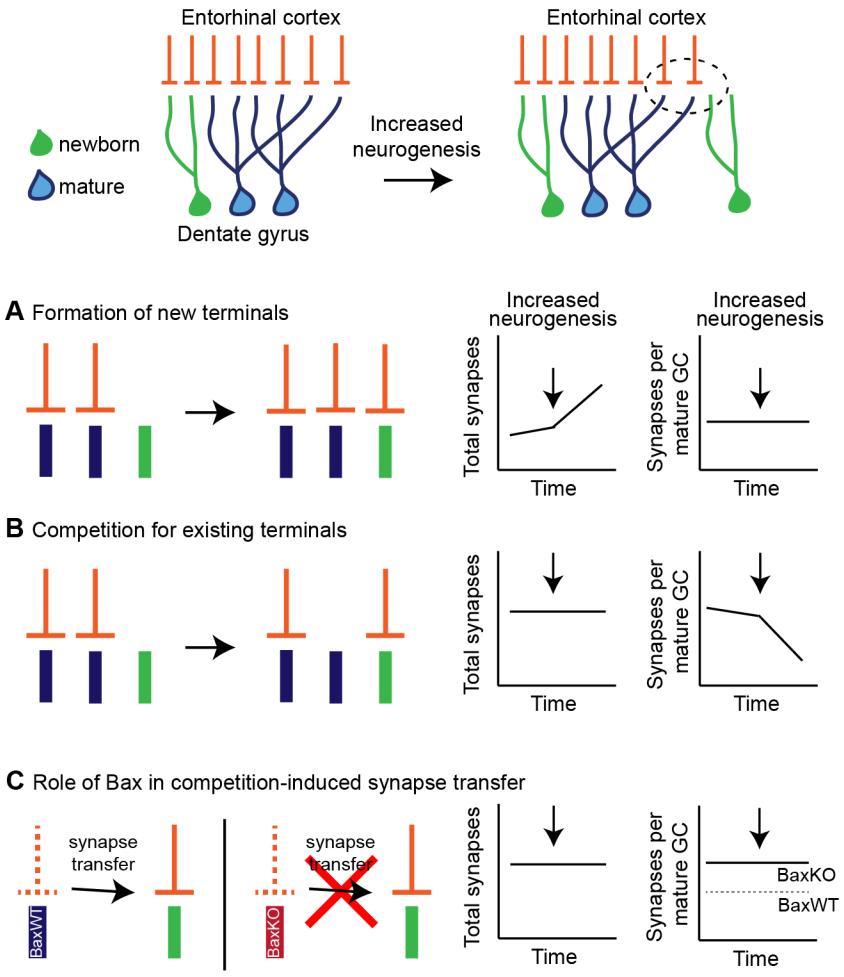

Figure 1. Synaptic integration of immature neurons in the EC-DG circuit.
Enhancing neurogenesis (arrows) requires that new GCs (green) gain EC synapses (orange) through two possible mechanisms. **A,** New EC terminals may form to innervate new GCs. In this case, increasing neurogenesis would increase the total number of synapses over time and the synapses per individual mature GC would remain constant. **B,** Alternatively, new GCs may compete for existing EC synapses on surrounding mature GCs. In this case, the total number of synapses would remain constant over time and the number of synapses per mature GC would decrease. **C,** If Bax expression in mature GCs is required for activity-dependent synapse loss, BaxKO mature GCs will not relinquish synapses in the face of competition. In this case, there would be no change in the total number of synapses and BaxKO GCs will maintain a higher number of synapses than BaxWT GCs (gray dotted line).

Increasing neurogenesis reduces synaptic strength of mature neurons

To first test how enhancing neurogenesis modifies the wildtype (WT) mature circuit, we increased the population of adult-born GCs by crossing inducible Nestin-CreER[20] and Bax$^{fl/fl}$ mice to selectively block apoptotic cell death in proliferating cells

and their progeny[14] **(Fig. S1)**. Four to six weeks after tamoxifen-induced recombination, these $BaxKO_{immature}$ mice have Bax-null immature GCs while the mature GC population is WT **(Fig. 2A, Fig. S1)**. To assess neurogenesis, we also crossed $BaxKO_{im}$ mice with a POMC reporter line that labels early postmitotic GCs[21]. As previously reported[14], four weeks after conditional deletion of Bax there were greater numbers of newborn GCs and overtly normal DG structure **(Fig. 2B)**.

To measure the strength of excitatory transmission across the population of GCs and onto individual mature GCs, we stimulated the middle molecular layer (MML) while simultaneously recording field excitatory postsynaptic potentials (fEPSPs) and EPSCs from mature GCs **(Fig. 2C)**. We targeted mature GCs located near the outer edge of the granule cell layer for whole cell recordings, and confirmed their maturity by their intrinsic membrane properties **(Fig. S2A-C)**. Both fEPSPs and EPSCs were normalized to the fiber volley (FV), a measure of axonal activation. There was no difference in the FV amplitude or fEPSP slope in slices from $BaxKO_{im}$ and control mice[14] **(Fig. S3A),** and synapse density is unaffected in germline BaxKO mice[13]. Thus, EC terminals appear to be a limiting factor in the total number of DG synapses. However, mature GCs in $BaxKO_{im}$ mice had significantly smaller EPSCs than mature GCs in controls at all FV amplitudes as well as overall lower EPSC/FV ratios **(Fig. 2D).** Thus for the same amount of axonal activation, mature GCs have reduced excitatory transmission. There was no difference in the paired pulse ratio (PPR) of evoked EPSCs, suggesting that the presynaptic release probability was unchanged **(Fig. S4A),** ruling out the possibility that adult-born neurons regulate transmission to mature GCs by secreting a factor that alters release probability. Enhanced neurogenesis in $BaxKO_{im}$ mice was also associated with a

lower frequency of spontaneous EPSCs (sEPSCs) in mature GCs with no change in sEPSC amplitude **(Fig. S4B)**, further suggesting a reduction in the number of synapses rather than a difference in postsynaptic responsiveness. Thus, enhanced neurogenesis reduces excitatory perforant path transmission to mature GCs in a manner consistent with a redistribution of synapses from mature to immature GCs **(Fig. 1B)**.

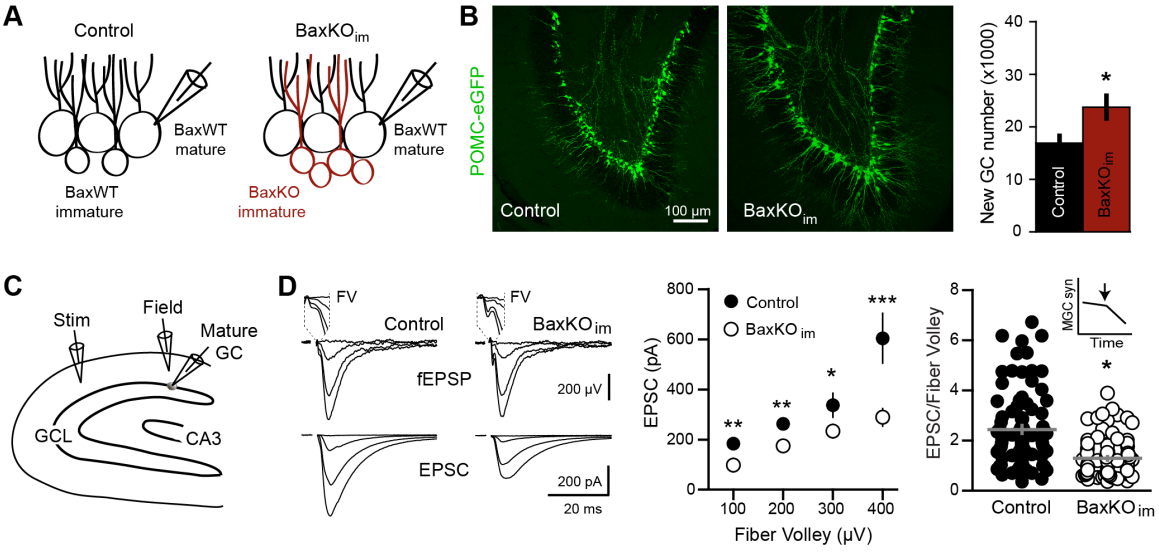

Figure 2. Increasing neurogenesis reduces synaptic strength of mature neurons
A. Schematic illustrating the genotype of mature and immature GCs in BaxKO$_{im}$ mice. **B.** Confocal images of POMC-eGFP expression used to assess neurogenesis. Stereological cell counts of GFP+ newborn cells at 4 weeks post-TMX injection revealed neurogenesis was enhanced by ~ 40% (control 16881 ± 1422 cells, n = 4; BaxKO$_{im}$ 23756 ± 2166 cells, n = 4; p=0.038 unpaired t-test). **C.** Schematic showing the recording strategy to simultaneously record fEPSPs and whole cell recordings from mature GCs to assay total synapses and synapses per mature GC. All experiments were performed in picrotoxin to isolate excitatory transmission. **D,** Examples of fEPSPs (top) and EPSCs (bottom) in slices from control and BaxKO$_{im}$ mice. The fiber volley (FV) was used to normalize fEPSPs and EPSCs across slices. A decrease in synaptic strength to individual mature GCs was revealed by the EPSC plotted against fiber volley amplitude (left; $p < 0.0001$ two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Bonferroni post hoc test, n=15 control, 14 BaxKO$_{im}$) and the ratios of EPSC to fiber volley for all stimulus intensities (right, control 2.44 ± 0.16, n = 86; BaxKO$_{im}$ 1.29 ± 0.07, n = 99; $p < 0.0001$ unpaired t-test). Inset shows predicted outcome from Fig 1A.

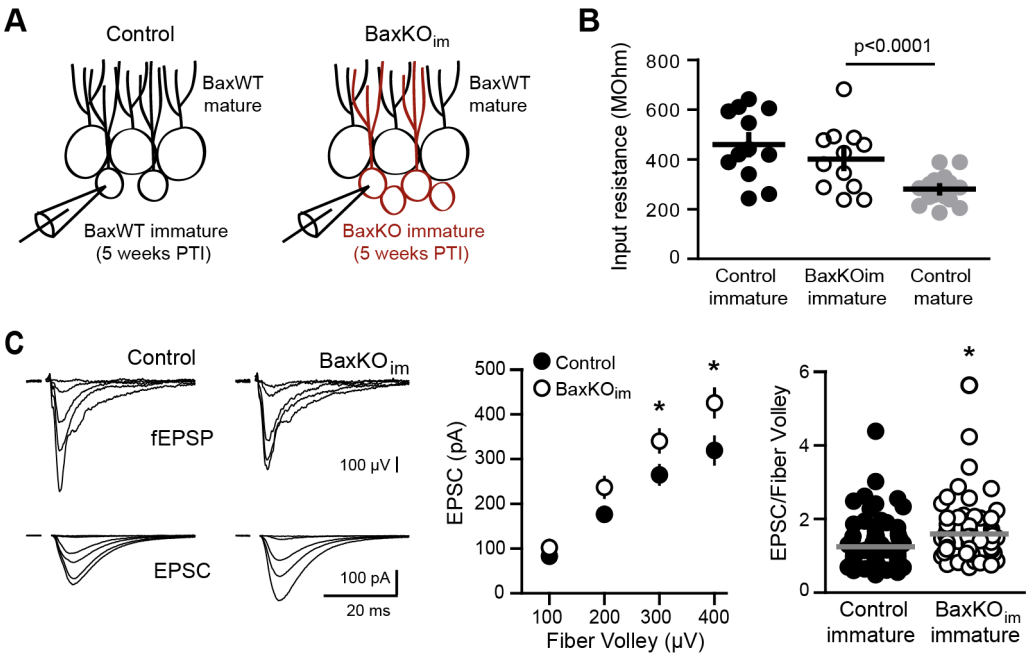

Figure 3. Bax deletion enhances synaptic strength of immature neurons
A. Schematic illustrating the genotype of mature and immature GCs in BaxKO$_{im}$ mice. **B.** Immature GCs in control and BaxKO$_{im}$ mice had a similar input resistance that was higher than mature GCs (immature control 459 ± 40, n=12, immature BaxKO$_{im}$ 400 ± 38, n=12, mature control 279 ± 15, n=16; $p < 0.0001$ between mature control and immature control). **C.** Examples of fEPSPs (top) and EPSCs (bottom) recorded in immature GCs in slices from control and BaxKO$_{im}$ mice. An increase in synaptic strength in immature GCs was revealed by the EPSC plotted against fiber volley amplitude (left; $p < 0.0001$ two-way ANOVA, * $p < 0.05$ Bonferroni post hoc test, n=12 immature control, 12 immature BaxKO$_{im}$) and the ratios of EPSC to fiber volley for all stimulus intensities (right, immature control 1.24 ± 0.07, n = 80; immature BaxKO$_{im}$ 1.59 ± 0.09, n = 75; $p = 0.0029$ unpaired t-test).

To show that loss of synaptic strength from mature GCs was associated with synaptic integration of newly generated GCs, we crossed BaxKO$_{im}$ and control mice with a Tdtomato reporter line (Ai14) to target 5 week-old BaxKO and BaxWT GCs for recordings **(Fig. 3A)**. The input resistance is a measure of cell maturity[22] and as expected, immature GCs had higher input resistance than mature GCs. But there was no difference in the input resistance between BaxKO and BaxWT immature GCs **(Fig. 3B)**, consistent with no effect on dendrite development in this model[14] and confirming immature GCs were at a similar stage of maturation. Simultaneously recorded fEPSPs and EPSCs

revealed that EPSCs in BaxKO immature GCs (recorded at 5 weeks post-tamoxifen injection) were significantly larger than EPSCs in BaxWT immature GCs at all FV amplitudes and the overall EPSC/FV ratio was greater **(Fig. 3C)**. Enhanced transmission is consistent with impaired activity-dependent synaptic pruning in Bax KO neurons[17], and suggests that Bax could also have a role in neurogenesis-induced loss of synapses in mature GCs.

Neurogenesis-induced loss of synaptic strength requires intact Bax signaling

To test the role of Bax in mature GCs during synaptic competition with new GCs, we generated a conditional Bax KO in postmitotic GCs ($BaxKO_{mature}$) using POMC-Cre[23] mice crossed with $Bax^{fl/fl}$ mice **(Fig. 4A)**. This strategy generates Bax-deficient immature and mature GCs **(Fig. 4B)**, while proliferating progenitors are BaxWT (not shown). To assess neurogenesis, we crossed $BaxKO_{mat}$ mice with POMC-GFP reporter mice and found that the number of newborn neurons was enhanced to similar degree as observed in $BaxKO_{im}$ mice, presumably due to a later period of cell death that occurs in newly postmitotic GCs[11] **(Fig. 4C)**. The intrinsic properties of mature GCs in $BaxKO_{mat}$ and control mice were the same as mature GCs in $BaxKO_{im}$ mice **(Fig. S2D)**, suggesting that Bax deletion does not affect these measures of cellular excitability.

Interestingly, neurogenesis-induced loss of synaptic transmission from mature GCs was absent when Bax was deleted from mature GCs. The EPSC was similar to controls across all stimulus intensities and the average EPSC/FV ratio was unchanged in $BaxKO_{mat}$ mice **(Fig. 4D)**. The paired pulse ratio as well as sEPSC frequency and amplitude were also similar between mature GCs in control and $BaxKO_{mat}$ mice **(Fig.**

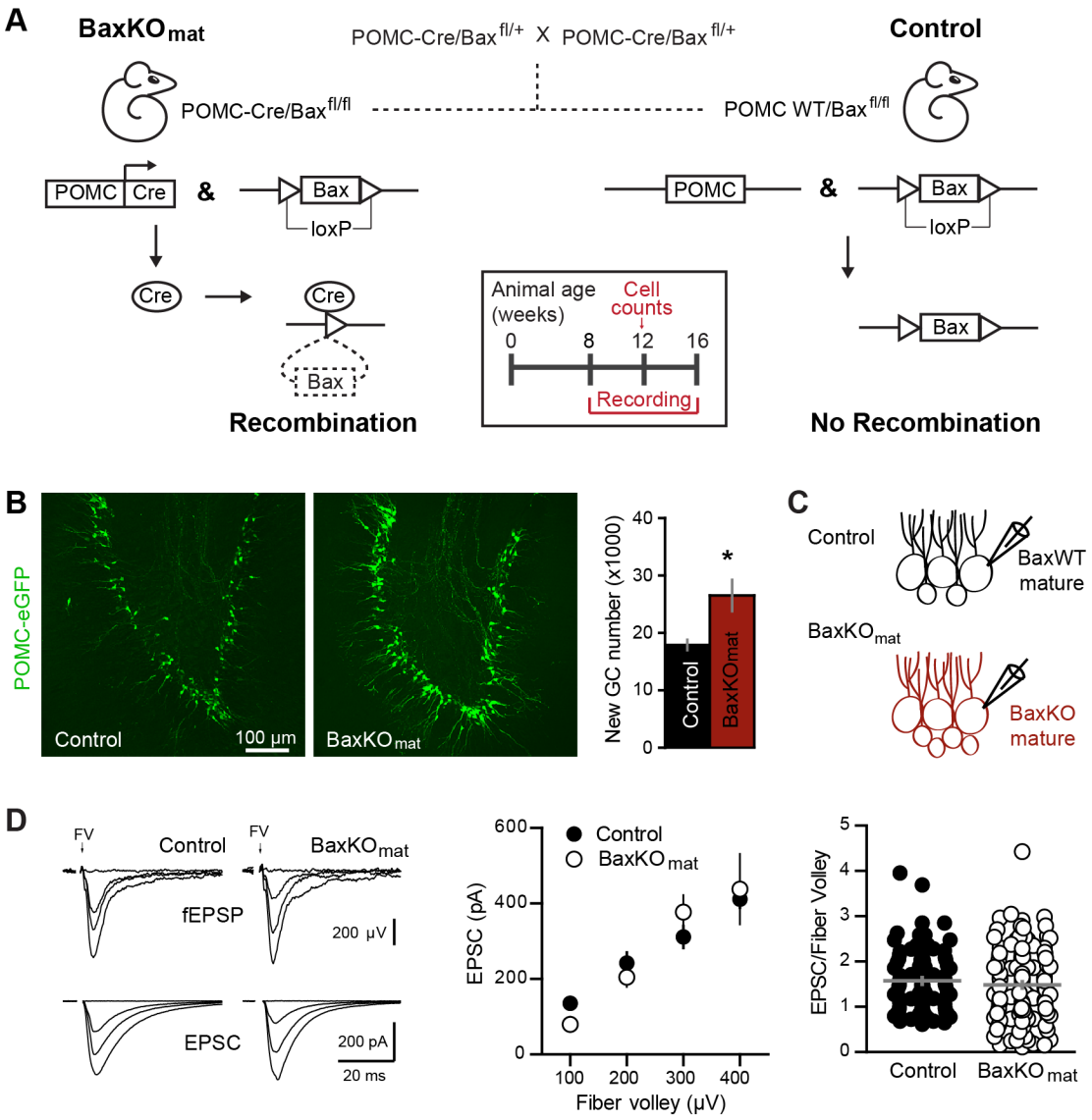

Figure 4. Neurogenesis-induced loss of synaptic strength requires intact Bax signaling
A. Schematic showing POMC-Cre dependent excision of the lox-p flanked Bax locus to produce $BaxKO_{mat}$ mice. Controls were Cre^{-}. The experimental timeline includes cell counts performed in mice at 12 weeks of age and recordings performed in 2-4 month-old mice. **B.** Confocal images of POMC-EGFP expression used to assess neurogenesis. Stereological cell counts of GFP+ newborn cells in revealed neurogenesis was enhanced by ~ 48% (control 17910±900 cells, n=7; $BaxKO_{mat}$ 26508±2728 cells, n=6; p=0.0085 unpaired t-test), similar to enhanced neurogenesis in $BaxKO_{im}$ mice. **C.** Schematic illustrating the genotype of mature and immature GCs in $BaxKO_{mat}$ mice. **D.** Examples of fEPSPs (top) and EPSCs in mature GCs (bottom) from control and $BaxKO_{mat}$ mice. There was no difference in EPSCs across FVs, although there was great variability in the $BaxKO_{mat}$ group (FV vs. EPSC amplitude, p=0.59 two-way ANOVA, CV= 52% vs. 43%, n=12 control, 19 $BaxKO_{mat}$; EPSC to fiber volley ratio p=0.39 unpaired t-test, n=88 control, 129 $BaxKO_{mat}$).

S4C, D). Thus, competition-induced loss of synaptic strength appears to require intact Bax signaling in mature GCs. Yet we also noticed considerable variability in EPSC/FV ratios and sEPSC frequencies in mature GCs from $BaxKO_{mat}$ mice, suggesting a heterogeneous population of BaxWT and BaxKO GCs with mixed susceptibility to competition-driven synapse loss.

Bax deletion in mature neurons increases synaptic strength

To determine if the variability in synaptic transmission correlated with Bax expression, we crossed $BaxKO_{mat}$ mice with the Ai14 reporter line to reveal the mixed population of recombined mature GCs **(Fig. 5A)**. We then directly compared EPSCs in simultaneous recordings from neighboring BaxWT and BaxKO mature GCs **(Fig. 5B)**. In this paradigm, normalizing the EPSC amplitudes to the fiber volley is unnecessary because the number of axons stimulated is the same for both cells. To compare EPSCs across cell pairs, we normalized to the EPSC in each BaxWT GC. Consistent with a role of Bax in competition-induced synapse loss, EPSCs in BaxKO GCs were larger than EPSCs in BaxWT GCs across a range of stimulus intensities **(Fig. 5C)**. To test whether Bax expression affected the strength of excitatory transmission by altering dendritic spines, during recordings we filled BaxKO and BaxWT cells with biocytin to visualize morphology. Posthoc analysis revealed a significant increase in the density of spines in BaxKO mature GCs, with no change in head diameter **(Fig. 5D)**. Importantly, the difference in EPSCs resulted from Bax expression rather than tdTomato expression or age/position of the cells, since EPSCs were similar in simultaneous recordings from $tdTomato^+$ and $tdTomato^-$ mature GCs from BaxWT mice **(Fig. 5E-G)**. Furthermore,

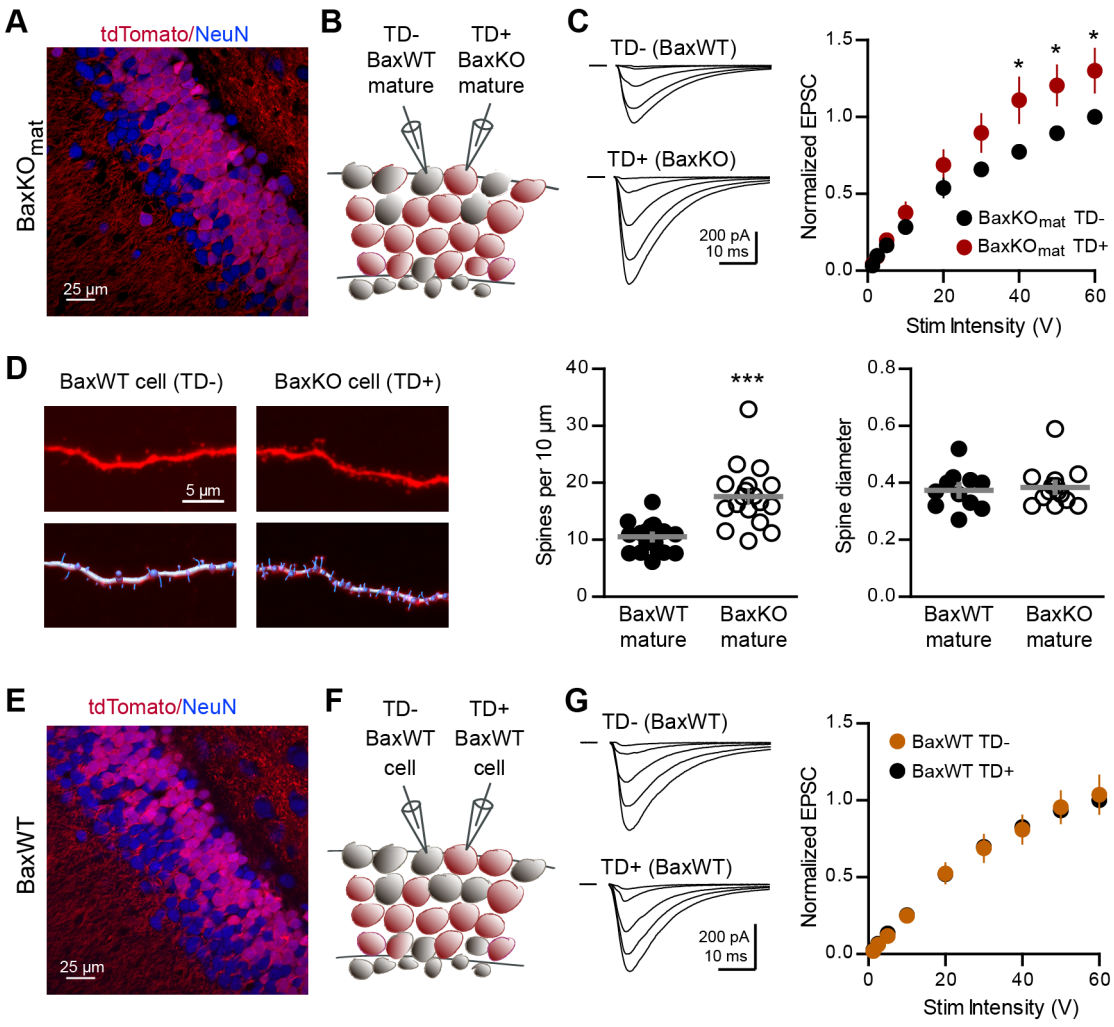

Figure 5. Bax deletion in mature neurons increases synaptic strength

A. Confocal image of fixed tissue from a $BaxKO_{mat}$/tdTomato mouse showing TD-expressing BaxKO cells (red) and NeuN (blue). **B.** Adjacent TD^- (BaxWT) and TD^+ (BaxKO) mature GCs were recorded simultaneously in the same slices. **C.** Examples of EPSCs in BaxKO and BaxWT mature GCs to the same stimuli. EPSCs were normalized to the maximum EPSC of the TD^- cell in each slice. EPSCs were larger in BaxKO GCs ($p<0.0001$ two-way ANOVA, * Bonferroni post hoc test, n=12 cell pairs). **D.** Posthoc dendrite reconstructions (top) revealed higher spine density in BaxKO GCs (10.51±0.53 spines/10 µm BaxWT, 17.60±1.3 BaxKO, $p<0.0001$ unpaired t-test) with no change in spine head diameter ($p=0.70$ unpaired t-test, n=21 segments from 5 images BaxWT, 18 segments from 9 images BaxKO). Lower images illustrate spine analysis. **E.** Confocal image of fixed tissue from a BaxWT/POMC-Cre^+/tdTomato animal, in which both TD^+ and TD^- cells are BaxWT (red tdTomato, blue NeuN). **F.** Adjacent TD^- (BaxWT) and TD^+ (BaxWT) mature GCs were recorded simultaneously in the same slices. **G.** There was no difference in EPSCs between TD^- and TD^+ cells ($p=1.0$ two-way ANOVA, n=8 cell pairs), confirming differences in panel C result from Bax expression.

repeating the same paradigm in $BaxKO_{im}$ mice crossed with Ai14 reporter mice likewise revealed increased excitatory transmission to BaxKO mature GCs compared to BaxWT mature GCs (recorded 16 weeks post tamoxifen; **Fig. S5**). Together these data show that Bax expression in mature GCs enhances synaptic strength and is required for neurogenesis-induced synapse loss.

Quantitative estimate of synapse transfer between mature and immature neurons

Immature GCs make up a small percent of total GCs, and yet the loss of synaptic strength to mature GCs when neurogenesis was modestly enhanced was surprisingly robust (~40% reduction based on the EPSC/fiber volley ratio in Fig. 2D). To determine whether the magnitude of reduced transmission to mature GCs in $BaxKO_{im}$ mice can be explained by synaptic competition with integrating new GCs, we made a quantitative estimate of the proportion of mature synapses that would be transferred to new GCs over the time course of our experiment **(Fig. S6A)**. Parameters were based on reported rates of neurogenesis[24,25], cell death[11] and excitatory synaptic integration[4], as well as the current finding that BaxKO GCs have more synapses than BaxWT GCs. The model showed a steep increase in the proportion of synapses occupied by immature GCs in the $BaxKO_{im}$ mice starting at the time point when immature BaxKO GCs start to integrate into the network (**Fig. S6B,** red line). The predicted reduction in mature synapse number (expressed as a %) at days 36-43 in the simulation was similar to the % change in mature EPSCs measured experimentally **(Fig. S6C)**. Thus, despite the relatively small proportion of immature GCs within the network (initially set at 5%), Bax depletion inflates the

number of new GCs and enhances their synaptic integration, which compounded over time, robustly attenuates excitatory transmission to the mature network.

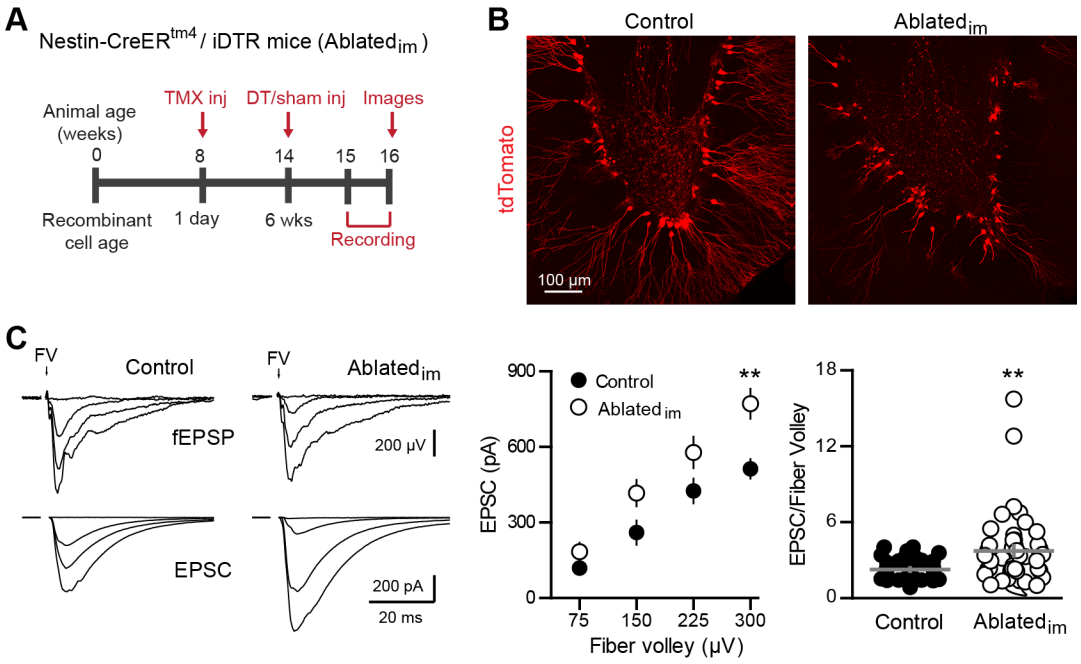

Figure 6. Ablation of immature neurons increases synaptic strength of mature neurons.
A. Experimental timeline showing ablation of immature GCs that are < 6 weeks of age. Recordings from mature GCs were done 1-2 weeks after ablation. **B.** Decreased number of tdTomato-expressing Cre^{+} immature GCs in $Ablated_{im}$ mice compared to controls that received a sham DT injection. **C.** Example of fEPSPs (top) and simultaneously recorded EPSCs from mature GCs (bottom) in control and $Ablated_{im}$ mice. The EPSC amplitude plotted against fiber volley was larger in mature GCs from $Ablated_{im}$ mice compared to controls (bins of 75 µV, $p<0.0001$ two-way ANOVA, ** Bonferroni post hoc test; n=7 control, 7 $Ablated_{im}$). The EPSC to fiber volley ratio was also larger in mature GCs from $Ablated_{im}$ mice (control 2.26 ± 0.12, n = 42; $Ablated_{im}$ 3.74 ± 0.40, n = 47; **p=0.001 unpaired t-test).

Ablation of immature neurons increases synaptic strength of mature neurons

Bax deletion in immature GCs likely provides a competitive advantage during synaptic integration that overestimates the neurogenesis-induced loss of synaptic transmission to mature GCs. To assess neurogenesis-induced competition without this potential confound, we tested whether ablating neurogenesis (independent of Bax

manipulation) was sufficient to increase excitatory transmission to mature GCs. We crossed Nestin-CreER^{tm4} mice[26] to Cre-inducible diphtheria toxin receptor (iDTR) mice [27,28] and ablated immature GCs in the offspring using DT injections 6 weeks after tamoxifen-induced recombination (termed Ablated$_{im}$ mice; **Fig. 6A**). Crossing Ablated$_{im}$ mice with a Cre-driven Rosa-tdTomato reporter line confirmed a reduction in the number of immature GCs **(Fig. 6B)**. Performing simultaneous field and whole-cell recordings from mature GCs in Ablated$_{im}$ mice and controls revealed no change in total synapse number assayed by the fEPSP slope **(Fig. S3C).** However, there was enhanced synaptic transmission to individual mature GCs, evidenced by larger EPSC amplitudes and EPSC/FV ratios **(Fig. 6C)**. Thus, the number of integrating immature GCs is inversely correlated with the strength of synaptic transmission to mature GCs.

Discussion

Our results support a model of adult neurogenesis wherein newly generated GCs modify the existing circuit through competition for pre-existing EC synapses. In BaxKO$_{im}$ mice, the redistribution of synapses from mature GCs was likely facilitated by providing immature GCs a competitive advantage through Bax deletion, since BaxKO GCs exhibited greater synaptic transmission than BaxWT GCs. Yet results from ablating immature GCs also confirm that manipulating the number of immature GCs is sufficient to alter excitatory transmission to mature GCs. We did not find functional evidence for an increase in the total number of synapses that would be expected from multi-synaptic boutons that have been anatomically identified[10], but a brief period of shared transmission from functional multi-synaptic boutons may be below detection using field

potential recordings. For the same reason, we cannot conclude from the negative fEPSP data **(Fig. S4)** that immature GCs fail to establish synapses with newly formed EC terminals **(Fig 1A)**. However, our results unambiguously demonstrate that neurogenesis is associated with a loss of synaptic strength existing mature GCs. Since Bax/caspase signaling is required for activity-dependent synapse pruning[15-17], the blockade of neurogenesis-induced synapse loss by Bax deletion in mature GCs supports the idea that neurogenesis provides a competitive environment for activity-dependent redistribution of existing EC terminals.

These results have implications for understanding the role of neurogenesis in DG function. For example, both enhancing neurogenesis and blocking output from mature GCs improves performance on the same context discrimination task[14,29], suggesting that neurogenesis could contribute to DG-mediated context discrimination by competition-induced reduction in mature GC activity. Since eliminating Bax in progenitors leads to greater innervation as well as greater survival of neural progeny, blocking the apoptotic pathway may promote competition to a greater extent than other methods of increasing neurogenesis, such as running[14].

In addition to providing distinct information processing capability to the dentate network based on their physiological properties, immature GCs could also modify dentate excitability via recruitment of local inhibitory circuits[30-32]. Since all our experiments were performed in GABA receptor antagonists, inhibition did not contribute to our observations. Rather, regulation of excitatory synaptic strength of mature GCs via neurogenesis-induced synaptic competition could potentially provide an alternative

explanation of the counter-intuitive finding that the number of excitable immature GCs is inversely related to overall network excitability[31].

Finally, our results showing that deletion of Bax signaling in mature GCs enhances synaptic transmission from EC is consistent with increased activation of DG observed in caspase-3$^{-/-}$ mice, which also show behavioral deficits in attending to relevant stimuli[33]. Thus, synaptic competition between immature and mature GCs may contribute to synaptic plasticity that allows novel and salient stimuli to receive precedence in hippocampal encoding.

Experimental procedures

Transgenic mice.

All animal procedures followed the Guide for the Care and Use of Laboratory Animals, U.S. Public Health Service, and were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Nestin-Cre/Bax$^{fl/fl}$ mice were generated by crossing heterozygous *loxP*-flanked *Bax* mice (Jackson #006329, the Bak null allele was bred out) with heterozygous Nestin-CreER mice (provided by Amelia Eisch, Jackson #016261). The positive offspring were crossed with each other to produce Nestin-Cre^{+} or $^{-}$/Bax$^{fl/fl}$, Bax$^{fl/+}$, or Bax$^{+/+}$ animals. Eight week-old mice were injected with tamoxifen (TMX, from a 20 mg/ml stock dissolved in sunflower seed oil, 75 mg/kg for three consecutive days) to induce recombination and experiments were done 4-6 weeks post-injection. Control Nestin-Cre^{-} or Bax$^{+/+}$ genotypes received TMX injections with the same protocol. A similar breeding strategy was used for conditional POMC-Cre/Bax$^{fl/fl}$ mice (Jackson #005965). Conditional knockouts were maintained on a mixed 129 and C57BL/6J background using sibling controls. For knockdown of neurogenesis, homozygous iDTR mice (Jackson #007900) were crossed with male heterozygous Nestin-CreER^{tm4} (provided by Chay Kuo) to obtain offspring that were iDTR het and either Nestin-Cre^{+} or $^{-}$ (control group). Nestin-CreER^{tm4}/iDTR mice were given TMX injections at 40-60 days old, then diphtheria toxin injections (DT, 16µg/kg in sterile saline for three consecutive days) 6 weeks later, and used in experiments 9-16 days after DT. For counting newborn GCs, mice were crossed with POMC-EGFP transgenic mice (Jackson #009593). To visualize Cre-expressing cells, conditional lines were crossed with Ai14 reporter mice (Jackson #007914). All experiments were performed in adult P60-P120 mice.

Electrophysiology
Mice were anesthetized and perfused intracardially with cold cutting solution containing (in mM): 110 choline chloride, 25 D-glucose, 2.5 $MgCl_2$, 2.5 KCl, 1.25 Na_2PO_4, 0.5 $CaCl_2$, 1.3 Na-ascorbate, 3 Na-pyruvate, and 25 $NaHCO_3$. The brain was removed and 300 μm horizontal slices were taken on a Vibratome 3000EP or Leica VT1200S in cold cutting solution. After recovery in artificial CSF (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, and 25 glucose, recordings were performed at 30°C in ACSF + 100 μm picrotoxin (PTX) to block $GABA_A$ receptors. Patch pipettes were filled with the following (in mM): 115 K-gluconate, 20 KCl, 4 $MgCl_2$, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, 7 phosphocreatine, 0.1 EGTA and 0.2% biocytin, pH 7.2 and 290 mOsm (2-4 MΩ). Field pipettes were placed in the middle molecular layer and filled with ACSF (1-2 MΩ). A patch pipette filled with 1M NaCl (1 MΩ) was used to stimulate the middle molecular layer, determined by paired-pulse depression of the EPSC response, using an isolated stimulator (Digitimer). EPSC amplitudes were normalized to the fiber volley amplitudes from simultaneously recorded fEPSPs for each cell.

Immunohistochemistry
Anesthetized mice were perfused intracardially with 0.9% NaCl or 0.1 M PBS and chilled 4% PFA before brains were removed and post-fixed overnight in PFA. Free-floating horizontal slices were taken on a Vibratome 1000 (50 μm). To enhance endogenous GFP expression, slices were blocked in TBS block buffer (0.1M TBS, glycine, 3% bovine serum albumin, 0.4% Triton X-100 and 10% normal goat serum) and incubated overnight with anti-GFP conjugated Alexa 488 (1:1000, Invitrogen). For NeuN staining, slices were washed in TBST (50 mM Tris, 0.9% NaCl and 0.5% Triton X-100) and treated with antigen retrieval solution (10 mM sodium citrate, 0.5% tween 20) and 0.3% hydrogen peroxide before being blocked in TBST+10% normal goat serum, followed by 48 hour incubation in rabbit anti-NeuN antibody (1:1000, Millipore) and subsequent 4 hour goat anti-rabbit Alexa 647. Slices were mounted with Prolong Gold or VectaShield mounting medium (Invitrogen). To visualize spines, acute brain slices containing biocytin-filled cells were post-fixed in 4% PFA for at least 24 hours then stained with streptavidin conjugated to Alexa Fluor 647 (1:1000, Invitrogen).

Stereology
POMC-GFP^+ cells were counted using the optical fractionator method from every sixth slice through the entire left dentate gyrus using StereoInvestigator software (MicroBrightField). Counting frame and SRS grid sizes were set to give a Gunderson coefficient of error of <0.1 by an investigator blinded to genotypes.

Spine counting
$TdTomato^+$ (BaxKO) or $tdTomato^-$ (BaxWT) cells were patched in alternating slices from a POMC-Cre/$Bax^{fl/fl}$/$tdTomato^+$ mouse with an internal solution that included 0.2% biocytin. After fixation, GC dendrites and spines were imaged on an Olympus Fluoview 300 confocal microscope with a 60X objective and a 3X digital zoom using a z-step of 0.1 μm. An investigator blinded to the genotypes analyzed density of spines per 10 μm, spine length and spine head width using Imaris software (Bitplane)[34].

Synaptic competition model
See supplemental material for description of quantitative estimate of synapse redistribution.

Author Contributions
E.W. A. and C.V.D. performed experiments and analysis. V. O. contributed to analysis and the simulation. J.I.W. and L.O-W. supervised the overall execution of the project and provided financial support. All authors contributed to the concept, design, and interpretation of the results, as well as writing the manuscript.

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Supplemental Figures

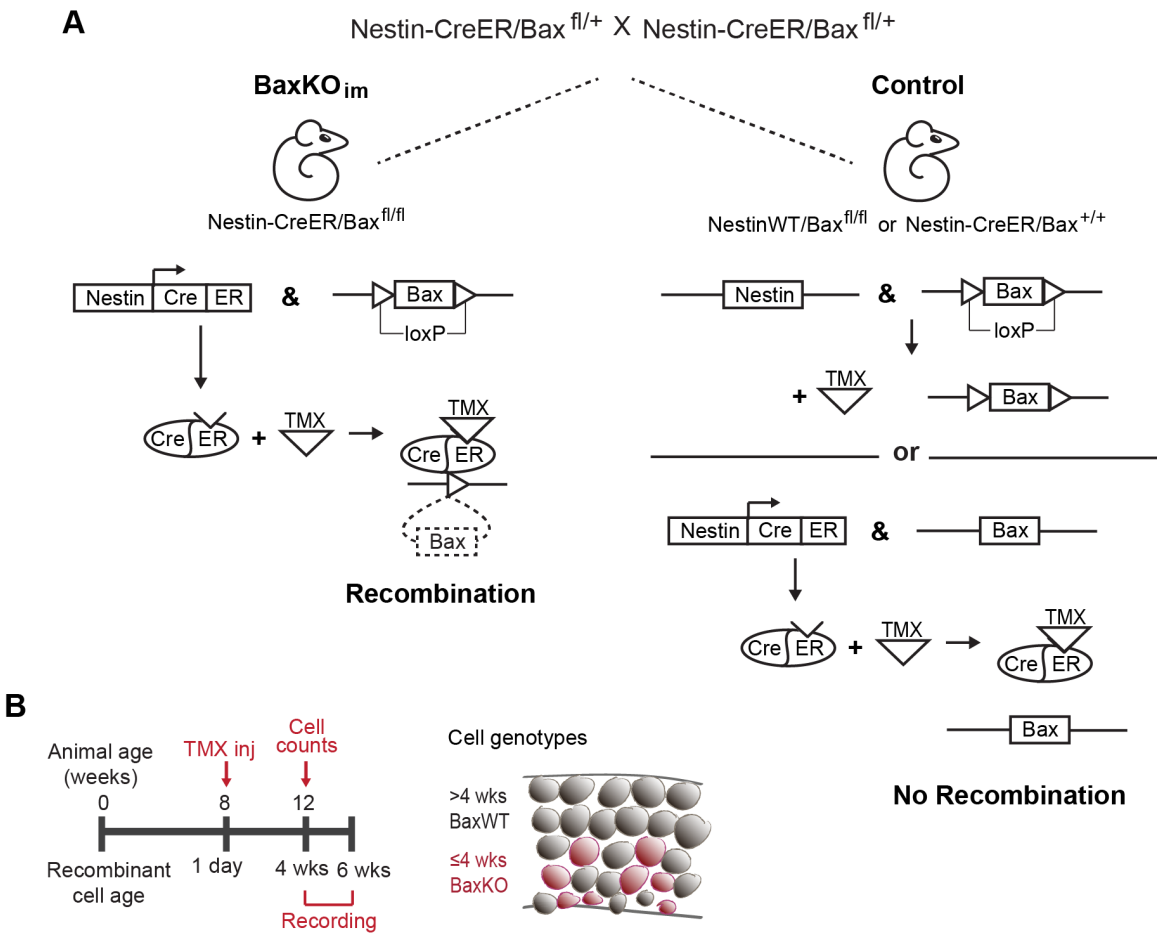

Figure S1. Generation of $BaxKO_{im}$ mice and experimental timeline.
A. Schematic illustrating tamoxifen (TMX)-induced excision of the lox-p flanked Bax locus to generate $BaxKO_{immature}$ mice ($BaxKO_{im}$). Controls included either $Bax^{+/+}$ or Nestin-$CreER^{-}$ mice that received TMX. **B.** The experimental timeline consisted of TMX injection at 8 weeks of age with experiments performed 4-6 weeks later. Cartoon of the granule cell layer shows that $BaxKO_{im}$ mice have BaxKO immature GCs and BaxWT mature GCs.

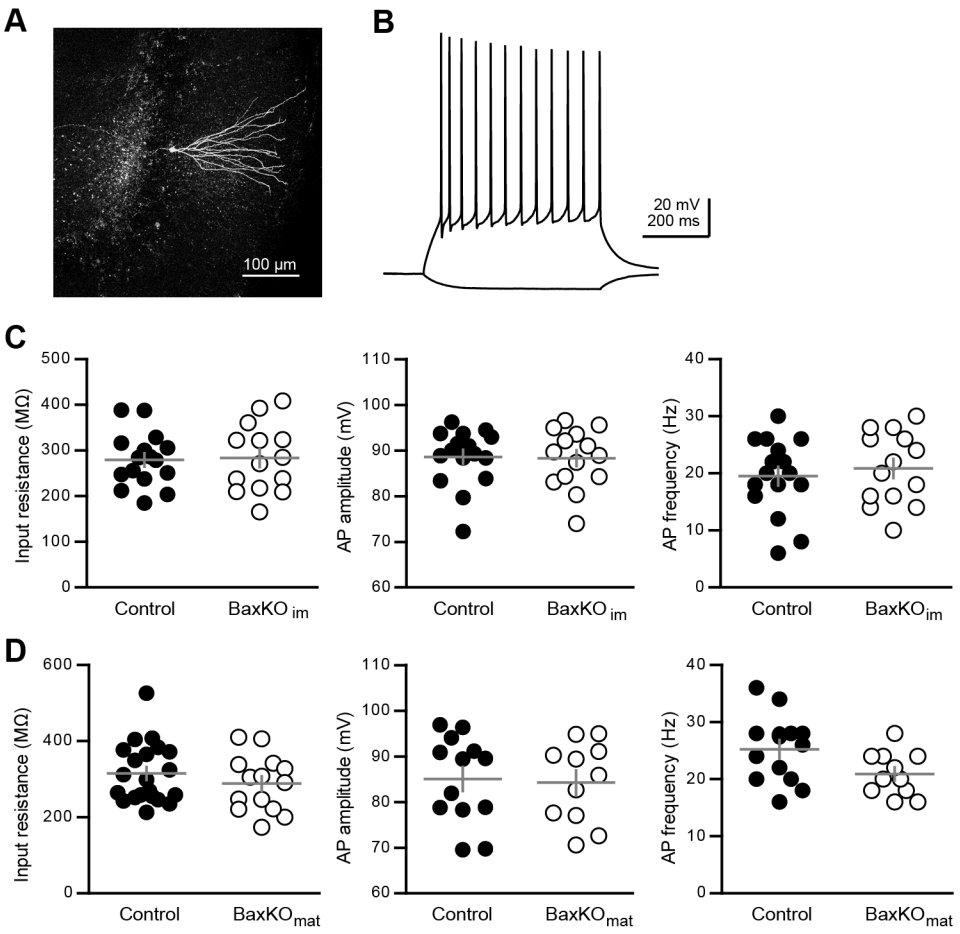

Figure S2. Unlabeled GCs in the outer 1/3 of the GCL have mature intrinsic properties.
A. A representative mature GC filled with biocytin during recording. **B.** Voltage responses to current injections were used to assess GC maturity (-20 and +100 current steps). In all experiments, the intrinsic properties confirmed the mature status of unlabeled GCs. **C.** The intrinsic properties of mature GCs were similar in $BaxKO_{im}$ and control mice, including input resistance (control 279 ± 15 MΩ, n=14; $BaxKO_{im}$ 283 ± 20 MΩ, n=16; p = 0.87 unpaired t-test), action potential (AP) amplitude measured from threshold (control 89 ± 1.5 mV, $BaxKO_{im}$ 88 ± 1.7 mV, p = 0.89 unpaired t-test) and AP frequency measured at 100 pA current injection (control 19.8 ± 1.7 Hz, $BaxKO_{im}$ 21.3 ± 1.7 Hz; p = 0.57 unpaired t-test). **D.** Mature GCs in $BaxKO_{mat}$ and control mice also had similar intrinsic properties, including input resistance (control 315 ± 17 MΩ, n = 21; $BaxKO_{mat}$ 289 ± 20 MΩ, n = 14; p = 0.32 unpaired t-test), AP amplitude (control 85 ± 2.6 mV, n = 13; $BaxKO_{mat}$ 84 ± 2.6 mV, n = 11; p = 0 .84 unpaired t-test) and AP frequency (control 25.2 ± 1.7 Hz, n =13; $BaxKO_{mat}$ 20.9 ± 1.2 Hz, n = 11; p = 0.051 unpaired t-test).

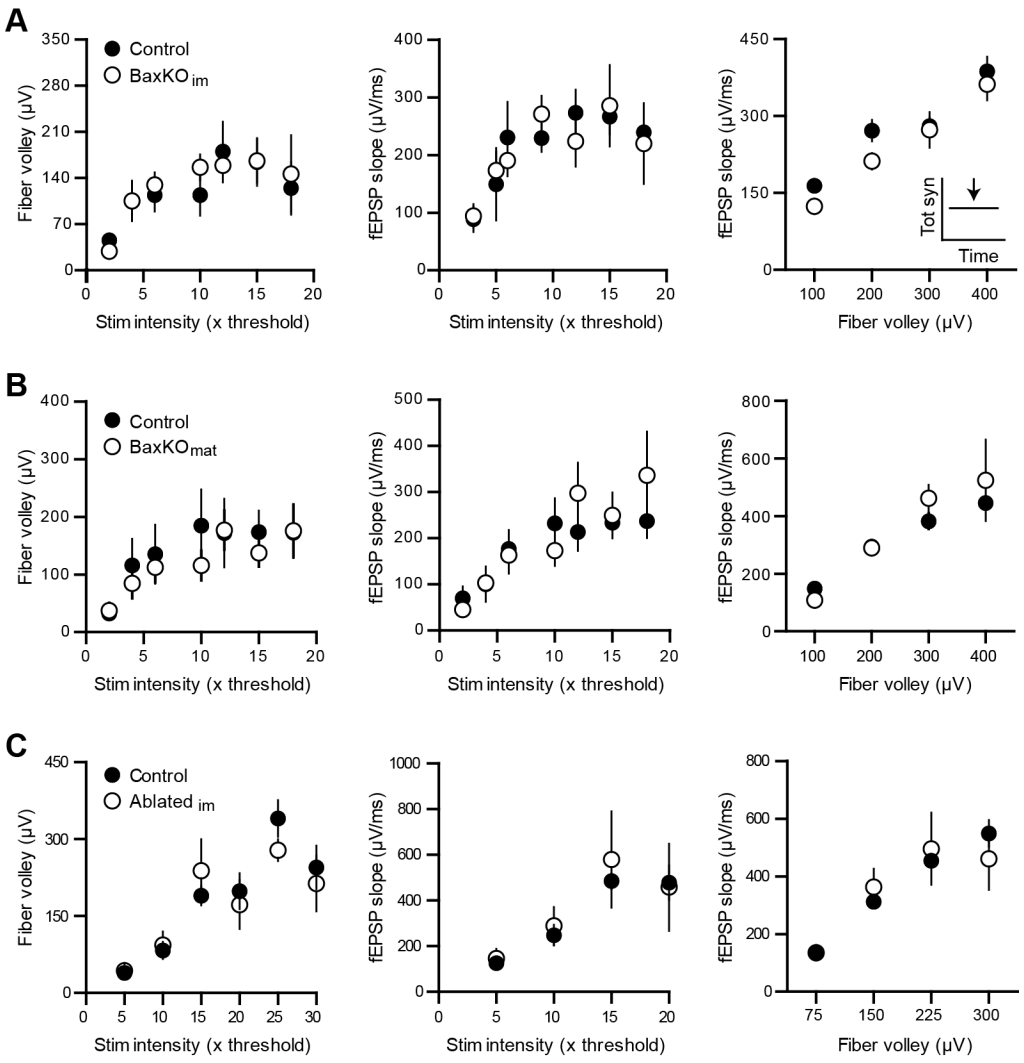

Figure S3. Neurogenesis does not alter functional measures of total DG synapses.
A. There were no differences in the FV amplitude (p=0.48) and fEPSP slope (p=1.0, two-way ANOVA) in slices from $BaxKO_{im}$ and control mice. The threshold stimulus intensity for evoking an EPSC was also similar (control 4.5 ± 0.5 V; $BaxKO_{im}$ 4.7 ± 0.7 V; p=0.77 unpaired t-test). Importantly, the fEPSP amplitude plotted against the FV also suggested no change in total synapse number (binned by 100 μV; p = 0.076 two-way ANOVA, n=15 control, 14 $BaxKO_{im}$). Inset shows predicted outcome from Fig 1a. **B.** There were no differences in the FV amplitude (p=0.13) and fEPSP slope (p=0.39) in slices from $BaxKO_{mat}$ and control mice. There was also no difference in fEPSP slope plotted against FV (bins of 100μV, p=0.21 two-way ANOVA, control n=13 cells, $BaxKO_{mat}$ n=18 cells). **C.** There were no differences in the FV amplitude (p=0.66) and fEPSP slope (p=0.63) in slices from $Ablated_{im}$ mice and controls. There was also no difference in fEPSP slope plotted against FV (bins of 75 μV, p=0.97 two-way ANOVA; n=7 control, 7 $Ablated_{im}$).

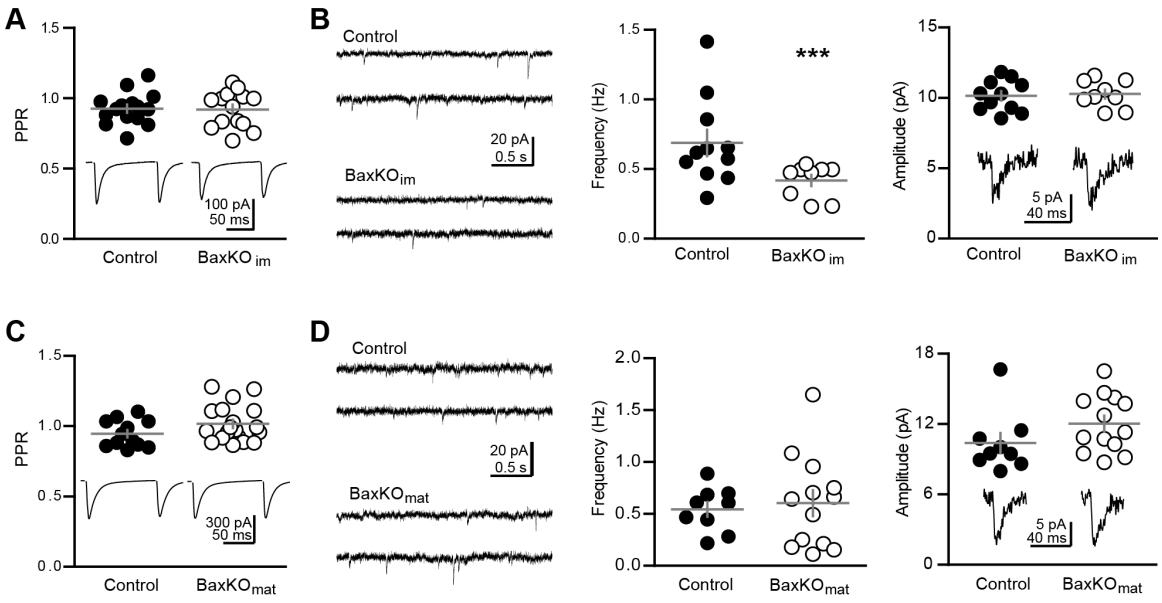

Figure S4. Increased neurogenesis leads to a Bax-dependent reduction in mature GC sEPSC frequency.

A. The paired pulse ratio of EPSCs (100 ms ISI) in mature GCs was similar in $BaxKO_{im}$ and control mice (p=0.90 unpaired t-test). **B.** Spontaneous EPSCs in mature GCs from $BaxKO_{im}$ mice had lower frequency (p=0.027 unpaired t-test) but similar amplitude as sEPSCs in mature GCs from control mice (p=0.79, n=11 controls, 9 $BaxKO_{im}$). **C.** The PPR of EPSCs in mature GCs was similar in $BaxKO_{mat}$ and control mice (p=0.12 unpaired t-test). **D.** The frequency of spontaneous EPSCs in mature GCs from $BaxKO_{mat}$ mice was similar to controls (p = 0.71), but with higher variance (p=0.04). The sEPSC amplitude was similar (p=0.14, unpaired t-tests, n=9 control, 13 $BaxKO_{mat}$).

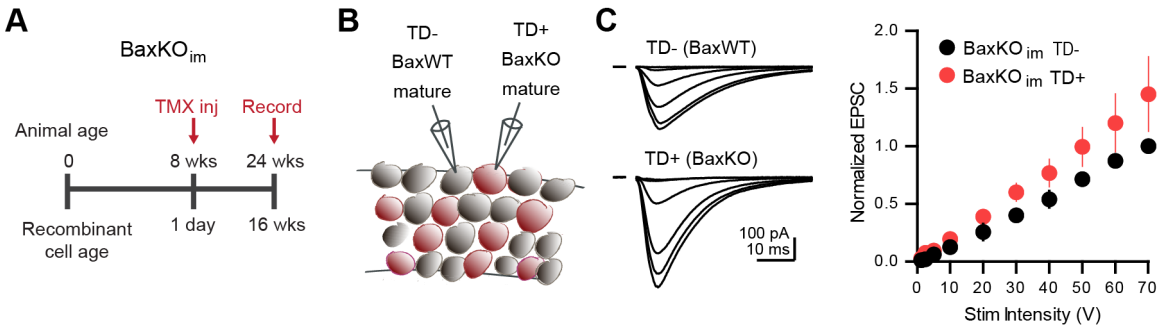

Figure S5. Adult-generated BaxKO mature GCs have more excitatory transmission than BaxWT cells.
A. Inducible $BaxKO_{im}$ animals crossed with a Cre-driven tdTomato reporter line were injected with tamoxifen (TMX) at 8 weeks of age and recordings from TD+ GCs were performed 16 weeks post-injection. **B.** Schematic showing the recordings from adjacent TD- (BaxWT) and TD+ (BaxKO) mature GCs in the same slice. **C.** Adult-generated BaxKO GCs had larger EPSCs than simultaneously recorded BaxWT mature GCs. Individual paired recordings were normalized to the maximum amplitude of the TD- cell in each slice, then averaged at each stim intensity ($p=0.001$ two-way ANOVA, $n=6$ pairs; scale bars: 10 ms, 100 pA).

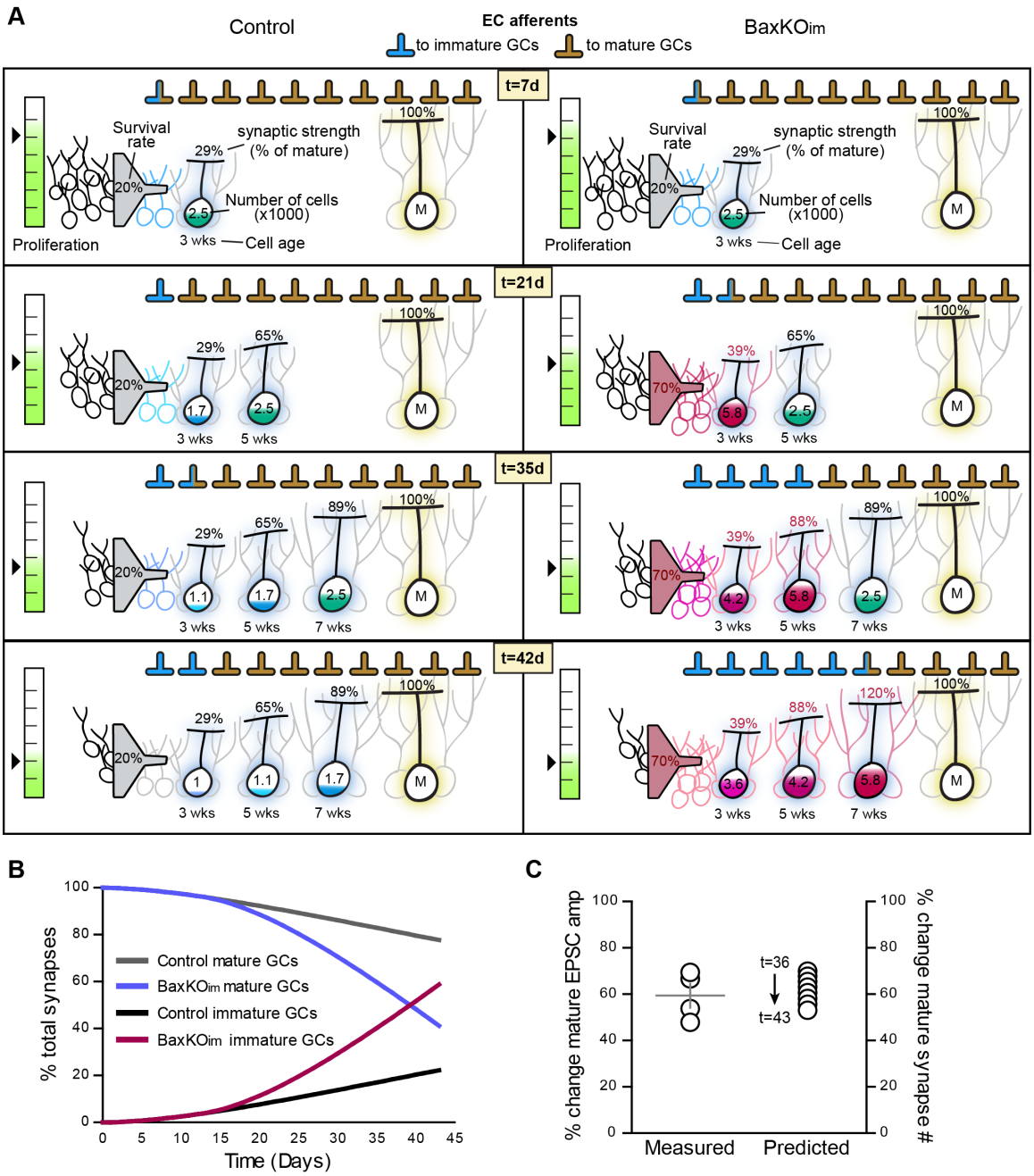

Figure S6. Synaptic competition predicts robust synaptic loss from mature GCs.
A. Graphical representation of a quantitative simulation of synapse redistribution. Control (left) and $BaxKO_{im}$ (right) conditions are illustrated at progressive time points (7, 21, 35 and 42 days), with t=0 being the day of tamoxifen-induced Cre recombination. There is a finite number of EC synapses defined at the beginning of the simulation, and synapses occupied by mature or immature GCs are portrayed as a percent of the total. Proliferation rate multiplied by survival determines the number of new GCs incorporating into the network on each day. As immature GCs age, they each increase in synaptic strength represented as the number of synapses relative to mature GCs. The sum of the number of immature GCs at each age multiplied by their synaptic strength determines the number of synapses appropriated by the immature population. The proliferation rate decreases steadily in both control and $BaxKO_{im}$ groups, and BaxKO GCs

have both increased survival and increased synaptic strength. **B.** Graph of total synapses occupied by mature and immature GCs using control or $BaxKO_{im}$ parameters between 0 and 43 days. There is a robust shift in the percent of synapses appropriated to immature GCs that initiates at t=14 when BaxKO GCs begin to acquire synapses. **C.** Experimentally measured change in EPSCs in mature GCs from $BaxKO_{im}$ mice (left axis) compared to the percent change in mature synapse number predicted by the simulation at time points t=36 through t=43 (right axis). Experimental data is the mean mature GC EPSC amplitude in $BaxKO_{im}$ mice normalized to control from each FV bin shown in Fig 2e. This simulation suggests that a redistribution of synapses to immature BaxKO GCs can account for the loss of synaptic transmission from mature GCs.

Supplemental Methods

Quantitative estimate of synapse redistribution

The purpose of the calculation is to predict the proportion of mature GC synapses that will be appropriated by immature cells over a 6-week time period in a control or $BaxKO_{im}$ DG. Time (t) is expressed in days, where t=0 represents the starting point when 8-week-old animals are injected with TMX. New GCs are continually added to an existing network comprised of mature and immature GCs. Each new GC gains synaptic strength beginning 2 weeks after cell birth[1-3], acquiring innervation from a finite pool of synapses with synaptic strength defined as the number of synapses per cell. The total number of GCs was initially set at 200,000 (unilateral cell count in the adult mouse DG[4]). The number of mature GCs was set at 95% of the total (190,000), while the initial number of immature cells was set at 5% of the total (10,000)[5]. The baseline number of mature GC synapses at t=0 was set at 100%, defined as 100 per cell, giving initial mature synapse number, S_M:

$$S_M = 100(190{,}000 \times 0.95)$$

We approximated the increase in synaptic strength, $Y(t)$, of developing GCs by fitting the amplitude of evoked EPSCs in immature GCs at progressive ages[2,3] by the equation:

$$Y(t) = 71.1 \ln(14 + t) - 187.7$$

For example, a 2-week-old control GC receives ~5% as many excitatory synapses as a mature GC, a 5-week-old GC contains ~65% as many excitatory synapses, and an 8-week-old GC achieves "mature" levels of 100% synaptic strength. To determine the initial number of immature synapses, $S_I(0)$, we divided the number of initial immature GCs by 43 (the number of days of maturation and thus the number of different synaptic strengths) and multiplied this quantity by the sum of all synaptic strengths:

$$S_I(0) = 10{,}000/43 \times \sum_{t=1}^{43} Y(t)$$

This result plus the initial number of mature synapses gives the total synapses in the system:

$$S_M + S_I(0)$$

which remains static throughout the simulation (~ 19.6 million).

To calculate the number of synapses appropriated by immature GCs each day, we considered cell proliferation $P(t)$, the rate of cell survival, and synaptic strength $Y(t)$. The rate of decrease in progenitor proliferation was defined by a best-fit equation[6], adjusted to give ~8000 progenitor cells at t=14, (stereological ki67 counts from 8-week-old mouse)[7], giving the available progenitor cell number, $P(t)$:

$$P(t) = 4 \times 10^6 (42 + t)^{-1.5}$$

The survival rate for new WT cells is 20%[8]. In the $BaxKO_{im}$ group, new GCs incorporating into the network at t=14 (2 weeks after TMX-induced recombination) have a survival rate of 70% (assuming partial efficiency of Cre expression)[9]. The number of immature GCs added to the system per day, $I(t)$, is:

$$I(t) = P(t) \times survival\ rate$$

All immature GCs will gain synaptic strength daily. The immature synapses appropriated each day, $S_I(t)$, is the cumulative sum of the surviving GCs times their respective synaptic strengths:

$$S_I(t) = (I(t) \times Y(1)) + (I(t-1) \times Y(2)) + (I(t-2) \times Y(3)) \ldots$$

Importantly, BaxKO GCs possess 35% more synapses than control due to lack of Bax-dependent synapse pruning (Supplemental Fig. 6).

In both groups, the cumulative number of immature synapses divided by the total synapses (multiplied by 100) equals the percent synapses appropriated by the immature population:

$$\%im = \frac{S_I(t)}{S_M + S_I(0)} \times 100$$

Since there is a static number of total synapses defined at the start of the simulation, the percent mature synapses remaining is:

$$\%mat = 100 - \%im$$

The synapses occupied by all groups across time is plotted in Supplemental Fig. 7b. Since the experiment is less than 8 weeks in duration, immature GCs never convert into mature GCs, and we did not account for the conversion of pre-existing WT immature GCs because that population would not differ between control and $BaxKO_{im}$ conditions. To calculate the predicted difference in mature synapse number in $BaxKO_{im}$ vs. control conditions, we took the ratio of $\%mat$ in $BaxKO_{im}$ to $\%mat$ in control at each time point from t=36 through t=43 (multiplied by 100).

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DISCUSSION

We have shown that altering neurogenesis alters the existing mature network through competition for excitatory inputs. While we used manipulation of the Bax gene initially as a tool to enhance neurogenesis, this project evolved over the last few years alongside the emerging field of "synaptic apoptosis," which addresses the localized actions of apoptotic machinery in the pruning of dendrites and synapses[61]. Consequently, the results of altering Bax in different GC populations have informed us about the mechanisms underlying activity-dependent survival and network plasticity in the DG. Importantly, as apparent from data shown in the main chapter and at the end of this discussion, different methods of altering neurogenesis are not created equal, and each one exerts unique changes on the DG milieu.

The results of this study indirectly address whether GCs require perforant path input to survive. Many studies have shown that synaptic activity promotes the survival of neurons. Inducing firing in cultured cortical cells prevents apoptosis triggered by external neurotoxic insults by suppressing apoptotic cascade proteins[62]. In the DG, newborn GCs with impaired NMDAR activation that exist next to normal newborn GCs have a higher rate of cell death[45], and increased sensory input from environmental enrichment increases both newborn GC survival and excitatory synaptogenesis[23]. Many have interpreted these findings as evidence that innervation by presynaptic terminals precedes cell death signals, and lack of innervation triggers apoptosis. In fact, when we began recording from animals with developmentally-generated Bax KO cells, we considered the possibility that

some mature GCs would not show an EPSC in response to perforant path stimulation. These would have been cells that were not selected to receive EC input during the critical period of development but lacked the mechanism to undergo apoptosis—morphologically normal, but receiving little or no excitatory drive. To our surprise, we never encountered a mature Bax KO GC that was not synaptically connected to the perforant path. This suggests that cell fate determination precedes innervation. In retrospect, it makes sense that absolute levels of afferent activity do not determine the targets of apoptosis, since spines initiate connections with existing boutons during synapse formation in the adult brain[47,51], and imbalances in activation but not global decreases in activity lead to pruning of cells and axons[45,46]. This means that EC axons do not discriminate, and as long as a GC can survive, it will be put to work. Thus, earlier determinants of cell fate may be major players in shaping the size and specificity of a circuit.

If all surviving GCs are innervated on a first-come-first-served basis, how would a massive accumulation of GCs, such as that seen in germline Bax KO mice, affect the adult network? With cell death completely blocked, there would be ~8000 cells added to the GCL per day (based on the number of ki67^{+} progenitor cells in one side of the DG in an adult mouse)[23]. 6-month-old germline Bax KOs have an unchanged density of perforant path synapses in the molecular layer, as measured by electron microscopy[40], indicating a finite number of terminals that is unaffected by age or number of efferents. We took simultaneous middle molecular layer field recordings and mature GC whole cell recordings in 3-month-old Bax KO mice and found that the overall number of synapses was the same (fEPSP slope vs. fiver volley, Fig. 1d), but the amount of excitatory input to individual mature GCs was larger than controls (EPSC vs. fiber volley, Fig. 1e),

consistent with a lack of Bax-dependent synaptic pruning. We assume this lack of synapse loss applies to all cells in the GCL, since they are all Bax KO.

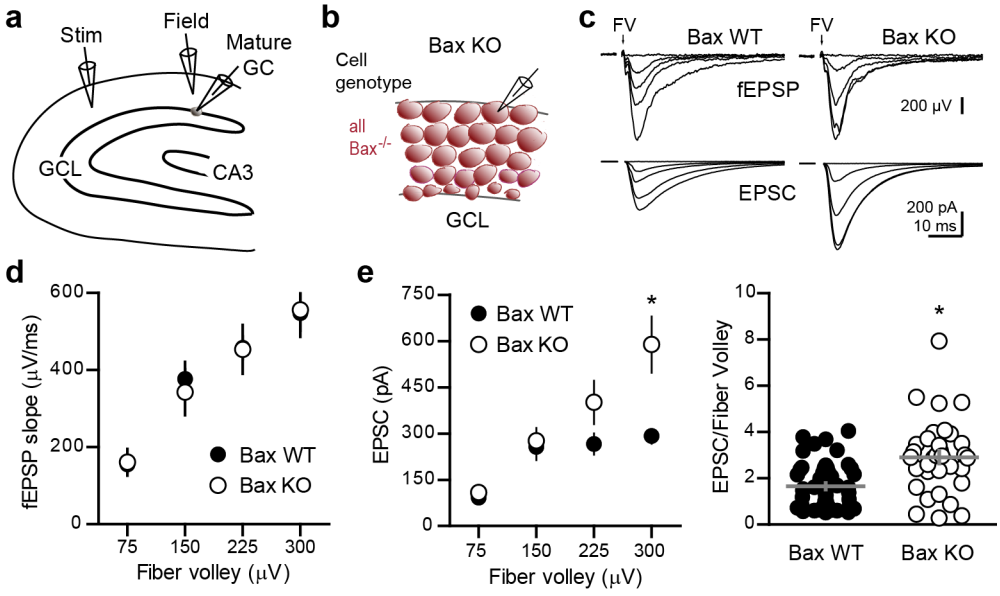

Figure 1. Mature GCs in germline Bax KO mice have higher excitatory transmission. **a,** Schematic showing the recording strategy for simultaneously measuring a dendritic field response and mature GC whole cell response while stimulating in the middle molecular layer. **b,** Cartoon showing genetically homogenous cell type in Bax KOs and the relative position of the patched GC in the GCL. **c,** Example traces from simultaneous field and mature GC whole cell recordings from Bax KO slices and controls (scale bars 5 ms, fEPSP 100 µV, EPSC 100 pA). **d,** The overall number of active synapses is unchanged between Bax KO and Bax WT slices, indicated by the fEPSP slope plotted against fiber volley amplitude in bins of 75 (p=n.s. two-way ANOVA, Bax WT n=7 cells, Bax KO n=6 cells). **e,** The amount of excitatory transmission from the perforant path onto individual mature GCs is increased in Bax KO slices, indicated by the higher mean EPSC plotted against fiber volley amplitudes in bins of 75 (p=0.0001 two-way ANOVA, bin 225-300 significantly different, Bonferroni post hoc test) and the average EPSC to fiber volley ratios from every cell at all stimulus intensities (control 1.66±0.14, n=49; Bax KO 2.92±0.27, n=34; p<0.0001 Student's t-test).

Since we have shown that new GCs gain synapses by appropriating terminals from older GCs, we would predict that once the EC terminals are occupied, adult-born GCs would not be able to incorporate into the excitatory network. This could lead to an accumulation

of GCs at an immature stage of development, unable to complete maturation without proper innervation. In fact, when comparing the numbers of newborn GCs (POMC-GFP$^+$ cells quantified by stereology) between Bax KO animals and controls at different ages, there is a 1.7-fold difference at 1 month, a 3-fold difference at 3 months and a 5.3-fold difference at 6 months (Fig. 2b).

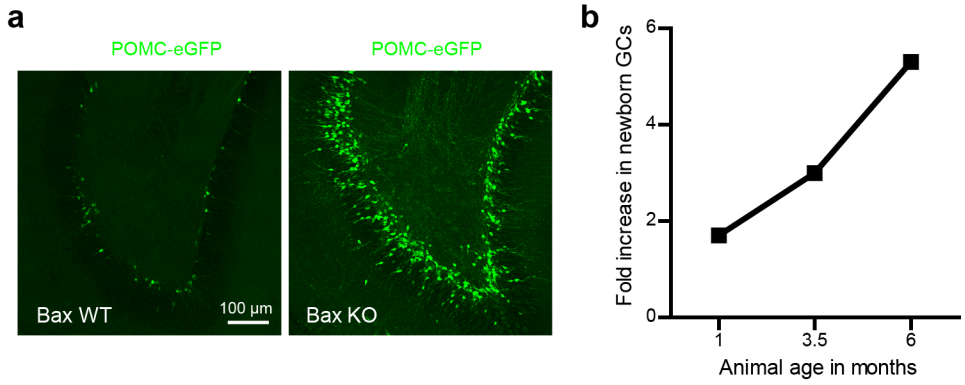

Figure 2. GCs increasingly accumulate at the newborn stage in older Bax KO mice. **a,** Confocal images showing the inflated presence of GFP$^+$ newborn GCs in the DG of a 3.5-month-old Bax KO animal. **b,** The fold increase in newborn GC number between Bax KO and Bax WT increases as animals age.

With age-dependent declines in the rates of proliferation[63] and cell death[39], the proportion of newborn GCs surviving compared to control should also decline. The dramatic increase in newborn GC number in older Bax KOs supports the idea that cells are not developing past the newborn GC stage, but recordings from immature GCs will need to be done in these mice to determine whether they are receiving excitatory input. Furthermore, impairments in circuit refinement and input specificity could start during development.

These network dysfunctions could contribute to the behavioral deficits exhibited by germline Bax KO mice. Several groups, including ourselves, have found that adult Bax KOs perform poorly on memory tasks such as the Morris Water Maze (Fig. 3a), even underperforming mice subjected to traumatic brain injury[41]. They are also hyperexcitable, as indicated by speed and distance traveled in the open field maze (Fig 3b).

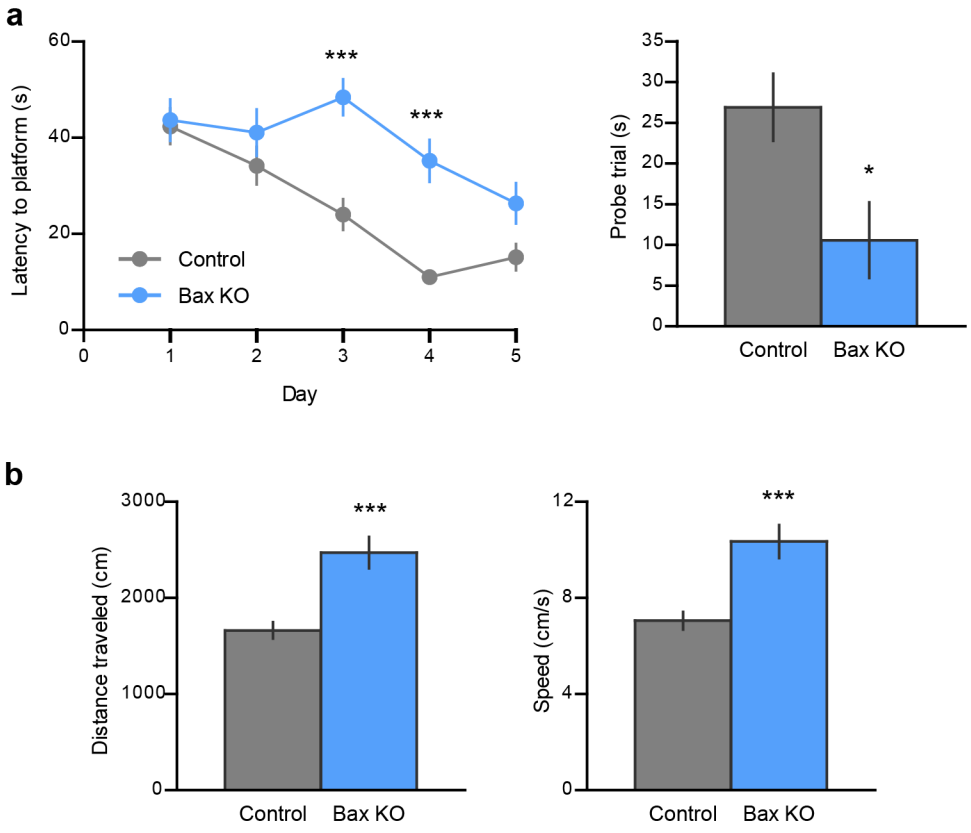

Figure 3. Bax KO mice are poor learners and exhibit hyperexcitability. **a,** 6-month-old Bax KOs took longer to find a hidden platform in the Morris Water Maze over a five-day training period ($p<0.0001$ two-way ANOVA, days 3 and 4 significantly different, Bonferonni post hoc test) and time spent in the target quadrant during a probe trial on the last day was lower ($p=0.03$, Student's t-test, Bax WT n=7, Bax KO n=5). **b,** Bax KOs show increased activity in the open field maze, both in distance traveled and speed ($p=0.0008$ and $p=0.001$, Student's t-test).

Because the change in cell density in these mice is most dramatic in the DG due to ongoing neurogenesis and cell death, we hypothesized that dentate-specific behavioral tasks may be affected in Bax KO mice. We used a spatial pattern separation task in which mice were trained to choose between two arms in an eight arm maze to find a food reward. The reward was always in the arm they were not exposed to immediately before (delayed non-matching), and the two arms were either separated by a large distance or small distance, the smaller distance being a more dependent on pattern separation ability (Fig. 4a).

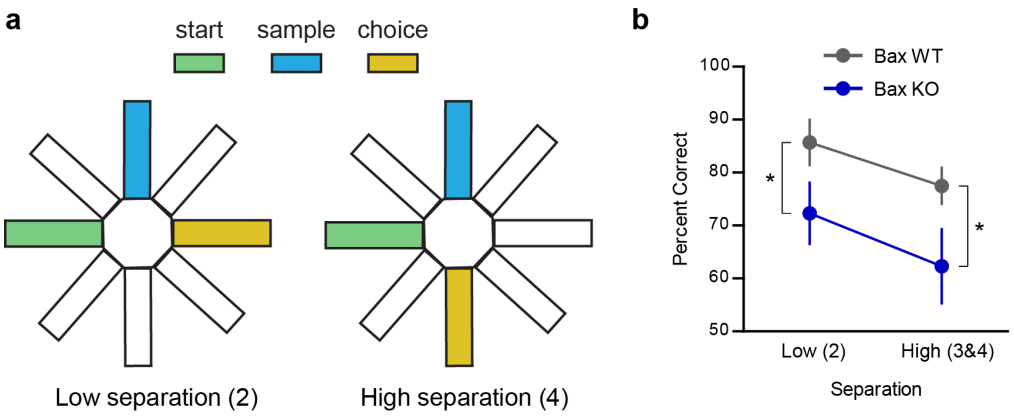

Figure 4. Bax KO mice show deficits in spatial memory and pattern separation. **a,** Examples of sample and choice arrangements in the eight-arm maze that constitute low separation (testing pattern separation) and high separation (testing spatial memory). **b,** Bax KO mice correctly chose the reward arm significantly fewer times than Bax WTs at both low and high separations (contingency Fisher's exact test, low, p=0.02, high, p=0.004).

Looking at the percent of correct choices, Bax KOs performed poorly at both large and small separations, indicating deficits in both pattern separation and general spatial memory (Fig. 4b). However, the KOs also acted distractable and unmotivated, often

taking upwards of five minutes to choose an arm, while controls took an average of 10 seconds. Interestingly, recent experiments in caspase-3 KOs showed increased activation of GCs in a novel environment (consistent with unpruned synapses) and impairment in tasks specifically designed to measure attention control and executive function[58], behaviors also attributed to the DG.

Enhancing adult neurogenesis by deleting Bax in progenitors creates an exaggerated environment of competition. Not only are certain GCs guaranteed to survive, but we see at least a 30% greater synaptic strength in immature GCs lacking Bax. Furthermore, since younger GCs receive depolarizing GABAergic input and excitatory hilar mossy cell input that is formative to their development, it is possible that heightened early innervation to Bax KO GCs accelerates their maturity and facilitates their selection into the network. Preventing cell death does not increase neurotrophic factors in the EC-hippocampal circuit. If anything, deleting Bax depletes existing neurotrophic factors. We were curious how this contrasted with another common method of enhancing neurogenesis, environmental enrichment with wheel running. Ten animals were placed in a larger cage with four running wheels and an assortment of shelters and other objects for 6 weeks (Fig. 5a). The objects were changed once a week to ensure a persistently novel environment. Electrophysiological recordings were performed in the same way as described in the main chapter of this thesis, by simultaneously recording a field potential in the middle molecular layer and whole cell current from a patched mature GC. Evoked EPSCs were dramatically enhanced in running/EE mice compared to mice kept in standard housing (Fig. 5b). Furthermore, there was a subtle but significant increase in number of total synapses (fEPSP slope vs. fiber volley, Fig. 5c).

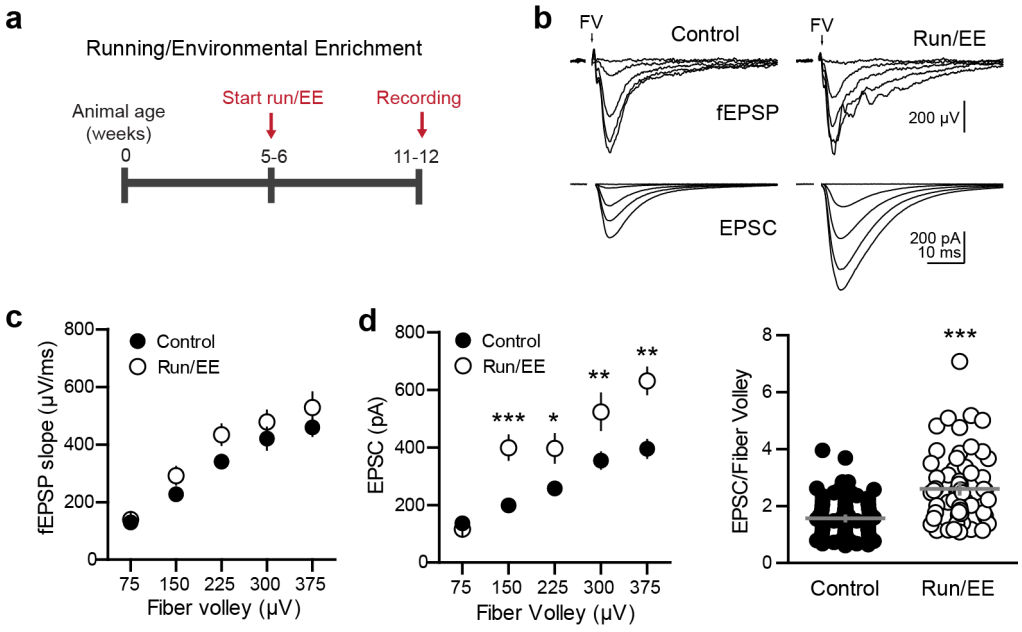

Figure 5. WT mice exposed to voluntary running and environmental enrichment (run/EE) show dramatically increased excitatory transmission to mature GCs. a, Mice were placed in EE at 5-6 weeks of age and sacrificed for experiments 6 weeks later. b, Example traces from control (standard housing) mice and run/EE showing simultaneous field and single-cell mature GC recordings (scale bars 10 ms, fEPSP 200 µV, EPSC 200 pA). **d,** The overall number of active synapses is higher in run/EE mice, indicated by the higher fEPSP slope plotted against fiber volley amplitude in bins of 75 (p=0.003 two-way ANOVA, control n=9 cells, run/EE n=10 cells). **e,** The amount of excitatory transmission from the perforant path onto individual mature GCs is increased in run/EE slices, indicated by the higher mean EPSC plotted against fiber volley amplitudes in bins of 75 (p=0.0001 two-way ANOVA, last four bins significant, Bonferroni post hoc test) and the average EPSC to fiber volley ratios from every cell at all stimulus intensities (control 1.6±0.07, n=88; run/EE 2.6±0.16, n=58; p<0.0001 Student's t-test).

What does this mean for the competition between immature and mature synapses? Many groups have shown an infusion of BDNF into the DG and increase in spine density on mature GCs after voluntary running. Would the immature GCs in this case have fewer spines because EC boutons are being occupied by mature GCs? Or does the increase in neurotrophic factors allow new terminals to be formed that can accommodate increases in both mature and immature synapses? A likely explanation is that standard mouse housing represents a form of sensory deprivation, and when presented with a stimulating

environment, baseline neurogenesis and activity increases. This is supported by the fact that running also increases spine density in the EC and CA1[64]. We predict that if Bax was eliminated from immature GCs in running/EE mice and compared to WT running/EE mice, there would still be a reduction in excitatory input to mature GCs from this new baseline. What is important to take away from these results is that altering neurogenesis modifies every part of the DG circuit, and different manipulations alter the circuit in distinct ways. Being aware of how immature and mature populations operate in balance during each manipulation will facilitate the discovery of the DG's overall function.

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APPENDIX

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 22-Jan-2015

TO: Wadiche, Linda S.

FROM: 

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 22-Jan-2015.

Protocol PI: Wadiche, Linda S.

Title: Newborn Neurons in the Adult Hippocampal Network

Sponsor: National Institute of Neurological Disorders and Stroke/NIH/DHHS

Animal Project Number (APN): IACUC-10134

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
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