Low excitatory innervation balances high intrinsic excitability of immature dentate neurons

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Persistent neurogenesis in the dentate gyrus produces immature neurons with high intrinsic excitability and low levels of inhibition that are predicted to be more broadly responsive to afferent activity than mature neurons. Mounting evidence suggests that these immature neurons are necessary for generating distinct neural representations of similar contexts, but it is unclear how broadly responsive neurons help distinguish between similar patterns of afferent activity. Here we show that stimulation of the entorhinal cortex in mouse brain slices paradoxically generates spiking of mature neurons in the absence of immature neuron spiking. Immature neurons with high intrinsic excitability fail to spike due to insufficient excitatory drive that results from low innervation rather than silent synapses or low release probability. Our results suggest that low synaptic connectivity prevents immature neurons from responding broadly to cortical activity, potentially enabling excitable immature neurons to contribute to sparse and orthogonal dentate representations.
The dentate gyrus (DG) has long been associated with the computational task of pattern separation, or the transformation of similar input patterns to output patterns that are less correlated. Sparse population coding is an essential component of pattern separation because the storage capacity for neural activity patterns is inversely related to the proportion of active principal neurons in a network. Accordingly, sensory stimulation or spatial memory tasks activate only a small fraction of DG granule cells (GCs) (<5%). Theories of DG pattern separation propose that strong inhibition selects small and distinct populations of active GCs in a manner that amplifies slight differences in inputs. Remarkably, manipulating the small population of adult born GCs is sufficient to alter behaviours that require discrimination of similar contexts, leading to the idea that adult born neurons have an important role in pattern separation. However, it is unclear how GCs of various developmental stages contribute to DG network function.

One way that newly generated GCs may contribute to DG function is if their distinct physiological properties confer unique contributions to coding processes. Ex vivo studies have shown that the morphological, intrinsic and synaptic properties of newly generated GCs undergo a protracted process of maturation during which immature GCs could transiently perform distinct network functions. Much attention has focused on a period when immature GCs are synaptically integrated within the circuit and also exhibit high intrinsic excitability that allow spiking in response to small current injections as well as distinctive integrative properties. In this developmental stage that occurs ~4 weeks after cell birth, immature GCs also exhibit less synaptic inhibition than mature GCs, and afferent stimulation preferentially generates spiking in (that is, ’recruits’) immature GCs over mature GCs. Thus, in comparison to mature GCs, immature GCs appear to be highly excitable and broadly responsive, acting as good integrators of afferent activity.

However, it is not clear how the physiological properties of immature GCs identified ex vivo contribute to putative higher order functions in vivo. From a theoretical standpoint it is surprising that broadly responsive neurons contribute to the computational task of pattern separation since neurons that act as integrators reduce sparse population coding. In fact, inclusion of excitable immature GCs in a realistic network model degrades rather than improves pattern separation. Furthermore, the contribution of immature GCs to network activity in vivo has been difficult to assess. Preferential recruitment of immature GCs in vivo was reported using cFos as a proxy for neural activation, but this conclusion has been disputed. In vivo recordings from the DG of rodents has identified distinct functional populations with overall sparse patterns of activity in presumed principal cells. The interpretation of how immature GCs contribute to this activity has been conflicting, ranging from the possibility that immature GCs make up the entire population of active DG neurons to more recent evidence that mature GCs are predominantly active during memory encoding.

At developmental stages when intrinsic excitability is high, immature GCs also exhibit features that suggest low glutamatergic synaptic connectivity, including small dendritic arbor, low spine densities and small evoked excitatory postsynaptic currents (EPSCs). Here we assess the role of synaptic connectivity in recruiting spiking in mature and immature GCs. Our results demonstrate that low excitatory connectivity from the entorhinal cortex (EC) prevents excitable immature GCs from spiking in response to afferent activity that is sufficient to generate spiking in mature GCs. Although immature GCs can spike with fewer active inputs than mature GCs (challenging the specific role of immature GCs in disambiguating input patterns), low innervation predicts that immature GCs sample a smaller component of EC afferent activity and thus exhibit lower correlations in synaptic inputs. Incorporating these results into a simple network model reveals that the combination of high excitability and low synaptic connectivity potentially provides an unexpected computational advantage wherein immature GCs enhance the range of EC activity levels that can be maintained with well-separated output representations.

**Results**

**Immature GC spiking is limited by low excitatory drive.** In adult hippocampal slices, immature GCs that are approximately 4 weeks post mitosis are more likely than mature GCs to spike in response to stimulation in the molecular layer (ML), a paradigm in which synchronized perforant path excitatory drive is above spike threshold hence spiking is largely determined by synaptic inhibition and almost all cells spike when inhibition is blocked. However, synchronous stimulation of a branch of perforant path axons in the ML may not provide excitatory drive representative of neuronal activity of EC projection neurons. Indeed, whole-cell (WC) recordings from mature GCs in vivo show a constant barrage of θ-modulated asynchronous EPSCs arising from EC that is associated with infrequent spiking. We thus sought to compare the relative spiking probably of mature and immature GCs in response to more diffuse afferent activity provided by direct stimulation of EC.

We used NestinCreER<sup>T2</sup> or NestinCreER<sup>TM4</sup> mice crossed with Ai14 Cre reporter mice at 30–36 days post tamoxifen injection to identify immature GCs (Fig. 1a). Consistent with prior characterization of intrinsic and synaptic maturation of GCs in NestinCreER<sup>T2</sup> mice, at this interval tdTomato (tdT)-labelled (immature) GCs displayed repetitive spiking in response to current injections (Fig. 1b,c), a characteristic that develops after 3 weeks of neuronal maturation in retroviral labelled GCs. Other intrinsic properties of immature GCs were consistent with ~4-week-old GCs identified by retroviral labelling, whereas unlabelled GCs with fully mature membrane properties were classified as ‘mature’ (Fig. 1b,c). The persistence of some immature intrinsic properties in GCs recorded up to 36 days post tamoxifen injection is consistent with the slow rate of maturation in the ventral hippocampus from mice housed under standard conditions.

We examined spiking probability in response to simultaneous stimulation of the medial and lateral EC (MEC/LEC) across a range of stimulus intensities (Fig. 2a,b). This paradigm allows activation of the perforant path while avoiding direct stimulation of local interneurons, and also mimics GC integration of spatial and sensory information arising in the MEC and LEC, respectively. We previously showed that focal stimulation in the MEC alone generates EPSCs with paired-pulse depression whereas LEC stimulation evokes EPSCs with paired-pulse facilitation, and the amplitude of dual-pathway evoked EPSCs are nearly the sum of the individual EPSCs suggestive of independent pathways. MEC/LEC stimulation generated spikes in 16% of mature GCs (5/32 cells) and blocking inhibition with gabazine increased the percentage of mature GCs that spiked to 50% (16/32 cells; Fig. 2b). Since MEC/LEC-evoked IPSCs are entirely blocked by NBQX, the gabazine-induced increase shows that feed forward inhibition contributes to GC sparse population activity. Yet the fact that only 50% of GCs spiked in gabazine also shows that excitatory drive is a limiting factor for GC spiking in this stimulating paradigm, unlike stimulation in the ML, in which essentially all nearby GCs spike when inhibition is blocked. Confirming this idea, stimulation in MEC/LEC...
evoked smaller EPSCs and excitatory postsynaptic potentials (EPSPs) compared with ML stimulation, presumably due to the spread of fibres from the distal location of the stimulating electrodes and cut fibres in the slice (Supplementary Fig. 1).

Since many initial attempts to evoke synaptic responses in immature GCs by MEC/LEC stimulation were unsuccessful (not shown), we first identified synaptic input to a mature GC and then, without moving the stimulating electrodes, recorded from a neighbouring immature GC. Using this sequential analysis, then, without moving the stimulating electrodes, recorded from a mature GC and then, without moving the stimulating electrodes, recorded from a neighbouring immature GC. Using this sequential analysis, we found that EPSCs evoked by the same MEC/LEC stimuli were dramatically smaller in immature GCs (Fig. 2c,d), and some immature GCs failed to respond altogether (n = 4/18; Supplementary Fig. 2a). Interestingly, EPSCs in immature GCs elicited smaller EPSPs in immature GCs (amplitude increased from 6.7 ± 1.3 mV to 8.1 ± 1.4 mV, n = 6, P = 0.03, Wilcoxon test), but still failed to elicit spikes despite the ability of immature GCs to spike with current injections (Fig. 2f). Thus, enhanced intrinsic excitability of immature GCs does not fully compensate for reduced excitatory drive in this paradigm. Importantly, sequential recordings from two mature GCs using the same paradigm resulted in identical EPSCs/EPSPs in the second mature GC, confirming that neighbouring mature GCs were sampling synaptic inputs from the same population of active fibres and that small EPSCs in immature GCs did not result from optimizing the stimulation for the first mature GC or other experimental bias (n = 9 pairs of mature GCs; Supplementary Fig. 3). Again, 3/18 (16.6%) of these mature GCs displayed spiking, confirming the spiking probability of mature GCs in this paradigm.

The small EPSCs in immature GCs and failure to spike under conditions where mature GCs could be recruited to spike suggests that synaptic connectivity plays a crucial role in selecting active
GCs. But to rule out the possibility that our WC recordings altered spiking behaviour by disrupting the intracellular milieu, we also examined spiking using noninvasive cell-attached (CA) recordings. Since we could not assess excitatory drive (synaptic responses) using CA recordings, we first used WC recording from a mature GC to confirm effective MEC/LEC stimulation. Then, without moving the stimulating electrodes, we assessed spiking using sequential CA recordings from multiple GCs within the field of view. In nine experiments where we evoked relatively large EPSCs monitored by WC recordings from mature GCs, we made a total of 57 CA recordings from nearby immature and mature GCs (Fig. 3a,b; note that there are many more mature GCs than immature GCs in each field of view). Similar to the WC recordings, 22% of mature GCs (9/41) exhibited spikes in CA mode, whereas none of the immature GCs displayed spikes (0/16; $\chi^2 = 4.1, P = 0.041$). The WC and CA results were not different, so we pooled all experiments to illustrate that immature GCs were less likely than mature GCs to spike in response to MEC/LEC stimulation (0/34 and 17/74, respectively, $P = 0.006$; Fig. 3b).

Some immature GCs could spike in response to current injection, the failure to spike in response to EC stimulation resulted from insufficient excitatory depolarization. Indeed, comparing EPSPs and spiking probability illustrated that EPSPs in immature GCs generated by MEC/LEC stimulation were too small to achieve threshold (Supplementary Fig. 1c). Thus low excitatory drive can prevent spiking of immature GCs in response to MEC/LEC stimulation. The lack of spiking in this stimulating paradigm, however, does not mean that immature GCs fail to spike to any stimulus. In fact, preferential afferent-induced spiking of immature GCs indicates that they spike efficiently when they receive sufficient excitatory drive$^{18,20,22}$. Rather, these results suggest that differential synaptic connectivity contributes to the spiking probability of mature and immature GCs.

Reduced excitatory drive monitored by AMPA and NMDA EPSCs. A potential caveat to the idea that immature GCs have less excitatory innervation than mature GCs is that newly generated GCs have silent synapses and a high ratio of N-methyl D-aspartic acid receptors (NMDARs) to $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPArs) that may underestimate synaptic connectivity measured exclusively by AMPAR EPSCs$^{36,37}$. We therefore assayed perforant path excitatory drive mediated by both AMPAR and NMDARs using simultaneous recordings of immature and neighbouring mature GCs during focal stimulation in the ML. In the presence of gabazine, we recorded AMPAR EPSCs at $-70 \text{ mV}$, using the depressing and facilitating paired-pulse ratio (PPR) to confirm medial perforant path (MPP) or lateral perforant path (LPP) stimulation, respectively.$^{22}$ Consistent with Fig. 2, AMPAR EPSCs in immature GCs were smaller than in neighbouring mature GCs, for both MPP and LPP stimulation (Fig. 4a). Since the number of fibres activated by the stimulating electrode was the same for each mature/immature GC pair, the smaller EPSCs in immature GCs likely reflect fewer active synapses. We also blocked AMPARs with NBQX and found that NMDAR EPSCs recorded at $-40 \text{ mV}$ were likewise smaller in immature GCs during simultaneous recordings (Fig. 4b). Thus, low excitatory drive of immature GCs is apparent with both NMDAR EPSCs as well as AMPAR EPSCs. To assess potential silent synapses, we quantified the NMDAR/AMPAR ratio by comparing AMPA EPSCs at $-70 \text{ mV}$ and NMDAR EPSCs at $+40 \text{ mV}$ in the same cells during simultaneous mature and immature GC recordings. For MPP stimulation the ratio was significantly higher for immature GCs, consistent with a higher proportion of NMDAR to AMPARs on developing GC dendrites$^{36,37}$ (Fig. 4c; $n = 8$, $P = 0.003$; and Tukey’s post-test $P < 0.05$; **$P < 0.01$).

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Release probability at mature and immature synapses. Low excitatory drive to immature GCs could result either from fewer perforant path synapses (reduced innervation) or from low release probability \( P_r \) across a similar number of synapses. To differentiate these possibilities, we compared \( P_r \) of perforant path synapses using the blocking rate of the NMDAR-EPSC by the irreversible open-channel blocker MK801 (ref. 38). After establishing a baseline of NMDAR EPSCs recorded at \(-40\) mV (stimulating either the MPP or LPP in NBQX and gabazine), we applied MK801 (40 \( \mu \)M) for 5 min and then resumed stimulation to compare the rate of EPSC block at immature and mature synapses (Fig. 5). Repeated synaptic stimulation in the presence of MK801 provides a relative measure of \( P_r \), since synapses with high \( P_r \) are blocked faster than synapses with low \( P_r \). The progressive block rate of NMDAR EPSCs was best described by two exponentials that we used to calculate a weighted decay time constant \( \tau_w \). For stimulation in the MPP, \( \tau_w \) of NMDAR EPSCs in immature GCs was \( 15.8 \pm 2.1 \) ms compared with \( 24.0 \pm 1.9 \) ms in mature GCs \( (n = 8 \) each; unpaired \( t \)-test \( P = 0.028 \)). The increased \( \tau_w \) resulted from an increase in \( \tau_{fast} \) with no change in \( \tau_{slow} \) (Fig. 5a, inset; \( \tau_{slow} : 32.5 \pm 3.7 \) compared with \( 37.2 \pm 6.1 \), \( n = 8 \), \( P = 0.32 \) unpaired \( t \)-test), similar to what has been observed at immature synapses in the developing hippocampus (Fig. 5b, inset; \( \tau_{fast} : 19.9 \pm 1.8 \) and \( 27.5 \pm 9.1 \) ms, \( n = 8 \) each, \( P = 0.44 \); \( \tau_{fast} : 9.4 \pm 2.6 \) versus \( 9.6 \pm 2.8 \), \( P = 0.98 \); \( \tau_{slow} : 35.4 \pm 5.2 \) versus \( 42.7 \pm 5.7 \), \( P = 0.34 \) unpaired \( t \)-tests). These results suggest that the release probability is higher rather than lower at immature MPP synapses. One potential caveat is that the MK801 blocking rate could be affected by different NMDAR subunit composition, since developing GCs have enriched expression of synaptic NMDAR2B receptors (Fig. 5b). However, the 2B-specific antagonist R0-256981 (1 \( \mu \)M) blocked MPP-evoked EPSCs in mature and immature GCs by a similar degree (27% in mature and 32% in immature GCs; \( n = 3 \), \( P = 0.7 \) Wilcoxon–Mann–Whitney test) and the MK801-induced acceleration of the EPSC decay \( \tau_r \), a measure of receptor open probability, was similar in mature and immature GCs (reduced by 31% in mature and 20% in immature GCs; \( n = 8 \), \( P = 0.3 \) paired \( t \)-test). Thus, immature GCs in our experiments have attained a largely mature complement of NMDARs.

The PPR also provides a relative measure of \( P_r \) that can be assayed by AMPARs, and we found no differences in the PPR of AMPAR EPSCs in immature and mature GCs. The PPR of MPP-evoked EPSCs in mature GCs was \( 0.89 \pm 0.03 \) compared with \( 0.84 \pm 0.03 \) in immature GCs \( (n = 10 \) each, \( P = 0.3 \) paired \( t \)-test), and the PPR of LPP-evoked EPSCs in mature GCs was \( 1.16 \pm 0.05 \) compared with \( 1.22 \pm 0.08 \) in immature GCs \( (n = 10 \), \( P = 0.4 \) paired \( t \)-test). Thus, the PPRs and MK801 blocking rates show that limited excitatory drive to immature GCs does not result from low \( P_r \) at a similar number of synapses.

Low overlap in perforant path synaptic inputs. As previously reported (Fig. 5a, inset; \( \tau_{slow} : 32.5 \pm 3.7 \) compared with \( 37.2 \pm 6.1 \), \( n = 8 \), \( P = 0.32 \) unpaired \( t \)-test), similar to what has been observed at immature synapses in the developing hippocampus (Fig. 5b, inset; \( \tau_{fast} : 19.9 \pm 1.8 \) and \( 27.5 \pm 9.1 \) ms, \( n = 8 \) each, \( P = 0.44 \); \( \tau_{fast} : 9.4 \pm 2.6 \) versus \( 9.6 \pm 2.8 \), \( P = 0.98 \); \( \tau_{slow} : 35.4 \pm 5.2 \) versus \( 42.7 \pm 5.7 \), \( P = 0.34 \) unpaired \( t \)-tests). These results suggest that the release probability is higher rather than lower at immature MPP synapses. One potential caveat is that the MK801 blocking rate could be affected by different NMDAR subunit composition, since developing GCs have enriched expression of synaptic NMDAR2B receptors (Fig. 5b). However, the 2B-specific antagonist R0-256981 (1 \( \mu \)M) blocked MPP-evoked EPSCs in mature and immature GCs by a similar degree (27% in mature and 32% in immature GCs; \( n = 3 \), \( P = 0.7 \) Wilcoxon–Mann–Whitney test) and the MK801-induced acceleration of the EPSC decay \( \tau_r \), a measure of receptor open probability, was similar in mature and immature GCs (reduced by 31% in mature and 20% in immature GCs; \( n = 8 \), \( P = 0.3 \) paired \( t \)-test). Thus, immature GCs in our experiments have attained a largely mature complement of NMDARs.

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Low overlap in perforant path synaptic inputs. As previously reported, we found that the frequency of mEPSCs in immature
GCs was lower than in mature GCs (0.52 ± 0.15 Hz versus 1.65 ± 0.25 Hz, n = 5, P = 0.008) with no difference in amplitude (6.25 ± 0.58 pA versus 6.88 ± 0.84 pA, P = 0.87). Together these results indicate that immature GCs receive less innervation from the perforant path compared with mature GCs, further suggesting that immature GCs retain high synaptic specificity since they sample only a fraction of the afferent axons arising from the EC population. To further assess this idea, we tested the ability to evoke EPSCs in immature and mature GCs using a modified paradigm previously used to define fine-scale specificity of cortical synaptic connectivity.42 We compared the probability of evoking EPSCs in simultaneously recorded pairs of GCs, using low-intensity stimulation of the MPP or LPP to activate small numbers of perforant path axons. We identified a stimulation location where focal stimulation (2 μA) was just sufficient to reliably evoke an EPSC in one GC, and then we quantified the percentage of trials where an EPSC was generated in the second GC as a function of stimulus intensity (Fig. 6a), where the % simultaneous success is defined as (number of trials with an EPSC in both cells/total number of trials) × 100. For recordings from two mature GCs or a mature and an immature GC, somata were located within 80–120 μm of each other. For pairs of mature GCs, increasing the stimulus intensity steeply increased the percentage of simultaneous successes (Fig. 6b,c, black symbols). This suggests that a largely overlapping population of afferent fibres innervate distinct mature GCs, that is, mature GCs have low synaptic specificity because there is high probability of activating fibres that synapse onto both cells as the stimulus intensity is increased. However, the percentage of simultaneous successes was significantly lower for pairs of a mature GC with an immature GC (Fig. 6b, teal symbols). At the lowest stimulus intensity, EPSCs were always observed in mature GCs with failures in immature GCs, consistent with lower innervation of immature GCs. There was no difference in the latency of EPSCs and the amplitude of EPSCs increased linearly with stimulation intensity, suggesting that increased stimulation recruited additional inputs to both cells rather than a separate population of inputs to the second cell (Supplementary Figs 4 and 5). The similar amplitude of successes at the lowest stimulus intensity also suggests that postsynaptic sensitivity (that is, receptor number) at immature synapses does not account for reduced excitatory drive (Supplementary Fig. 4), in accordance with the similarity of mEPSC amplitudes noted above. Furthermore, pairs of two immature GCs with somata within 80 μm of each other likewise displayed lower percentage of simultaneous successes compared with mature GC pairs within the same slices for both MPP and LPP stimulation, confirming that the overlap in synaptic input to immature GCs is lower than the overlap in input to mature GCs (Fig. 6c). Finally, we found that simultaneous recordings of medial and lateral perforant path-evoked EPSCs between immature GCs at 39–52 days post tamoxifen treatment and neighbouring mature GCs displayed similar % successes as two mature GCs (Supplementary Fig. 6). As described previously, many immature GCs at this later developmental stage exhibit excitatory synaptic currents, intrinsic excitability and spiking behaviour that approaches mature values.22 Thus as synaptic innervation progresses across the first 2 months of new GC maturation,21 overlap in synaptic input also increases.

Reduced overlap in synaptic inputs could occur either if immature GCs receive fewer synaptic contacts per EC fibre, or if immature GCs receive innervation from a smaller number of fibres. It is difficult to discriminate these possibilities because the large variance of quantal parameters in GCs obscures quantal analysis of evoked EPSCs.43 However, we favour the latter option because the former requires that unitary EPSCs in mature GCs are generated at synapses comprised of many release sites. The small amplitude of K+ -evoked unitary EPSCs from perforant path is consistent with only a few release sites,35 supporting the idea that excitatory projection cells typically innervate each other at a small number of sites.43,44 Furthermore the amplitude of eEPSCs in mature GCs was similar to the amplitude of mEPSCs (in the same cells, P = 0.1, paired t-test, n = 10), and the small amplitude of low-intensity-evoked EPSCs (Supplementary Fig. 5) further support small numbers of release sites per fibre. Thus, we predict that that immature GCs sample the activity of fewer perforant path fibres (and EC projection neurons) than mature GCs.

**Simulation of distinct connectivity in network functions.** It is generally thought that DG contributes to hippocampal memory encoding by orthogonalizing cortical activity patterns using very sparse population coding. Paradoxically, immature GCs with high...
Intrinsic excitability and low inhibition (that are preferentially recruited byafferent activity) are expected to reduce population sparseness and degrade pattern separation. Our results suggest that low excitatory innervation could limit the recruitment of immature GCs into active neural ensembles, thereby counteracting neurogenesis-induced degradation of orthogonalization. To test this idea, we fit the experimental data shown in Fig. 6 to a simple statistical model designed to assess the overlap in GC output patterns across different levels of EC input (Fig. 7; see Methods). To isolate the contribution of differing levels of excitatory connectivity, the model did not include inhibition (which is known to differ between mature and immature GCs). We assumed that the same aged GCs have equivalent number of synapses that sample input from the same total set of perforant path fibres (with $P_e = 1$). We constrained the ratio of excitatory connectivity (modelled as the number of synapses) to GCs according to the average ratio of EPSC amplitudes that we measured in simultaneous recordings of immature and mature GCs across a large range of stimulus locations and intensities (0.35; Fig. 7a). The EPSC amplitude depends on the number of fibres activated by the stimulating electrode, the release probability of each fibre, the quantal size and the number of active fibres that innervate each cell. In these experiments, the number of fibres activated by the stimulating electrode, the presynaptic release probability and mEPSC amplitude are the same for mature and immature GCs, thus the relative size of EPSCs reflects the likelihood that activate fibres innervate each cell. Assuming random connectivity, binomial statistics can be used to estimate the density and overlap of synaptic connectivity for mature and immature neurons. Using a fitting approach (see Methods), we observed strong fits with a $P_{\text{min}}$ of 0.39% and $N_{\infty}$ of 1,296 fibres for MPP stimulation. For mature GCs, we estimated an average of 219 MPP inputs, of which 37 were shared between pairs of neurons, and for immature GCs we estimated 77 inputs, of which five were shared (note that this analysis is meant to replicate our experimental paradigm rather than to recapitulate total synapse number). Most combinations of input values with good fits provided similar outcomes (the top 20% of random parameter fits are shown in Supplementary Table 1). We then generated networks of neurons obeying these statistics, and observed that the randomly connected neurons exhibited comparable overlapped synaptic inputs as observed experimentally (Fig. 7b). Next, we generated a simple network similar to that used previously to simulate DG function, whereby different GC neurons had connection densities representative of either all mature, all immature or a mixture of both. These networks shared input connection statistics comparable to the observed slice results. To incorporate the higher intrinsic excitability of immature GCs, we dictated that each GC would fire if 20% of their synapses were active, allowing immature GCs to fire with lower numbers of active synaptic inputs.

Our results and others suggest that relative spiking of mature and immature GCs depended on the strength of the input, thus we did not vary the input correlations (keeping the inputs random) but rather examined the effects of different input levels on the output correlations. Using this approach, we assessed how differing levels of afferent stimulation (corresponding to different levels of EC activity) affects the overlap in GC output. Consistent with previous modelling, networks with immature neurons exhibited higher correlations (reduced orthogonality) than networks without neurogenesis when the input activity was on average below threshold for GCs to fire (dotted line in Fig. 7c). Recruitment of excitable immature GCs decreases sparseness and increases output overlap, apparently detrimental to the proposed role of immature GCs pattern separation. In this input range, the response curve of the mature only network was steep; if EC activity was well below threshold, the mature-only DG could orthogonalize within a limited range of active EC inputs, but the network became ineffective as threshold was approached. Interestingly, networks of all immature neurons more gradually increased overlap as input activity approached threshold, and also displayed reduced overlap at higher levels of EC activity. Thus, excitable but poorly connected immature neurons are less sensitive to changes in input levels than can be maintained with low overlap in outputs, we further tested how the network responded across a large spectrum of young neuron densities (0–100% immature neurons) with EC activity levels (0.1–0.22 of EC neurons active). This analysis requires that we define a tolerable range of overlap, which we set as the difference between the EC activity level that provided at least a normalized dot product (NDP) of 0.005 and the EC level that provided at least an NDP of 0.05 (that is, between 0.5 and 5% overlap). Although somewhat arbitrary, this low range of overlap is consistent with the generally accepted idea that the point of pattern separation in the DG is to provide near-orthogonal inputs to downstream CA3 (ref. 10).

Figure 7d illustrates the responses of four networks with different fractions of immature neurons with the tolerable range...
**Figure 6 | Less overlap of synaptic inputs to immature GCs.** (a, top) Reconstructions of two mature GCs (black, left) or a mature and an immature GC (teal, right) showing the approximate placement of the stimulating electrode. Scale bar, 50 µm. (bottom) The percentage of trials with simultaneous EPSCs in both cells (% simultaneous success) was measured at increasing stimulus intensities (20 trials at each intensity). Examples are from MPP stimulation. Scale bar, 20 pA, 40 ms. (b) The percentage simultaneous success versus stimulus intensity is shown for MPP stimulation (left) and LPP stimulation (right). The percentage simultaneous success for pairs of mature with immature teal symbols) was different from pairs of two mature GCs (black symbols) at multiple stimulation intensities. This suggests more active fibres were required to recruit overlapping synaptic inputs in pairs with an immature GC. Repeated measure two-way ANOVA, MPP, n = 8–11 cell pairs per group: factor cell age, F(1,17) = 14.6, P = 0.0013; factor stimulus, F(9,153) = 173.2, P < 0.0001; Interaction, F(9,153) = 10.6, P < 0.0001; LPP, n = 6–7 cell pairs per group: factor cell age, F(1,11) = 6.3, P = 0.04; factor stimulus, F(9,99) = 98.7, P < 0.0001; Interaction, F(9,99) = 3.2, P = 0.031. (c) The % simultaneous success for pairs of two immature GCs (teal) was different from pairs of two mature GCs (black symbols) at multiple stimulation intensities, indicated less overlap in synaptic inputs between immature GCs. Repeated measure two-way ANOVA, MPP, n = 10–8 cell pairs per group: factor cell age, F(1,16) = 16.1, P = 0.001; factor stimulus, F(9,144) = 131.9, P < 0.0001; and interaction, F(9,144) = 4.8, P = 0.0001. LPP, n = 9–7 cell pairs per group: factor cell age, F(1,14) = 16.7, P = 0.001; factor stimulus, F(9,126) = 138.2, P < 0.0001; and interaction, F(9,126) = 4.8, P < 0.0001. ANOVA, analysis of variance. Inner molecular layer (IML); middle molecular layer (MML); outer molecular layer (OML).
of NDP highlighted by blue shading. Networks without immature neurons showed a small range of allowable input levels, with little difference between the level of EC inputs that was insufficient to drive DG activity and levels that induced high correlations. In contrast, networks of all immature neurons and those with equal mix of immature and old showed a larger range of allowable input levels given the more gradual recruitment of immature neurons into activated populations. Notably, the greatest range of allowable input levels was observed for networks that were mostly mature with a small fraction of immature neurons. In effect, these networks captured the best of both populations; the upper bound of permissible input level was increased by the lower excitability of the mature neurons and the gradual recruitment of immature neurons, whereas the lower bound of permissible inputs was reduced by excitable immature neurons that can be recruited by the small number of active inputs. This effect is seen more directly when the tolerable range of inputs for all neurogenesis levels from 0 to 100% are compared (Fig. 7e).

**Discussion**

Here we assess the role of excitatory drive in afferent-induced spiking of immature and mature GCs. First, we show that the relative probability of perforant path-induced spiking depends on the stimulus paradigm. Although strong beam-like stimulation paradigms with supra-threshold excitatory drive generate preferential spiking of immature GCs because of their reduced inhibition, we found that weaker (and potentially diffuse) stimuli preferentially recruited mature GCs. Our results suggest that low excitatory drive from the perforant path provides a previously underappreciated mechanism that prevents broad responsiveness of immature GCs. Second, we show that low excitatory drive to immature GCs results from less innervation rather than functional differences at immature synapses. Low innervation is consistent with the low frequency of mEPSCs and low spine density in retroviral labelled immature GCs as well as the small dendritic trees of transgenic-labelled immature GCs. Finally, we extend our experimental results to predict how poorly connected immature GCs could contribute to network functions. Using a simple statistical model, we show that excitable immature GCs with low innervation enhance pattern overlap at low input levels yet decrease pattern overlap at high input levels, potentially enhancing the range of input levels that can maintain well-separated output representations. Together these results suggest that low innervation counteracts high intrinsic excitability and contributes to distinct input–output transformations than expected for high excitability alone.

Our results suggest that the small dendritic structure of developing GCs has functional significance in limiting innervation. Since immature GCs are in a transient period of...
cell growth, the magnitude of excitatory drive is correlated with morphological maturation and post-mitotic cell age\cite{18,22,29,30,48}. Functional and morphological maturation of newly generated GCs is heterogeneous as well as progressive, and depends on diverse factors including animal age, housing condition, septal-temporal location and local network activity\cite{19,30,34,48-50}. In young adult rodents, newly generated GCs exhibit relatively rapid dendritic and spine development during the first month after cell birth that continues over many subsequent weeks and is paralleled by the development of functional excitatory synapses\cite{18,22,28}. Since developing GCs progress through immature stages when innervation is inversely correlated with intrinsic excitability\cite{22}, our conclusions are relevant to understanding immature GC function across various stages of maturation with the caveat that the timing of a particular stage varies according to specific conditions. Developing GCs appear to undergo the same sequence of maturation regardless of the age of the animal, thus we also expect that the factors contributing to immature GC spiking in young adult mice are relevant to understanding the function of immature GCs generated in older adult mice that are typically used to assess behavioural functions is subject to the caveat that

The model primarily serves to illustrate the main point that immature GCs with high excitability and low synaptic connectivity differentially affect NDP overlap across input levels, whereas high intrinsic excitability alone would be predicted to reduce population sparseness (and increase NDP overlap) across all input levels.

Our results can be incorporated into a broader view of the DG's function in hippocampal coding\cite{10,55}. If the DG is relevant in driving CA3, it is necessary that its outputs have some minimal level of activity—perfect separation is meaningless if no information is communicated. Even a very low activity level necessitates some minimal level of neuronal overlap; however, too much overlap presumably leads to interference in CA3 memory formation. In our simple model, maintaining low overlap requires that the EC's activity level can only be tolerated within a small range that is more than doubled by the inclusion of immature neurons (Fig. 7d). The low percentage of immature GCs that is optimized for expanding this range could imply that small numbers of excitable but sparsely innervated immature neurons facilitate input–output transformations by promoting discrete network representations across variable levels of EC activity.

Finally, it is important to consider that ‘what’ young and mature neurons encode is likely as important as ‘how’ they encode it\cite{15}. One implication of differential synaptic connectivity is that old and young GCs could represent different aspects of information incoming from EC. Because they are sampling more cortical space, mature GCs may encode and separate based on complex characteristics formed by many features of representation. Because of their limited sampling of EC, immature GCs may codify selective features of a representation with high fidelity due to their intrinsic excitability and low inhibition. In this manner, immature GCs could encode a singular aspect of a representation, potentially providing selectivity within fewer dimensions, which may help in contextualizing information incoming from EC based on combinations of concurrent spatial or temporal features\cite{56,57}. Thus immature GCs in the network could increase memory resolution or acuity as well as contribute to associating a contextualizing event to CA3 during memory formation\cite{23}.

**Methods**

We used male and female ~8-week-old tamoxifen-inducible nestin-based reporter mice. Nestin-CreERT\textsuperscript{2} mice\cite{32} were maintained on the C57Bl/6J background.
Jackson Labs, # 016261) and Nestin-CreERTM (provided by Kuo

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solution (100

Synaptic responses were evoked using patch pipettes filled with extracellular

elevation after recording using streptavidin conjugated to Alexa Fluor 647.

expected ‘shared’ inputs ( Jackson Labs, # 007914) to obtain offspring used in experiments. All animal

immature GCs in slices and the degree of overlapping synaptic inputs, we fit a basic

110 chloride, 2.5 g-glucose, 2.5 MgCl2, 1.25 KCl, 1.25 Na2PO4, 0.5 CaCl2, 13 Na-ascorbate, 3 Na-pyruvate and 25 NaHCO3, bubbled with 95% O2/5% CO2. The brain was removed and 300-μm-thick horizontal slices were prepared using a vibratome (Leica VT1200, Leica Instruments). Slices were incubated at 37°C for ~30 min in recording solution containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 Na2PO4, 2 CaCl2, 1 MgCl2, 25 NaHCO3 and 25 g-glucose bubbled with 95% O2/5% CO2, and then transferred to room temperature in the same solution. Slices were visualized using a 40 x water immersion objective on an upright microscope (Scientifica) equipped with a custom-made contrast imaging gradient (Dowd optics), a mercury burner, and a Texas Red filter set. In most experiments, patch pipettes were filled with the following (mM): 150 K-gluconate, 1 MgCl2, 1.1 EGTA, 5 HEPES and 10 phosphocreatine, pH 7.2 and 300 mosM. In experiments to measure NMHDR EPSCs, we used a pipette internal with the following (mM): 97.5 Cs-glucuronate, 17.5 CsCl, 10 BAPTA, 10 HEPES, 2 MgATP, 0.3 Na-GTP, 7 phosphocreatine and 5 QX-314, pH 7.2 and 290 mosM. Biocytin (0.2%) was included in the pipette in some experiments for morphological visualization after recording using streptavidin conjugated to Alexa Fluor 647. Synaptic responses were evoked using patch pipettes filled with extracellular solution (100 μS; 2–12 Pa at 100–300 μM). All recordings were done at room temperature and the holding potential of ~70 mV unless otherwise noted. EPSC latencies were measured from the onset of the stimulus artifact to the onset of the EPSC. Miniature EPSCs were recorded in 0.5 μM TTX. Series resistance was uncompensated (10–25 MΩ) and experiments were discarded if substantial changes (>20%) were observed. Voltages were not corrected for junction potentials and currents were filtered at 2 kHz and sampled at 10 kHz (MultiClamp 100C/Molecular Devices). Action potential threshold was detected when the slope of the spikes was calculated from the train of action potentials elicited by the highest intensity current injection of 20 Pa for mature GCs and 10 Pa for immature GCs. Bridge balance was automatically adjusted in the Multiclamp commander. CA recordings were performed with a patch pipette filled with artificial cerebral spinal fluid (ASC) in voltage-clamp mode at current = 0 Pa. Recordings were made in pClamp10 (Molecular Devices) and analysed using Axograph X (Axograph Scientific). Drugs and chemicals were obtained from Sigma-Aldrich, Tocris Bioscience, or Ascent Scientific.

Confocal images were taken from biocytin-filled mature and newborn GCs in acute slices after overnight fixation. GC morphology was reconstructed from image stacks using the tracing program Neuro lucida (MicroBrightfield).

Statistical analysis. Data were expressed as mean ± s.e.m. To minimize type I error, we set the stricter level of P<0.005 and accepted results with P<0.05 as well statistical tests. Normality was estimated using Shapiro-Wilk test, Kolmogorov-Smirnov test and Lilliefors test. When data sets satisfied normality criteria, we used two-tailed t-tests or two-way analysis of variance repeated-measures to evaluate differences among two or multiple samples, respectively (Statistica, StatSoft and GraphPad Prism). We evaluated the effect of drug (mabavine and control), the difference between GCs at the same or different ages (mature or immature) and between multiple pathways (MPP/LPP and MEC/LEC), across increasing stimulus difference between GCs at the same or different ages (mature or immature) and differences among two or multiple samples, respectively (Statistica, StatSoft and GraphPad Prism).

Molecular Devices). Action potential threshold was detected when the slope of the spikes was calculated from the train of action potentials elicited by the highest intensity current injection of 20 Pa for mature GCs and 10 Pa for immature GCs. Bridge balance was automatically adjusted in the Multiclamp commander. CA recordings were performed with a patch pipette filled with artificial cerebral spinal fluid (ASC) in voltage-clamp mode at current = 0 Pa. Recordings were made in pClamp10 (Molecular Devices) and analysed using Axograph X (Axograph Scientific). Drugs and chemicals were obtained from Sigma-Aldrich, Tocris Bioscience, or Ascent Scientific.

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Model methods. Estimating perforant path connection densities from slice
dentates GCs receive thousands of excitatory synaptic inputs from the lateral and medial EC, however, the number of viable inputs that are potentially activated using focal stimulation in the slice preparation is a small fraction of the total number. To estimate the number of active synaptic inputs onto mature and immature dentates GCs with the degree of synaptic inputs estimated from a statistical model to the data shown in Fig. 6. Given a pair of neurons, the number of expected ‘shared’ inputs (Nshared) is that, source fibres both neurons receive an input from) and ‘independent’ inputs (Nind that is, source fibres unique to one of

Nshared = Ntotal × Nind

Nind = Ntotal − Nshared

Ntotal is the total number of functional synapses on a GC (from that

Ppost shared = (1 − p)Ntotal

Ppost ind = (1 − p)Nind

Pboth ind = (1 − p)Nind

Finally, we are concerned with the probability that both neurons receive EPSCs simultaneously, since that is what we can measure. To compute this, we must subtract from one the mutual probability that the two neurons are neither activated by independent inputs nor by shared inputs:

Pboth = 1 − Ppost both ind − Ppost shared

Substituting equations (3)–(6) into equation (7) gives the following expanded form

Pboth = 1 − (1 − (1 − p)Nind) × (1 − p)Ntotal

In this equation, Pboth is measurable for different experimental multiples of p. Notably, we do not know the absolute value of p for any given stimulation, but we can assume that if we are far enough below saturation, increases in the experimental stimulation intensity yield a proportional increase in input fibres activated. As a result, the proportion of synaptic inputs activated for the minimal experimental stimulation in Fig. 6 is considered to be the p parameter (with higher amplitude stimulations resulting in a multiple of p), with Nshared and Ntotal being the other parameters necessary to fit. On the basis of Fig. 7a, we use the constraint that the ratio of intact synapses on young neurons to mature neurons (Ntotal-young/Ntotal-mature) is 0.35. Further, we constrained the Ntotal-mature to be no more than five times Nshared-mature. Our goal was to minimize the squared error between equation (8)’s estimate for overlapping outputs between two neurons and our experimental measurements in Fig. 6b, per the following equation

err = ∑ (Estimated stim − Measured stim)^2

Where Pestimatedstim is the output of equation (8) and Pmeasuredstim refers to the measured overlap for a given stimulation level in Fig. 6b.

Since the binomial relationship in equation (8) was not well suited for an analytical optimization of the parameters that globally minimize the error in equation (9), we used a Monte Carlo exploration of the space to find combinations of Nshared-mature and p that gave good fits. We structured our Monte Carlo search to have 250 000 combinations of the three independent parameters: 0.001 < P < 0.005, 20 < Nshared < 100 and 100 < Ntotal < 500. Identifying which set of parameters produced a good fit per equation (9). Notably, there were a number of solutions with approximately equivalent errors for which we selected P = 0.0039;

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ARTICLE

Simple neural network model. We generated a simple perceptron-based neural network model of the EC to DG circuit21. This model clearly is a considerable
determined above. Any connection resulted in a synapse of weight 1, and there was

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We tested each network on 100 sets of random EC inputs, and then computed the
correlation of the average overlap between GC outputs, which is given by

\[ DG_{\text{NDP}} = \frac{1}{N-1} \sum_{i=1}^{N} \frac{G_{\text{Output}}^{i} - G_{\text{Output}}^{\text{mid}}}{|G_{\text{Output}}^{i}|} \]

(12)

As the level of neurogenesis affects the dynamic range of permissible EC inputs, we ran 101 neurogenesis levels (networks containing from 0–100% of
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abstraction from the biological system and lacks spiking and long-timescale dynamics; however, it can illustrate how neuron variation can influence output
correlations of the system. The model consisted of 13,000 GC neurons and 1,300 EC neurons. The 1,300 EC neurons scale was selected to be comparable to the

established within a slice, and the 13,000 GCs scale was selected to allow us to investigate the large, close to 1:10, expansion ratio from EC to DG. Each GC neuron was either considered mature or immature and randomly

connected to neurons in the source EC population based on the frequencies determined above. Any connection resulted in a synapse of weight 1, and there was

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**Author contributions**  
C.V.D., R.P., J.B.A., J.I.W. and L.O.-W. designed, performed or analysed the experiments and simulations. C.K. provided technical expertise and reagents. C.V.D., J.I.W. and L.O.-W. wrote the manuscript. All authors contributed scientific insights and provided critical readings of the manuscript.

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