Increased Cell-Intrinsic Excitability Induces Synaptic Changes in New Neurons in the Adult Dentate Gyrus That Require Npas4

Shuyin Sim,^{1*} Salome Antolin,^{2*} Chia-Wei Lin,¹ Yingxi Lin,¹ and Carlos Lois²

¹Brain and Cognitive Sciences Department, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and ²Neurobiology Department, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Electrical activity regulates the manner in which neurons mature and form connections to each other. However, it remains unclear whether increased single-cell activity is sufficient to alter the development of synaptic connectivity of that neuron or whether a global increase in circuit activity is necessary. To address this question, we genetically increased neuronal excitability of *in vivo* individual adult-born neurons in the mouse dentate gyrus via expression of a voltage-gated bacterial sodium channel. We observed that increasing the excitability of new neurons in an otherwise unperturbed circuit leads to changes in both their input and axonal synapses. Furthermore, the activity-dependent transcription factor Npas4 is necessary for the changes in the input synapses of these neurons, but it is not involved in changes to their axonal synapses. Our results reveal that an increase in cell-intrinsic activity during maturation is sufficient to alter the synaptic connectivity of a neuron with the hippocampal circuit and that Npas4 is required for activity-dependent changes in input synapses.

Introduction

The addition of new granule cell (GC) neurons to the adult dentate gyrus (DG) of the hippocampus may serve as a substrate for memory throughout life (Imayoshi et al., 2008). These excitatory neurons display enhanced synaptic plasticity (Schmidt-Hieber et al., 2004) and integrate into existing circuitry (Jessberger and Kempermann, 2003). The production, survival, and wiring of GCs into the DG circuit are affected by activity (Kempermann et al., 1997; van Praag et al., 1999; Kee et al., 2007). Importantly, seizures alter the maturation and connectivity of adult-born DG GCs (Parent et al., 1997; Jessberger et al., 2007).

Understanding the factors underlying activity-dependent maturation and connectivity of adult-born neurons is important to understand the physiological basis of learning and the pathological basis of epilepsy. In a seizure or in behavioral paradigms used to stimulate DG activity, general levels of activity in the brain are increased, so it is unclear whether the observed changes in maturation and connectivity result directly from the increased

activity of an individual new neuron, indirectly via elevated activity of other neurons in the circuit in which the new neurons are embedded, or a combination of both.

To investigate how the level of neuronal activity of a single developing neuron affects its maturation and integration into an unperturbed circuit, we have developed a system to genetically increase excitability in individual neurons by introducing a voltage-gated sodium channel (Kelsch et al., 2008; Lin et al., 2010). We recently used this system to investigate the effects of genetically increased excitability on the maturation and integration of the GCs of the olfactory bulb, a type of inhibitory neuron produced during adulthood. We demonstrated that genetically increased intrinsic excitability was sufficient to enhance the survival of the new granule neurons of the bulb but, surprisingly, did not affect their synaptic organization (Kelsch et al., 2009; Lin et al., 2010). The plasticity of excitatory and inhibitory neurons differs in many respects (Bi and Poo, 1998), and it is plausible that electrical hyperexcitability affects the morphology of excitatory but not inhibitory neurons.

To test this hypothesis, we genetically raised the intrinsic excitability of individual new GCs in the DG, a type of excitatory neuron also produced during adulthood. We observed that elevating neuronal excitability of individual new neurons during their maturation is sufficient to induce changes in synaptic connectivity such as aberrant localization of synapses and enlarged spines. Cell-autonomous hyperexcitability leads to both input and output connectivity alterations that increase inhibition on the hyperexcitable neuron and dampen its excitatory influence on its downstream targets. We then examined the genetic basis for these alterations by deleting the transcription factor Npas4 in individual new neurons in conditional NPas4 knock-out mice.

Received March 30, 2012; revised May 9, 2012; accepted March 19, 2013.

Author contributions: S.S., S.A., Y.L., and C.L. designed research; S.S., S.A., C.-W.L., and C.L. performed research; S.A., Y.L., and C.L. contributed unpublished reagents/analytic tools; S.S., S.A., C.-W.L., and C.L. analyzed data; S.S., S.A., C.-W.L., and C.L. wrote the paper.

This work was supported by a NIDCD R01 Grant to C.L. and funded in part with a grant from the MIT Simons Initiative on Autism and the Brain (Y.L. and C.L.). We thank Alberto Stolfi and Drew Friedman for help with the engineering of the viral constructs, David Clapham (Harvard Medical School, Boston, MA) for providing us with the NaChBac cDNA, and Karl Deisseroth (Stanford University, Palo Alto, CA) for providing the double-floxed inverse ORF vector.

The authors declare no competing financial interests.

*S.S. and S.A. contributed equally to this work.

Correspondence should be addressed to Carlos Lois, Neurobiology Department, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605. E-mail: carlos.lois@umassmed.edu.

DOI:10.1523/JNEUROSCI.1571-12.2013

Copyright © 2013 the authors 0270-6474/13/337928-13\$15.00/0

We observed that the transcription factor *Npas4* is required for the activity-induced changes in synaptic inputs to these neurons but not for changes to output synapses in their axons. These observations indicate that cell-autonomous increases in excitability during neuronal maturation can effect profound changes in neuronal connectivity and that separate genetic programs regulate activity-dependent changes in input and output synapses.

Materials and Methods

Retroviral vectors. Cloning of the different constructs was performed using standard molecular techniques. The cDNA for NaChBac was obtained from David Clapham (Howard Hughes Medical Institute, Children's Hospital, Harvard Medical School, Boston, MA). NaChBac E191K was generated by PCR based on previously published sequences (Ren et al., 2001; Yue et al., 2002). Retroviral vectors were derived from a Moloney leukemia virus with an internal promoter derived from the Rous sarcoma virus (Molar) (Kelsch et al., 2007). Retroviral particles were produced and stored as described previously (Lois et al., 2002). The viral titers were $\sim 10^7$ infectious units/ μ l. Viral constructs were generated as follows. For NaChBac–EGFP, the stop codon of NaChBac was eliminated by PCR and fused in-frame to the cDNA of EGFP. For NaChBac–Cre, the stop codon of the NaChBac–EGFP fusion was eliminated by PCR and linked by a foot-and-mouth disease (FMDV) virus 2A sequence to the cDNA of Cre recombinase. For PalmEGFP–NaChBac, the palmitoylation sequence from the GAP43 gene was first added to the N terminus of EGFP. The stop codon of the palmitoylated version of EGFP was eliminated by PCR and linked by an FMDV 2A picornavirus sequence to the cDNA of NaChBac. For Synaptophysin–GFP, the stop codon of Synaptophysin was eliminated by PCR and fused in-frame to the cDNA of EGFP. For Synaptophysin–GFP–NaChBac, the stop codon of the Synaptophysin–GFP fusion was removed by PCR and linked by a 2A sequence to the cDNA of NaChBac. For PSD-95–GFP, the stop codon of PSD-95 was eliminated by PCR and fused in-frame to the cDNA of EGFP. For PSD-95–GFP–NaChBac, the stop codon of the PSD-95–GFP fusion was removed by PCR and linked by a 2A sequence to the cDNA of NaChBac. For invertible palmitoylated mCherry (PalmMCherry), the double-floxed inverse open reading frame vector was obtained from Karl Deisseroth (Stanford University, Palo Alto, CA). The Chr2–EYFP cDNA was excised from the vector and replaced with the cDNA of PalmMCherry, which was made by adding the palmitoylation sequence from the GAP43 gene to the N terminus of the mCherry cDNA.

Retroviral labeling in vivo. Nine- to 12-week-old female BL6 mice (Charles River), floxed NMDA receptor subunit 1 mice (Tsien et al., 1996), floxed *Npas4* mice (Lin et al., 2008), and their respective wild-type littermates were stereotactically injected at two sites per DG with 0.5 μ l/site retroviral vectors, after anesthesia with avertin solution. The stereotaxic coordinates were 2.0 mm posterior from bregma, 1.5 mm lateral from the midline, and 1.95 mm ventral from the brain surface, and 2.7 mm posterior from bregma, 1.9 mm lateral from the midline, and 2.05 mm ventral from the brain surface.

Histology. Mice were administered an overdose of avertin before they were perfused intracardially, first with PBS and then with 3% paraformaldehyde (PFA). The brains were incubated with 3% PFA overnight and cut with a Leica vibratome into 40 μ m frontal sections. For immunocytochemistry, the sections were first blocked with blocking solution containing bovine serum albumin (3 mg/ml PBS) and 0.25% Triton X-100 in PBS and incubated overnight in the relevant primary antibody diluted in blocking solution: polyclonal rabbit anti-GFP antibody (AB3080; 1:2000; Millipore Bioscience Research Reagents), rabbit anti-RFP antibody (LS-C60076; 1:200; Lifespan), mouse anti-vesicular GABA transporter (VGAT) antibody (catalog #131 002; 1:500; Synaptic Systems), mouse anti-parvalbumin (P3088; 1:500; Sigma), and rabbit anti-*Npas4* (1:10,000; gift from Y.L.). Sections were washed four times in PBS, for 10 min each time, before a 2 h incubation at room temperature with Alexa Fluor 488 or 555 goat anti-rabbit or anti-mouse secondary antibody (Invitrogen) diluted 1:700 in blocking solution. The sections were washed four times in PBS, for 10 min each time, before being mounted on slides with mounting medium (Vectashield; Vector Laboratories).

Survival ratio analyses. Two viruses were mixed at an approximate 1:1 ratio for survival analysis. One of the viruses carried the construct encoding mCherry, whereas the other carried either NaChBac or NaChBac E191K fused to EGFP (NaChBac–EGFP or NaChBacE191K–EGFP). Fluorescently labeled cells were quantified with the aid of the NeuroLucida software (MicroBrightField). The survival ratio is defined as the total number of EGFP-positive cells (including double-labeled cells) divided by the number of single-labeled mCherry⁺ cells. The ratio of EGFP⁺ to mCherry⁺ neurons at 7 d post-infection (dpi) was used to normalize all data at subsequent time points for comparison; hence, ratios at all subsequent time points were relative to the 7 dpi ratio. Ten to 20 entire sections per DG were analyzed to collect at least 100 counted cells in each DG. The mean survival ratio from each DG was treated as a single sample.

Electrophysiology. For electrophysiology, viruses in which NaChBac is directly fused to GFP were used because they produce strong fluorescent signals in the soma, which is useful for targeting neurons for fluorescence-guided whole-cell recordings. Animals were given an overdose of ketamine/xylazine and then perfused intracardially with ice-cold slicing solution containing the following (in mM): 212 sucrose, 3 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 7 MgCl₂, and 10 glucose, pH 7.3 (308 mOsm). Brain slices were incubated in ice-cold cutting solution and cut into 350 μ m frontal slices with a Leica microtome at a speed of 0.08 mm/s. Slices were incubated for 30 min at 35°C, for recovery, in carbogenated recording solution containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 1 MgCl₂, 2 CaCl₂, and 20 glucose, pH 7.3 (312 mOsm). Fluorescent-guided whole-cell patch-clamp recordings were performed with a MultiClamp 700B amplifier (Molecular Devices). The pipette solution contained the following (in mM): 2 NaCl, 4 KCl, 130 K-gluconate, 10 HEPES, 0.2 EGTA, 4 Mg-ATP, 0.3 Tris-GTP, and 14 Tris-phosphocreatine, pH 7.3. Successful patching onto the target cell was confirmed by identifying a fragment of fluorescent membrane trapped inside the pipette tip during or after the recording. Pipette resistance ranged from 5 to 8 M Ω , and the pipette access resistance was always <16 M Ω after series resistance compensation. The junction potential was not corrected throughout the study. For spontaneous EPSC (sEPSC) recording, the neurons were held at -77 mV, and synaptic events were collected at 25°C. sEPSCs contributed to the majority of spontaneous events because $\sim 98\%$ of events could be blocked by 100 μ M D,L-AP-5 and 20 μ M NBQX (Sigma) at the end of the recording. Furthermore, we observed that the spontaneous synaptic events recorded with bicuculline and TTX in both control and NaChBac⁺ neurons have a reversal potential at approximately -4 mV. These values are consistent with those of sEPSCs mediated by the opening of glutamate receptors (Cull-Candy and Usowicz, 1989), as reported previously for sEPSPs in GCs of the rat DG (-5.5 ± 1.1 mV; Crunelli et al., 1984).

Inhibitory blockers, such as bicuculline, were not included during sEPSC recording because they triggered frequent EPSC bursting input in granule neurons, which precluded additional analysis. To record sIPSCs, intracellular 130 K-gluconate was replaced with 130 CsCl and included 20 μ M NBQX and 50 μ M AP-5 in the recording bath to increase the driving force for chloride efflux, enabling us to record spontaneous GABAergic input at -77 mV.

Analysis of electrophysiological data. Data were acquired and analyzed with pClamp9 software (Molecular Devices), and 2 min traces of sIPSCs and sEPSCs were analyzed with Mini Analysis Program (Synaptosoft). Overall current was calculated by multiplying the average charge area per spike of each individual neuron by frequency of spikes of the same neuron.

Morphological analyses. For confocal microscopy, confocal image stacks of 40- μ m-thick DG sections were acquired by using an Olympus Fluoview confocal microscope (60 $\times$ oil-immersion lens; numerical aperture 1.4; pixel size, 0.23×0.23 μ m) and with z-step 0.25 μ m. Ten to 30 neurons were analyzed in each DG for dendritic length, density, spine size, and perisomatic inhibitory analysis, and data from four to seven DGs were collected for each experimental condition. A typical image stack consisted of ~ 80 – 150 image planes each of 1024×1024 pixels.

Figure 1. Expression of NaChBac increases spontaneous neuronal activity in adult-born DG GCs. **A**, Top traces, In current-clamp mode, 9 dpi wild-type ($mCherry^{+}$) DG neurons did not fire spontaneous action potentials. In contrast, NaChBac expression resulted in spontaneous depolarizations of 9 dpi DG neurons (middle trace). The NaChBac depolarization marked by the red bar is shown magnified at the bottom right of the panel. In addition, NaChBac expression also resulted in oscillations of the resting membrane potential (indicated by blue bar and shown magnified at the bottom left), which were not present in control cells. **B**, Top left, Control, $mCherry^{+}$ neurons did not show any spontaneous firing at any time tested (between 9 and 36 dpi). In contrast, NaChBac expression in DGs induced spontaneous depolarizations at all times tested (0.037 ± 0.017 Hz at 7–14 dpi and 0.005 ± 0.004 Hz at 24–36 dpi; both $n = 10$). Top right, NaChBac expression did not affect the resting membrane potentials [-62.4 ± 2.2 mV for wild-type ($mCherry^{+}$) neurons, -60.9 ± 2.9 and -60.1 ± 3.1 mV for 7–14 and 24–36 dpi NaChBac-expressing (Figure legend continues.)

Figure 2. Expression of NaChBac in adult-born DG GCs results in additional perisomatic GABAergic inputs. **A**, NaChBac⁺ neurons displayed increased numbers of perisomatic VGAT⁺ inhibitory terminals from 13 dpi onward (9 dpi GFP, 5.341 ± 0.269 VGAT⁺ puncta/soma, $n = 94$ neurons from 5 DGs; NaChBac, 6.57 ± 0.733 VGAT⁺ puncta/soma, $n = 72$ neurons from 5 DGs, $p = 0.176$; 13 dpi GFP, 4.794 ± 0.322 VGAT⁺ puncta/soma, $n = 100$ neurons from 8 DGs; NaChBac, 6.648 ± 0.217 VGAT⁺ puncta/soma, $n = 109$ neurons from 8 DGs, $***p = 0.0005$; 17 dpi GFP, 5.6098 ± 0.342 VGAT⁺ puncta/soma, $n = 56$ neurons from 4 DGs; NaChBac, 7.96 ± 0.339 VGAT⁺ puncta/soma, $n = 115$ neurons from 4 DGs, $**p = 0.0045$; 28 dpi GFP, 5.65 ± 0.325 VGAT⁺ puncta/soma, $n = 121$ neurons from 6 DGs; NaChBac, 7.76 ± 0.258 VGAT⁺ puncta/soma, $n = 115$ neurons from 6 DGs, $***p = 0.0005$). **B**, Confocal z-stack images of parvalbumin (Parv) staining of control and NaChBac⁺ neurons. **C**, Consistent with the increase in VGAT⁺ perisomatic contacts (**B**), NaChBac⁺ GCs have more parvalbumin- and GAD65-positive contacts on their cell bodies than control cells expressing the E191K pore-dead mutant channel. Two-tailed *t* test used for statistical analysis. Error bars represent SEM.

←

(Figure legend continued.) neurons; each condition, $n = 10$). Bottom left, NaChBac expression increases the capacitance of DG neurons. The capacitance increased from 3.42 ± 0.21 pF in wild-type (mCherry⁺) 9 dpi neurons ($n = 4$) to 7.8 ± 1.3 pF in NaChBac⁺ 7–14 dpi cells and from 6.03 ± 0.61 pF in wild-type cells to 10.2 ± 1.2 pF in NaChBac⁺ cells in between 24 and 36 dpi ($n = 12$ and $n = 10$, respectively). Bottom right, NaChBac expression did not affect the input resistance of DG neurons. Between 7 and 14 dpi, input resistance was 837 ± 345 and 792 ± 105 M Ω for wild-type and NaChBac⁺ neurons, respectively. Input resistance was also comparable between 24 and 36 dpi, 291 ± 67 M Ω for wild-type, and 271 ± 45 M Ω for NaChBac⁺ neurons. **C**, Left traces, Current injection in NaChBac⁺ cells (top) induced long-lasting depolarizations in 9 dpi DG neurons. In contrast, mCherry⁺ 9 dpi neurons (bottom) only showed passive membrane responses to the same amount of injected current. Middle and right traces, Current injection induced long depolarizations in 28 and 36 dpi NaChBac⁺ neurons and trains of repetitive action potentials in control, mCherry⁺ neurons. **D**, Left, Steps of current injection, up to 100 pA, were not able to elicit sodium action potentials in 9 dpi control cells ($n = 4$). In contrast, current injection as low as 20 pA triggered sustained depolarizations in NaChBac⁺ cells, whose amplitude did not significantly increase on higher levels of current injection (65.5 ± 3.1 mV; $n = 10$). From 24 to 36 dpi, in wild-type, mCherry⁺ neurons, sodium spikes can be triggered by an average current of 40 ± 7.3 pA, ~ 20 pA above the threshold necessary to elicit an NaChBac depolarization ($n = 6$). During this period, the NaChBac peak amplitude gradually increased from 54.5 ± 12.2 to 73.7 ± 7.8 mV during 20–80 pA current injections ($n = 10$). Right, The duration of the NaChBac response was assessed in 10–90% for the rise and decay time of the depolarization. This typically fell within the range 1076 ± 69 ms for 7–14 dpi and 485 ± 33 ms for 24–36 dpi.

For image processing and quantification, after acquisition, maximal intensity projections were prepared for each image stack by using the MetaMorph analysis software (Universal Imaging).

Measurements of PSD-95:GFP clusters were performed as described in our previous publication (Kelsch et al., 2007). To attribute the GFP puncta to a particular neuron, we took advantage of the presence of low levels of diffuse PSD-95:GFP protein in the cytoplasm not detectable by its endogenous fluorescence. This diffuse PSD-95:GFP protein could be visualized by amplifying its signal with antibodies raised against GFP (coupled to a red fluorophore to distinguish it from the intrinsic green fluorescence of PSD⁺ puncta) and allowed us to attribute PSD⁺ puncta to the neurites belonging to a particular neuron. For the projection images, the threshold was set so that any possible diffuse GFP fluorescence at the dendritic shaft was below this threshold. The number of PSD-95:GFP⁺ clusters in a region of interest was counted by using the integrated morphometry analysis function of the MetaMorph software. The length of the respective segment of the dendritic arbor was then measured, and the density of PSD-95:GFP⁺ clusters was determined. All datasets were manually supervised to prevent the inclusion of nonspecific green specks.

Spine size in palmitoylated EGFP (PalmG) neurons were measured as follow. For each stack, laser intensity and detector sensitivity were set so that the fluorescence signal from the spines occupied the full dynamic range of the detector. This meant that some pixels in the dendritic branch were saturated, but no pixels were saturated within the spines. Maximum density projections of the confocal stacks were prepared. Only the areas of the spine heads flanking the dendrites were measured. Spines

above or below the respective dendrite were not included in the analysis. The cross-sectional area of spine heads in a region of interest was obtained from the integrated morphometry analysis function in MetaMorph and expressed in square micrometers. To calculate spine density, we divided the number of spines by the length of the respective segment of the dendritic arbor.

Presynaptic sites in PalmG neurons were measured as follows. First, we traced (with NeuroLucida) the axons present in each section and marked their presynaptic sites. On each axon, we identified these presynaptic sites as boutons or large mossy fiber terminals (LMTs) based on their characteristic morphology. Second, we divided the number of boutons or LMTs by the length of the axons traced and expressed their density as “number of presynaptic sites per micrometer” or “number of LMTs per micrometer.”

Statistical analyses. The Mann–Whitney test from OriginPro 8 (OriginLab) was used for comparing the frequency of spontaneous firing in NaChBac⁺ and control neurons at resting membrane potential to determine statistical significance. All other data were analyzed with the two-sample two-tailed Student's *t* test in Prism 5 (GraphPad Software). Data were reported as mean $\pm$ SEM.

Results

Expression of NaChBac in adult-born DG GCs elevates neuronal excitability

Changes in brain activity, such as those triggered by increased behavioral demands or seizures, affect the maturation and con-

nectivity of new neurons born in the adult DG (Overstreet-Wadiche et al., 2006; Kron et al., 2010). These changes in connectivity could be attributable to the increased activity of the new neurons, of the circuit in which the neurons are embedded, or a combination of both. To isolate the contribution of elevated activity in new neurons, we increased the activity of individual new neurons cell autonomously with the ion channel NaChBac. NaChBac is a bacterial voltage-gated sodium channel that has both a more negative activation threshold than native sodium channels in GCs (~ 15 mV more negative) and a longer inactivation time (hundreds of milliseconds compared with <1 ms in mammalian sodium channels) (Ren et al., 2001; Bean, 2007). Because of its unique electrical properties, NaChBac was used previously to induce hyperexcitability in *Drosophila* pacemaker neurons (Nitabach et al., 2006). More recently, we took advantage of NaChBac-induced depolarization to investigate the maturation of the GCs of the olfactory bulb, a type of inhibitory neuron generated throughout life. Genetically increasing intrinsic excitability by NaChBac expression is sufficient to enhance the survival of the new granule neurons of the bulb but, surprisingly, does not affect their synaptic organization (Kelsch et al., 2009; Lin et al., 2010). The plasticity responses of excitatory and inhibitory neurons differ in many respects (Bi and Poo, 1998), and it is plausible that electrical hyperexcitability by NaChBac affects the morphology of excitatory but not inhibitory neurons.

To investigate whether cell-autonomous increases in excitability are sufficient to alter neuronal connectivity of excitatory neurons, we used oncoretroviruses to introduce NaChBac into individual adult-born DG GCs, a type of excitatory neuron. Because this class of retroviruses cannot transport their genetic material across the intact nuclear envelopes of nondividing cells (Lewis and Emerman, 1994), they can selectively infect dividing cells in the hilus region of the DG, labeling and effectively birth-dating new GCs. We used a titer of oncoretrovirus that sparsely labeled GCs [~ 100 hyperexcitable cells among $\sim 400,000$ wild-type cells of the DG in the adult B6/57mouse (Abusaad et al., 1999)] in the DG, thus keeping the vast majority of the circuit unaltered. We performed patch-clamp electrophysiological recordings of labeled GCs and found that control, wild-type cells (mCherry⁺) did not exhibit any type of spontaneous activity at any times tested, between 9 and 36 d post-injection (dpi). In contrast, NaChBac⁺ cells exhibited spontaneous, long-lasting depolarizations starting as early as 7 dpi (Fig. 1A,B). NaChBac expression did not affect the resting membrane potential or input resistance of the neurons at any times, but it increased their capacitance (Fig. 1B).

Current injection failed to elicit action potentials in control cells at 9 dpi but triggered trains of action potentials between 24 and 36 dpi (Fig. 1C,D). In contrast, current injection triggered long-lasting depolarizations in NaChBac⁺ cells at all times examined (9, 28, and 36 dpi) (Fig. 1C,D). Finally, whereas the threshold to fire action potentials in wild-type, mCherry⁺ cells between 26 and 36 dpi is 40 pA, 20 pA is sufficient to trigger a full depolarization in NaChBac⁺ neurons (Fig. 1D).

In summary, NaChBac expression renders new DG hyperexcitable, confirming our previous observations in the olfactory bulb (Kelsch et al., 2009; Lin et al., 2010).

To investigate whether, as in the olfactory bulb, NaChBac expression enhances the survival of new neurons, we measured the ratio of mCherry⁺ (wild-type) to NaChBac⁺ (hyperexcitable) cells at different time points after viral injection. However, we did not find any deviations from the 1:1 ratio at any time point (7–36 dpi; data not shown). Similarly, we did not find any devi-

Figure 3. Cell-intrinsic hyperexcitability induces increased synaptic inhibition and accelerates neuronal maturation. **A**, In voltage-clamp mode, control neurons (top panel, left trace) experienced few spontaneous inhibitory synaptic events, whereas NaChBac⁺ neurons (top panel, right trace) had frequent spikes of inhibitory inputs that were of higher amplitudes. Both frequency (bottom panel, left) and amplitude (bottom panel, middle) of spontaneous IPSCs received by NaChBac⁺ neurons are higher than those received by control neurons (mCherry control frequency, 0.248 ± 0.054 Hz, $n = 7$ neurons; NaChBac frequency, 1.571 ± 0.381 Hz, $n = 9$ neurons, $**p = 0.009$; mCherry control amplitude, 26.42 ± 2.68 pA, $n = 7$ neurons; NaChBac amplitude, 69.28 ± 8.9 pA, $n = 9$ neurons, $**p = 0.001$). Overall current received by NaChBac⁺ neurons is ~ 10 times that received by control neurons (mCherry control, 0.281 ± 0.071 pA, $n = 7$ neurons; NaChBac, 2.9 ± 0.63 , $n = 8$ neurons; bottom panel, right). **B**, A larger proportion of neurons expressing NaChBac are KCC2 positive compared with E191K control neurons at 13 dpi, suggesting that NaChBac accelerates the upregulation of KCC2 in GCs. $*p = 0.023$. **C**, Significantly fewer NaChBac⁺ neurons expressed PSA-NCAM at both 16 and 21 dpi compared with controls (16 dpi E191K control, $83.5 \pm 1.35\%$, $n = 2$ DGs; NaChBac, $33.57 \pm 10.5\%$, $n = 3$ DGs, $*p = 0.0347$; 28 dpi E191K control, $32.67 \pm 3.6\%$, $n = 3$ DGs; NaChBac, $11.81 \pm 5.9\%$, $n = 3$ DGs, $*p < 0.05$). Two-tailed t test used for statistical analysis. Error bars represent SEM.

Figure 4. Increased electrical activity via NaChBac results in ectopic localization of DG GCs, reduced migration, and persistence of basal dendrites. **A**, NaChBac⁺ GCs were occasionally found in the hilar region of the DG (arrowhead) in which control neurons are never found. Stippled line represents the boundary between the GC layer and hilus. **B**, NaChBac⁺ GCs have cell bodies that are located lower within the GC layer of the DG compared with control neurons expressing the pore-dead NaChBac E191K. *** $p = 0.0004$. Two-tailed t test used for statistical analysis. Error bars represent SEM. **C**, Basal dendrites on NaChBac⁺ GCs displayed PSD-95:GFP⁺ clusters. Image on the right (labeled GFP) shows immunocytochemistry against the diffuse, unclustered GFP that filled the cytoplasm with a red secondary antibody. Middle image (labeled NaChBac/PSD95) shows the PSD-95:GFP-positive clusters identified by the direct green fluorescence from GFP. Stippled line represents the boundary between the GC layer and hilus.

ations in the ratio of mCherry⁺ to E191K⁺ [a nonconducting variant of NaChBac (Yue et al., 2002)] cells at any time points after injection. These observations suggest that NaChBac expression does not significantly affect the proliferation of neuronal progenitors or the survival advantage of young neurons in the DG, in contrast with our observations in the olfactory bulb (Lin et al., 2010).

We did not observe any seizures or any other gross behavioral changes in the animals injected with NaChBac viruses, which is not surprising given the very low percentage of cells expressing NaChBac (approximately one NaChBac⁺ cell for every 4000 wild-type cells in the DG). These results confirm that expression of NaChBac in small numbers of new DG GCs increases their excitability in a cell-autonomous manner, without grossly perturbing the function of the DG.

NaChBac-induced excitability results in additional GABAergic input to the cell body

In many circuits, surrounding neurons react to individual neuron activity to keep circuit activity within a range that prevents disruption of function (Turrigiano and Nelson, 2004). DG GCs only start receiving glutamatergic input late in development, ~21 d after birth, but receive GABAergic input much earlier. These cells first receive extrasynaptic input by ambient (extrasynaptic) GABA starting 3 d after they are generated, followed by GABA-mediated synaptic inputs as early as 7 d after their birth (Ge et al., 2006). For this reason, we hypothesized that, when hyperexcitable adult-born GCs are introduced into the DG circuit, one of the earliest responses of the surrounding circuit would be to alter the GABAergic input targeted to NaChBac⁺ neurons. To test this

hypothesis, we performed immunostaining against the VGAT, which is present in the vast majority of the presynaptic terminals of inhibitory interneurons (Chaudhry et al., 1998). We quantified VGAT⁺ puncta on cell bodies, because this measurement was more reliable than counting the number of contacts on dendrites.

At 9 dpi, there was no significant difference in the density of VGAT⁺ contacts with the soma of either control or NaChBac⁺ neurons (Fig. 2A). However, by 13 dpi, there were significantly more VGAT⁺ contacts on the soma of NaChBac⁺ neurons compared with neurons expressing NaChBac E191K, a nonconducting variant of NaChBac (Yue et al., 2002), and this effect persisted until at least 28 dpi (Fig. 2A). To further investigate this increased innervation, we then used antibodies against parvalbumin and GAD65. Parvalbumin⁺ cells are a subset of inhibitory interneurons that preferentially synapse onto the cell bodies of DG granule neurons (Freund and Buzsáki, 1996), whereas GAD65 is an isoform of glutamic acid decarboxylase (GAD), an enzyme present in a large proportion of inhibitory interneurons (Erlander and Tobin, 1991). We confirmed the trend of increased GABAergic contact in NaChBac⁺ neurons using parvalbumin (Fig.

2B,C) and GAD65 immunolabeling (Fig. 2C). To verify whether our observations regarding perisomatic GABAergic contact corresponded to a functional increase in inhibitory input, we performed electrophysiological recordings to measure sIPSCs of individual neurons. We coinjected a mixture of retroviruses, one carrying the construct for NaChBac fused to GFP and the other carrying the construct for mCherry, into the DG and recorded from control neurons (mCherry-only) and NaChBac⁺ neurons in the same DG at 17 dpi. mCherry was used to label control neurons because these cells would appear red and could be easily distinguished from the GFP-expressing NaChBac⁺ neurons. Indeed, there was an increase in both the frequency and amplitude of sIPSCs received by NaChBac⁺ GCs relative to control GCs (Fig. 3A). These results indicate that individual adult-born DG GCs with elevated neuronal excitability receive more GABAergic inputs than age-matched wild-type GCs.

GABAergic innervation to adult-born GCs is initially depolarizing as a result of high levels of expression of the Na⁺/K⁺/Cl[−] cotransporter NKCC1 relative to the K⁺/Cl[−] cotransporter KCC2 (Plotkin et al., 1997; Clayton et al., 1998). The subsequent upregulation of KCC2 as cells mature lowers the intracellular concentration of Cl[−] and eventually makes the GABA reversal potential more negative than the resting membrane potential, rendering GABAergic innervation hyperpolarizing (Rivera et al., 1999; Wang et al., 2002). This switch from depolarizing to hyperpolarizing GABAergic inputs occurs after 14 dpi in adult-born GCs (Ge et al., 2006). As mentioned above, we observed an increase in inhibitory input to NaChBac⁺ GCs that occurs by 13 dpi (Fig. 2A). At 13 dpi, we also observed an increase in the number of KCC2-

positive NaChBac⁺ GCs compared with controls (Fig. 3B). The increase in the percentage of KCC2⁺ cells induced by NaChBac expression suggests a premature reduction in Cl[−] concentration, which would in turn result in an earlier switch to inhibition by GABA. This accelerated maturation could enable the increase in GABAergic input to prematurely become inhibitory and thus dampen the hyperexcitable neurons earlier in development. In agreement with the notion of hyperexcitability accelerating the maturation of new GCs, we observed that polysialylated neural cell adhesion molecule (PSA-NCAM), a marker for immature neurons (Seki and Arai, 1993), is also downregulated earlier in NaChBac⁺ GCs compared with control neurons (Fig. 3C).

Our results indicate that NaChBac activity impacts the maturation and early synapse formation of DG GCs. From an early developmental stage, hyperexcitable GCs start receiving more GABAergic input from surrounding interneurons. In addition, cell-autonomous hyperexcitability speeds up development of newly born GCs.

Increased excitability leads to changes in excitatory glutamatergic input

Having discovered that NaChBac-induced hyperexcitability induces marked changes in neuronal maturation and an increase in inhibitory inputs early on, we proceeded to study how NaChBac affects the next phase of development of these GCs when they start to receive excitatory input synapses. DG GCs normally begin receiving excitatory inputs from 21 d after birth via spines along their apical dendrites that acquire mature morphology by ~28 d. To examine the changes in excitatory input received by a neuron rendered hyperexcitable by NaChBac, we infected neural progenitors in the DG with a bicistronic retroviral vector that expresses both PalmG and NaChBac. PalmG is localized into the membranes of infected neurons, allowing us to visualize the detailed morphology of the neurons, including dendritic spines.

Using the bicistronic PalmG:NaChBac construct, we observed that NaChBac⁺ GCs exhibited some connectivity changes that were similar to those observed in immature DG GCs after seizures. DG GCs migrate a small distance, ~5–10 μ m, from the hilar border in the DG, in which neural progenitors reside, to the granule layer of the GC, in which they settle and integrate into the DG circuit. Seizures induce the ectopic migration of GCs to either the outer third of the GC layer or the hilar region (Parent et al., 1997; Jessberger et al., 2009). The majority of NaChBac⁺ neurons had cell bodies correctly localized within the granule layer of the DG. We observed no NaChBac⁺ neurons in the outer third of the GC layer but found occasional neurons in the hilar

Figure 5. Elevated neuronal excitability in DG granule neurons leads to morphological changes in excitatory glutamatergic inputs. **A**, Low-magnification confocal images showing decreased dendritic length of NaChBac⁺ neurons compared with control neurons (pore-dead NaChBac E191K) (left panels). The distance between the furthest dendrite tip to the base of the apical dendrite is significantly lower in NaChBac⁺ neurons than controls at 28 dpi (far right panel; GFP control, 238.6 $\pm$ 3.23 μ m, n = 71 neurons; NaChBac, 200.8 $\pm$ 4.35 μ m, n = 56 neurons, $***p$ < 0.0001). **B**, High-magnification confocal images showing the decreased spine density and increased spine size in NaChBac⁺ neurons (left panels). NaChBac⁺ neurons have significantly fewer spines per length of dendrite than control (NaChBac E191K) neurons (far right panel; 28 dpi E191K control, 1.1 $\pm$ 0.05 spines/ μ m, n = 21 images; NaChBac, 0.478 $\pm$ 0.044 spines/ μ m, n = 40 images, $***p$ < 0.0001; 42 dpi E191K control, 1.29 $\pm$ 0.052 spines/ μ m, n = 17 images; NaChBac, 0.316 $\pm$ 0.024 spines/ μ m, n = 9 images, $***p$ < 0.0001; 28 vs 42 dpi E191K, $*p$ = 0.014). **C**, All dendritic protrusions on both control and NaChBac⁺ neurons cluster PSD-95:GFP, suggesting that they represent functional excitatory input synapses. Larger spines also exhibit correspondingly larger PSD95:GFP⁺ puncta (left panels). NaChBac⁺ neurons have significantly larger spines than control neurons at both 28 and 42 dpi (far right panel; 28 dpi E191K control, 0.636 $\pm$ 0.073 μ m², n = 52 images from 4 DGs; NaChBac, 1.298 $\pm$ 0.15 μ m², n = 24 images from 4 DGs, $***p$ < 0.0001; 42 dpi E191K control, 0.513 $\pm$ 0.042 μ m², n = 75 images from 5 DGs; NaChBac, 2.618 $\pm$ 0.18 μ m², n = 35 images from 8 DGs, $***p$ < 0.0001; 28 vs 42 dpi NaChBac, $***p$ < 0.0001).

region (Fig. 4A), whereas no wild-type neurons were ever found there. The morphology of these ectopic neurons was similar to their counterparts in the GC layer. They were polarized and had dendrites extending in the opposite direction of their axon. The neurons were entirely in the hilus, and their dendrites were also located solely in the hilus instead of the molecular layer. Because of the location of their dendrites, the connectivity of these neurons is likely to be perturbed. As mentioned above, NaChBac activity induces premature downregulation of PSA-NCAM (Fig. 3C), which has been implicated in neuronal migration either through its role in decreasing cell–cell adhesion (Johnson et al., 2005) or in sensing growth factor gradients (Muller et al., 2000). Thus, it is possible that the downregulation of PSA-NCAM may be responsible for the ectopic location of some of these NaChBac⁺ neurons. In addition to

Figure 6. Elevated neuronal excitability in DG granule neurons leads to electrophysiological changes in excitatory glutamatergic inputs. **A**, sEPSCs were recorded in control (mCherry⁺) neurons and NaChBac⁺ neurons at 28 dpi (top panel). At 28 dpi, the frequency of sEPSCs was significantly lower for NaChBac⁺ neurons than control (mCherry control, 0.876 ± 0.158 Hz, $n = 6$ neurons; NaChBac, 0.408 ± 0.098 , $n = 5$ neurons, $*p = 0.041$; right panel, leftmost), whereas the average amplitude was higher (mCherry control, 5.653 ± 0.387 pA, $n = 6$ neurons; NaChBac, 25.46 ± 7.4 pA, $n = 5$ neurons, $**p = 0.016$; right panel, middle). The overall excitatory current received by NaChBac was not significantly different from controls (mCherry control, 0.0266 ± 0.0059 pA, $n = 6$ neurons; NaChBac, 0.0224 ± 0.0043 , $n = 4$ neurons, $p = 0.62$; right panel, rightmost). **B**, sEPSCs for NaChBac⁺ and mCherry⁺ neurons reverse polarity at the same membrane potential (24–36 dpi). Left, sEPSCs recorded at six different membrane potentials in the same NaChBac⁺ neuron. Recordings were made in the presence of $10 \mu\text{M}$ bicuculline and $3 \mu\text{M}$ TTX and with a CsCl-filled electrode. Right, The amplitude of sEPSCs for NaChBac⁺ and control mCherry⁺ neurons recorded in the same slice are plotted against the membrane potential. The reversal potential was -4.25 ± 0.18 and -4.4 ± 0.06 mV for NaChBac⁺ and mCherry⁺ neurons, respectively ($n = 2$). Two-tailed t test used for statistical analysis. Error bars represent SEM.

cells found in the hilus, we also observed that cell bodies of NaChBac⁺ GCs reside closer to the hilar border than wild-type controls at 28 dpi (Fig. 4B). These observations suggest that hyperexcitability in young neurons affects the migration and settling of the GCs within the GC layer.

Mature GCs in the DG display apical dendrites that branch into the molecular layer of the DG. During their development, newly generated GCs in the adult DG transiently display basal dendrites extending into the hilus, which disappear by 4 or 5 d after the birth of the neuron (Shapiro and Ribak, 2006). Approximately 20% of NaChBac⁺ neurons displayed these aberrant basal dendrites, whereas control neurons expressing the E191K

nonconducting channel never did (Fig. 4C). Interestingly, the presence of spiny basal dendrites emanating from the cell bodies of fully mature GCs and extending into the hilus is one of the hallmarks of seizure-related changes in the DG (Shapiro and Ribak, 2006; Jessberger et al., 2007). To investigate whether the basal dendrites of NaChBac⁺ neurons contained postsynaptic sites, we infected new DG GCs with viral vectors encoding both NaChBac and a fusion between GFP and PSD-95, a scaffolding protein selectively localized to the postsynaptic density of glutamatergic input synapses (Kelsch et al., 2008). We performed immunocytochemistry against the diffuse, unclustered GFP that filled the cytoplasm with a red secondary antibody to visualize the dendritic morphology, whereas PSD-95⁺ clusters were identified by the direct green fluorescence from GFP (Kelsch et al., 2008). We observed that the basal dendrites of NaChBac⁺ cells had PSD-95:GFP⁺ clusters (Fig. 4C). The persistence of basal dendrites suggests that hyperexcitable neurons receive additional synaptic inputs to their cell bodies, and this input is likely to be excitatory (Ribak et al., 2000; Thind et al., 2008).

Increase in neuronal activity during seizures also affects the formation of apical dendrites and their synapses. When we examined the morphology of NaChBac⁺ GCs in the granule layers, we observed that they had shorter apical dendrites on average (Fig. 5A). In addition, the density of protrusions on apical dendrites of NaChBac⁺ neurons was half of the spine density of control neurons expressing the pore-dead NaChBac E191K channel at 28 dpi (Fig. 5B). There was an increase in spine density from 28 to 42 dpi for control neurons (Fig. 5B, far right panel) but no additional change for NaChBac⁺ neurons. The average spine size at 28 dpi of NaChBac⁺ neurons was twice of that of NaChBac E191⁺ neurons (Fig. 5C, far right panel). Interestingly, the increase in spine size of NaChBac⁺ neurons resembles the increased proportion of mushroom spines observed in GCs after seizure (Jessberger et al., 2007).

To investigate whether the large protrusions on NaChBac⁺ neuron apical dendrites were indeed synaptic spines, we infected new DG GCs with GFP constructs fused to PSD-95 (Kelsch et al., 2008).

Control neurons were infected with a virus expressing only the PSD-95–GFP fusion, whereas hyperexcitable neurons were infected with PSD-95–GFP:NaChBac, a bicistronic construct encoding both GFP-tagged PSD-95 and NaChBac. All protrusions present on the dendrites of labeled neurons expressed GFP:PSD-95 (Fig. 5C, left panels), confirming that the larger protrusions in NaChBac⁺ cells are postsynaptic sites. Furthermore, larger protrusions exhibited

larger GFP:PSD-95⁺ clusters, suggesting that any observed change in spine size could possibly indicate larger postsynaptic densities and, in effect, larger synapses.

The morphological alterations we report here suggest that NaChBac⁺ neurons experience an overall decrease in the number of excitatory inputs. However, although fewer in number, each individual spine in NaChBac⁺ neurons was larger on average than those of control neurons. To examine how these morphological changes translated into functional differences, we measured the sEPSCs of individual NaChBac⁺ neurons by electrophysiological recording and found that overall frequency of sEPSCs is significantly reduced in NaChBac⁺ neurons (Fig. 6A, left graph), whereas the average amplitude of sEPSCs was increased (Fig. 6A, middle graph). These results are consistent with NaChBac⁺ neurons having fewer but larger synapses. We observed that these spontaneous synaptic events, in both control and NaChBac⁺ neurons, could be blocked by glutamate blockers such as D,L-AP-5 and NBQX and had a reversal potential at approximately −4 mV (Fig. 6B). These values are consistent with those of sEPSCs mediated by the opening of glutamate receptors (Cull-Candy and Usowicz, 1989), as reported previously for sEPSPs in GCs of the rat DG (−5.5 ± 1.1 mV; (Crunelli et al., 1984).

Because the frequency and amplitude of sEPSCs in NaChBac⁺ neurons changed in opposing directions, to find out what the resultant current was, we calculated the overall excitatory current that the neurons received by multiplying the average charge area per spike of each individual neuron by the frequency of spikes of the same neuron. The overall excitatory current received by NaChBac⁺ neurons was not significantly different from that received by controls (Fig. 6A, right graph), hinting at the existence of a mechanism that maintains a set level of excitatory drive into these neurons.

Elevated excitability leads to changes in excitatory outputs at CA3

To quantify the changes in outputs of NaChBac-expressing DG GCs, we examined the morphology of presynaptic terminals on their axons in the CA3 region, in which their main output is.

The axons of DG GCs synapse on multiple targets on CA3, on both excitatory pyramidal cells and inhibitory interneurons. The axon collaterals of dentate GCs form specialized presynaptic sites called LMTs. LMTs measure between 3 and 8 μm in their greatest dimension and form complex interdigitating connections with CA3 pyramidal cells. DG axons also have two other types of smaller output synapses that contact inhibitory neurons at CA3 called en passant boutons and filopodial terminals (Acsády et al., 1998). En passant boutons are varicosities 0.5–2 μm in diameter

Figure 7. Elevated neuronal excitability in DG granule neurons leads to changes in excitatory output targets at CA3. **A**, Confocal maximal projection images showing LMTs and en passant boutons (white arrows) on the axons of NaChBac⁺ and control neurons. **B**, Axons of NaChBac⁺ neurons showed an overall decrease in the total number of presynaptic sites compared to controls at both 28 and 42 dpi (28 dpi E191K control: 0.0087 ± 0.0011 sites/m, *n* = 11 images; NaChBac: 0.0059 ± 0.00068 sites/m, *n* = 11 images, **p* < 0.05; 42 dpi E191K control: 0.01 ± 0.00075 sites/m, *n* = 10 images; NaChBac: 0.0056 ± 0.00059 sites/m, *n* = 12 images, ****p* = 0.0001). **C**, NaChBac⁺ neurons had significantly fewer LMTs on their axons compared to controls at both 28 and 42 dpi (28 dpi E191K control: 0.0079 ± 0.0011 LMT/m, *n* = 11 images; NaChBac: 0.0031 ± 0.00043 LMT/m, *n* = 11 images, ***p* = 0.0013; 42 dpi E191K control: 0.0074 ± 0.00068 LMT/m, *n* = 10 images; NaChBac: 0.0007 ± 0.00029 LMT/m, *n* = 12 images, ****p* < 0.0001; 28 vs 42 dpi NaChBac ****p* = 0.0001). **D**, The percentage of presynaptic sites that were LMTs at 28 dpi was much lower in NaChBac⁺ neurons than neurons expressing the pore-dead channel (E191K control: 88.24 ± 1.95%, *n* = 17 images; NaChBac: 40.66 ± 7.05%, *n* = 19 images, **p* < 0.03). **E**, Structures resembling presynaptic terminals on the axons of both control and NaChBac⁺ neurons accumulated synaptophysin:GFP, suggesting the change in density of the different structures correlates with a change in presynaptic sites. Two-tailed *t* test used for statistical analysis. Error bars represent SEM.

distributed along the axons of GCs, and filopodial terminals are thin protrusions emanating from the LMT. We focused on the effects of hyperexcitability on LMTs because, as a result of their characteristic morphology, these presynaptic sites can be unambiguously identified by membrane-bound GFP labeling. Expression of NaChBac decreased the overall density of presynaptic terminals present on the axons of adult-born dentate GCs at CA3 (Fig. 7A,B), which suggests that the hyperexcitable neurons downregulated their overall output to CA3. We observed that the overall density of LMTs in the axons of NaChBac⁺ neurons was significantly decreased compared with control neurons (Fig. 7A,C). In addition, NaChBac expression also appears to reduce the size of the LMTs (Fig. 7A). Finally, the proportion of total presynaptic sites that are LMTs is also significantly reduced in NaChBac⁺ neurons (Fig. 7D). These observations indicate that the output from hyperexcitable NaChBac⁺ DG GCs onto CA3 is significantly decreased.

To confirm that we were quantifying actual presynaptic sites in our measurements, we injected adult mice with retroviral vectors expressing Synaptophysin–GFP, a protein selectively local-

Figure 8. Strategy to selectively delete *Npas4* in hyperexcitable DG neurons. *Npas4* conditional mice (**A**) are simultaneously infected with two independent viruses (**B**, **C**). The **B** virus carries a bicistronic cassette encoding both a membrane-bound form of GFP (PalmGFP) and the recombinase Cre. The **C** virus carries (in a reversed 3' to 5' orientation) a bicistronic cassette encoding both a membrane-bound form of the red fluorescent protein mCherry and NaChBac (PalmMCherry2ANaChBac). The PalmMCherry2ANaChBac cassette (arranged in the reverse 3' to 5' orientation with respect to the viral promoter) is flanked by a set of mutually incompatible double loxP sites (loxP and lox2722). Cells infected only with virus **C** are not detectable because they do not express either PalmMCherry or NaChBac. Cells from the conditional *Npas4* mouse infected only by the virus **B** express PalmGFP and the protein Cre (**D**), which induces the deletion of the *NPAS4* gene flanked by loxP sites (**E**). In cells infected by both viruses **B** and **C**, the Cre protein (**D**) from the **B** virus leads to the simultaneous deletion of *NPAS4* (**E**) and to the reversion of orientation of the PalmMCherry2ANaChBac cassette from the **C** virus (**F**). Thus, only cells doubly infected by viruses **B** and **C** display both green and red fluorescence (because they express both PalmGFP and PalmMCherry), and they are hyperexcitable because of NaChBac expression and become deficient in *NPAS4* as a result of Cre expression.

ized to presynaptic neurotransmitter vesicles (Wiedenmann and Franke, 1985). Neurons were infected with a bicistronic virus encoding both Synaptophysin–GFP and NaChBac to visualize the presynaptic sites on axons of hyperexcitable cells. Cells in a separate DG infected with a virus encoding Synaptophysin–GFP were used as controls. Using a red fluorescent secondary antibody against the diffuse, unclustered GFP that filled the cytoplasm, we were able to visualize the full morphology of the axons at CA3, as well as determine the location of Synaptophysin-positive presynaptic sites, which showed up as green GFP-positive clusters. All structures resembling presynaptic terminals as labeled by PalmGFP were positive for GFP:synaptophysin in both wild-type neurons and those expressing NaChBac (Fig. 7E). This confirms that the structures we quantified corresponded to presynaptic terminals on the axons of these adult-born GCs.

Our observations of output connectivity at CA3 suggest that an increase in intrinsic excitability in adult-born GCs leads to a decrease in excitatory output at CA3. However, the output does not depend exclusively on the number of synaptic contacts but also on the properties of each of these contacts. For instance, the properties of voltage-gated potassium channels in the terminal are critical in determining the amount of release (Geiger and Jonas, 2000). Additional experiments involving electrophysiological recordings from the postsynaptic neurons in CA3 may allow to investigate whether the output is indeed reduced.

Activity-induced changes in input connectivity are dependent on cell-autonomous *Npas4* signaling

Two of our observations in NaChBac⁺ neurons led us to hypothesize that the early increase of GABAergic synapses triggered by hyperexcitability could be related to the later changes in synaptic connectivity observed in dendrites and axons. First, one of the

earliest changes observed in the development of NaChBac⁺ neurons was the increase in perisomatic GABAergic inputs at ~13 dpi (Fig. 2). At 17 dpi, the overall current of sIPSCs received by NaChBac⁺ neurons was 10 times that of controls (Fig. 3A, bottom panel, right). An alteration of this magnitude early in neuronal development could have a significant impact on subsequent integration. Second, the premature upregulation of KCC2 (Fig. 3B) suggests that the action of GABA could be hyperpolarizing earlier in the maturation of NaChBac⁺ neurons. Rendering GABA hyperpolarizing on immature neurons by altering chloride concentration is known to affect the dendritic development of adult-born GCs in the DG (Ge et al., 2006).

The transcription factor *Npas4* was a likely candidate underlying the increase of inhibition in NaChBac⁺ neurons, because it is regulated by activity and is involved in the activity-dependent regulation of inhibitory synapses in hippocampal neurons (Lin et al., 2008). We hypothesized that the increased inhibition observed in hyperexcitable new DG GCs could be attributable to the expression of *Npas4*.

To study the effects of expressing NaChBac in the absence of *Npas4*, we expressed Cre recombinase, NaChBac, and a fluorescent protein in individual neurons in the DG of *Npas4* conditional knock-out mice. The expression level of a tricistronic vector containing the three abovementioned genes was too low for visualization of the labeled neurons. To achieve stronger expression of fluorescent proteins, we injected a mixture of viruses into the DG of *Npas4* conditional knock-out mice. The first virus carried a bicistronic construct expressing GFP and Cre recombinase, and the second virus carried an invertible cassette with a bicistronic construct encoding both PalmMCherry and NaChBac. The invertible cassette is in the reverse 3' to 5' orientation with respect to the retroviral promoter except in the presence of Cre recombinase when it flips to the correct 5' to 3' orientation and expresses both mCherry and NaChBac (Fig. 8). In this manner, the presence of Cre leads to the expression of mCherry and NaChBac and, simultaneously, to the deletion of the *Npas4* locus in individual GCs in the *Npas4* conditional knock-out mice (Fig. 8). The PalmMCherry protein localizes to the membranes of such neurons, enabling the identification of fine structural features, such as synaptic spines. In this experiment, we used the same dual virus strategy in both wild-type mice and conditional knock-out mice and compared results between them.

As described above, expression of NaChBac in wild-type adult mice results in an increase in VGAT⁺ perisomatic inhibitory terminals on new DG GCs (Fig. 2A). In contrast, the deletion of *Npas4* in individual NaChBac⁺ new GCs blocked the increase in VGAT terminals triggered by NaChBac at both 17 and 28 dpi (Fig. 9A). Knocking out *Npas4* alone in DG GCs, using a virus carrying only a GFP–Cre recombinase construct, has no effect on the number of VGAT⁺ puncta at 17 dpi and leads to a very small increase at 28 dpi (Fig. 9A). Furthermore, *Npas4* signaling within individual adult-born neurons in the DG is necessary to trigger

Figure 9. Excitability-induced changes in input connectivity are dependent on cell-autonomous Npas4 signaling. **A**, Deletion of Npas4 blocked the increase in VGAT⁺ perisomatic contacts observed in NaChBac⁺ neurons at both 17 and 28 dpi (17 dpi: GFP control, 5.61 ± 0.34 VGAT⁺ puncta/soma, $n = 56$ neurons from 4 DGs; NaChBac⁺, 7.96 ± 0.34 VGAT⁺ puncta/soma, $n = 115$ neurons from 4 DGs; NaChBac⁺Npas4⁻, 6.64 ± 0.32 VGAT⁺ puncta/soma, $n = 84$ neurons from 5 DGs; GFP control vs NaChBac⁺, $**p < 0.005$; NaChBac⁺ vs NaChBac⁺Npas4⁻, $*p = 0.03$; 28 dpi: GFP control, 5.65 ± 0.33 VGAT⁺ puncta/soma, $n = 121$ neurons from 6 DGs; NaChBac⁺, 7.8 ± 0.26 VGAT⁺ puncta/soma, $n = 115$ neurons from 6 DGs; NaChBac⁺Npas4⁻, 5.33 ± 0.64 VGAT⁺ puncta/soma, $n = 70$ neurons from 5 DGs; GFP control vs NaChBac⁺, $***p = 0.0005$; NaChBac⁺ vs NaChBac⁺Npas4⁻, $*p = 0.017$). Absence of Npas4 alone did not decrease the number of contacts and caused a slight increase at 28 dpi (17 dpi: Npas4⁻, 5.84 ± 0.37 VGAT⁺ puncta/soma, $n = 85$ neurons from 4 DGs; E191K control vs Npas4⁻, $p = 0.67$; 28 dpi: Npas4⁻, 6.61 ± 0.26 VGAT⁺ puncta/soma, $n = 130$ neurons from 5 DGs; E191K control vs Npas4⁻, $*p < 0.05$). **B**, High-magnification confocal maximal projection images showing that eliminating Npas4 signaling from NaChBac⁺ neurons effectively restored spine density and size to resemble that of controls. **C**, Deletion of Npas4 from NaChBac⁺ neurons had no change on the decrease in LMT density observed (NaChBac⁺, 0.002 ± 0.00046 LMT/ μ m, $n = 11$ images; NaChBac⁺Npas4⁻, 0.0037 ± 0.00086 LMT/ μ m, $n = 18$ images, $p = 0.1$; E191K control vs NaChBac⁺, $***p = 0.0003$; E191K control vs NaChBac⁺Npas4⁻, $**p = 0.006$; NaChBac⁺Npas4⁻ vs Npas4⁻, $**p = 0.008$). **D**, Absence of Npas4 signaling prevented decrease in spine density resulting from NaChBac activity; there was no significant difference between the spine density on control

the changes in dendritic morphology induced by NaChBac⁺ neurons (Fig. 9B,D,E). Deletion of Npas4 in new GCs blocked the changes caused by NaChBac expression in dendritic length, spine density, and size. Dendritic length and spine density in NaChBac⁺Npas4⁻ neurons are indistinguishable from those of control neurons expressing the pore-dead version of NaChBac (Fig. 9B,D).

Interestingly, Npas4 was not required for activity-dependent changes in the output synapses, because NaChBac⁺Npas4⁻ neurons still exhibited a dramatic reduction in density of LMTs at CA3 (Fig. 9C). Knocking out Npas4 alone has no effect on either input or output synapses (Fig. 9B–E). These results demonstrate that Npas4 signaling specifically regulates activity-dependent formation of synaptic inputs to DG GCs.

Discussion

Increase in electrical activity of a single new neuron in the DG is sufficient to induce changes in maturation and connectivity

Global manipulations of brain activity via behavioral paradigms or seizures have demonstrated the influence of neuronal activity on the maturation, integration, and connectivity of adult-born neurons in the DG (Kee et al., 2007; Kron et al., 2010). However, it is unclear whether these changes resulted directly from cell-autonomous increased firing of new neu-

neurons and that of NaChBac⁺ neurons lacking Npas4 (E191K control, 1.05 ± 0.001 spines/ μ m, $n = 21$ images from 4 DGs; NaChBac⁺Npas4⁻, 1.03 ± 0.075 spines/ μ m, $n = 47$ images from 4 DGs, $p = 0.83$; E191K control vs NaChBac⁺, $**p = 0.007$; NaChBac⁺ vs NaChBac⁺Npas4⁻, $*p = 0.0235$). Deletion of Npas4 alone did not increase spine density (Npas4⁻, 1.19 ± 0.063 spines/ μ m, $n = 51$ images from 5 DGs; E191K control vs Npas4⁻, $p = 0.158$). **E**, Deletion of Npas4 from NaChBac⁺ neurons decreased dendritic spine size to almost as low as control levels (E191K control, 0.6 ± 0.009 μ m², $n = 24$ images from 3 DGs; NaChBac⁺Npas4⁻, 0.69 ± 0.018 μ m², $n = 37$ images from 4 DGs, $*p = 0.01$). Deletion of Npas4 alone did not decrease spine size but led to a very small increase (Npas4⁻, 0.71 ± 0.027 μ m², $n = 50$ images from 5 DGs; E191K control vs Npas4⁻, $*p = 0.02$; NaChBac⁺Npas4⁻ vs Npas4⁻, $p = 0.69$). Two-tailed *t* test used for statistical analysis. Error bars represent SEM. **F**, The electrical signatures of NaChBac action were similar regardless of the status of NPAS4. Left, Current injection steps in NaChBac⁺:Npas4^{-/-} neurons triggered long-lasting depolarizations in a similar mode as they did in NaChBac⁺:Npas4^{+/+} cells (compare with traces in Fig. 1C). Right, There was no significant difference between the average amplitude of NaChBac depolarizations in Npas4^{+/+} and NPAS4^{-/-} neurons: 66.3 ± 3.6 mV for 7–14 dpi, NaChBac⁺:Npas4^{+/+}; 66.12 ± 2.3 mV for 24–36 dpi, NaChBac⁺:Npas4^{+/+}; and 58.9 ± 10.36 mV for 21 dpi, NaChBac⁺:Npas4^{+/+}.

rons, indirectly through the elevated activity in the surrounding circuit, or from a combination of both. Here, we genetically modulate the electrical activity in individual adult-born DG GCs and show that an increase in cell-intrinsic activity of new neurons is sufficient to cause dramatic changes in their maturation and connectivity. NaChBac activity-induced connectivity changes in the adult DG appear to be homeostatic, because NaChBac induces an increase in inhibitory inputs and decrease in excitatory outputs. Furthermore, at 13 dpi, when NaChBac⁺ neurons display a significantly higher number of perisomatic VGAT⁺ puncta, more NaChBac⁺ neurons express KCC2 than control neurons (Fig. 3B). This observation is consistent with previous reports indicating that the timing of KCC2 expression is activity dependent (Ganguly et al., 2001). Because the upregulation of KCC2 relative to NKCC1 is correlated with the switch between GABAergic inputs being depolarizing to hyperpolarizing (Rivera et al., 1999; Wang et al., 2002), this suggests that the GABAergic input to NaChBac⁺ neurons becomes inhibitory earlier in their development than in control cells, which could serve to dampen the heightened excitability of these neurons.

The results of a previous study *in vitro* seemed to suggest that global activity alterations are necessary to affect changes in GABAergic terminals because suppression of single-cell activity in dissociated hippocampal cultures using the potassium channel Kir2.1 did not alter GABAergic inputs (Hartman et al., 2006). Our results show that, in new DG neurons *in vivo*, elevating single-cell activity is sufficient to induce changes in GABAergic input (Fig. 2). It is not possible to directly compare the results from these two experiments because they were produced in different conditions and systems, namely adult-generated DG neurons rendered hyperexcitable *in vivo* versus embryonic hippocampal neurons silenced *in vitro*. However, in agreement with this previous study, when we silenced adult-generated DG neurons *in vivo* via retroviral expression of the Kir2.1 channel, we did not observe any changes in either dendritic or axonal morphology (data not shown). These observations suggest that the regulation of synaptic input to a single adult-born DG GC may be modulated by increases, but not decreases, in intrinsic activity.

In our study, we observed that sEPSCs received by NaChBac⁺ neurons were of a lower frequency but of increased amplitude than those received by control neurons, and this was consistent with the decreased spine density but increased spine size on their dendrites (Fig. 5B,C). Interestingly, the overall excitatory current received was not significantly different compared with controls (Fig. 6A, right graph). In contrast, the overall GABAergic current received by NaChBac⁺ neurons was ~10 times that of control neurons (Fig. 3A, right graph). These findings suggest that there may exist mechanisms to ensure that new DG neurons receive a set value of excitatory input and that modulating inhibition may be the primary method by which the activity of an adult-born neuron in the DG is regulated.

An activity-dependent genetic program involving immediate early gene Npas4 governs neuronal connectivity of adult-born neurons to the mature DG circuit

Our results reveal an intermediate step between neuronal activity and changes in synaptic connectivity, which involves a transcription factor whose expression is activity dependent. The connectivity changes triggered by an increase in cell-intrinsic excitability in neurons are dependent on the immediate early gene Npas4. The role of Npas4 in these connectivity changes is activity dependent; deletion of Npas4 does not affect the formation of synapses in control cells that have baseline excitability, but it blocks synaptic alterations triggered by NaChBac-induced hyperexcitability.

Although Npas4 has been shown to regulate the formation of inhibitory inputs to CA1 pyramidal cells *in vitro* (Lin et al., 2008), our results in DG GCs suggests that knocking out Npas4 alone in individual neurons does not decrease the number of inhibitory VGAT⁺ contacts on its soma (Fig. 9A). Npas4 is expressed at extremely low levels at baseline in DG GCs, but it is upregulated by strong stimuli, such as during kainic acid-induced seizures (Ramamoorthi et al., 2011). These observations suggest that, in young DG GCs, the role of Npas4 in inducing an increase in inhibitory contacts may require exceeding a threshold of activity that is only achieved by hyperexcitable neurons.

Finally, Npas4 is involved in activity-dependent changes to input connectivity to adult-born DG GCs but not to their output connectivity in CA3 (Fig. 9C). This observation suggests that there are independent programs governing input and output synapses, and this finding could have important implications for the structural alterations triggered by epilepsy.

References

- Abusaad I, MacKay D, Zhao J, Stanford P, Collier DA, Everall IP (1999) Stereological estimation of the total number of neurons in the murine hippocampus using the optical disector. *J Comp Neurol* 408:560–566. [CrossRef Medline](#)
- Acsády L, Kamondi A, Sik A, Freund T, Buzsáki G (1998) GABAergic cells are the major postsynaptic targets of mossy fibers in the rat hippocampus. *J Neurosci* 18:3386–3403. [Medline](#)
- Bean BP (2007) The action potential in mammalian central neurons. *Nat Rev Neurosci* 8:451–465. [CrossRef Medline](#)
- Bi GQ, Poo MM (1998) Synaptic modifications in cultured hippocampal neurons: dependence on spike timing, synaptic strength, and postsynaptic cell type. *J Neurosci* 18:10464–10472. [Medline](#)
- Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH, Storm-Mathisen J (1998) The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. *J Neurosci* 18:9733–9750. [Medline](#)
- Clayton GH, Owens GC, Wolff JS, Smith RL (1998) Ontogeny of cation-Cl[−] cotransporter expression in rat neocortex. *Brain Res Dev Brain Res* 109:281–292. [CrossRef Medline](#)
- Crunelli V, Fonda S, Kelly JS (1984) The reversal potential of excitatory amino acid action on granule cells of the rat dentate gyrus. *J Physiol* 351:327–342. [Medline](#)
- Cull-Candy SG, Usowicz MM (1989) Whole-cell current noise produced by excitatory and inhibitory amino acids in large cerebellar neurones of the rat. *J Physiol* 415:533–553. [Medline](#)
- Erlander MG, Tobin AJ (1991) The structural and functional heterogeneity of glutamic acid decarboxylase: a review. *Neurochem Res* 16:215–226. [CrossRef Medline](#)
- Freund TF, Buzsáki G (1996) Interneurons of the hippocampus. *Hippocampus* 6:347–470. [CrossRef Medline](#)
- Ganguly K, Schinder AF, Wong ST, Poo M (2001) GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. *Cell* 105:521–532. [CrossRef Medline](#)
- Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H (2006) GABA regulates synaptic integration of newly generated neurons in the adult brain. *Nature* 439:589–593. [CrossRef Medline](#)
- Geiger JR, Jonas P (2000) Dynamic control of presynaptic Ca(2+) inflow by fast-inactivating K(+) channels in hippocampal mossy fiber boutons. *Neuron* 28:927–939. [CrossRef Medline](#)
- Hartman KN, Pal SK, Burrone J, Murthy VN (2006) Activity-dependent regulation of inhibitory synaptic transmission in hippocampal neurons. *Nat Neurosci* 9:642–649. [CrossRef Medline](#)
- Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R (2008) Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. *Nat Neurosci* 11:1153–1161. [CrossRef Medline](#)
- Jessberger S, Kempermann G (2003) Adult-born hippocampal neurons mature into activity-dependent responsiveness. *Eur J Neurosci* 18:2707–2712. [CrossRef Medline](#)
- Jessberger S, Zhao C, Toni N, Clemenson GD Jr, Li Y, Gage FH (2007) Seizure-associated, aberrant neurogenesis in adult rats characterized with

- retrovirus-mediated cell labeling. *J Neurosci* 27:9400–9407. [CrossRef Medline](#)
- Jessberger S, Clark RE, Broadbent NJ, Clemenson GD Jr, Consiglio A, Lie DC, Squire LR, Gage FH (2009) Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. *Learn Mem* 16:147–154. [CrossRef Medline](#)
- Johnson CP, Fujimoto I, Rutishauser U, Leckband DE (2005) Direct evidence that neural cell adhesion molecule (NCAM) polysialylation increases intermembrane repulsion and abrogates adhesion. *J Biol Chem* 280:137–145. [CrossRef Medline](#)
- Kee N, Teixeira CM, Wang AH, Frankland PW (2007) Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. *Nat Neurosci* 10:355–362. [CrossRef Medline](#)
- Kelsch W, Mosley CP, Lin CW, Lois C (2007) Distinct mammalian precursors are committed to generate neurons with defined dendritic projection patterns. *PLoS Biol* 5:e300. [CrossRef Medline](#)
- Kelsch W, Lin CW, Lois C (2008) Sequential development of synapses in dendritic domains during adult neurogenesis. *Proc Natl Acad Sci U S A* 105:16803–16808. [CrossRef Medline](#)
- Kelsch W, Lin CW, Mosley CP, Lois C (2009) A critical period for activity-dependent synaptic development during olfactory bulb adult neurogenesis. *J Neurosci* 29:11852–11858. [CrossRef Medline](#)
- Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. *Nature* 386:493–495. [CrossRef Medline](#)
- Kron MM, Zhang H, Parent JM (2010) The developmental stage of dentate granule cells dictates their contribution to seizure-induced plasticity. *J Neurosci* 30:2051–2059. [CrossRef Medline](#)
- Lewis PF, Emerman M (1994) Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol* 68:510–516. [Medline](#)
- Lin CW, Sim S, Ainsworth A, Okada M, Kelsch W, Lois C (2010) Genetically increased cell-intrinsic excitability enhances neuronal integration into adult brain circuits. *Neuron* 65:32–39. [CrossRef Medline](#)
- Lin Y, Bloodgood BL, Hauser JL, Lapan AD, Koon AC, Kim TK, Hu LS, Malik AN, Greenberg ME (2008) Activity-dependent regulation of inhibitory synapse development by Npas4. *Nature* 455:1198–1204. [CrossRef Medline](#)
- Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295:868–872. [CrossRef Medline](#)
- Muller D, Djebbara-Hannas Z, Jourdain P, Vutsits L, Durbec P, Rougon G, Kiss JZ (2000) Brain-derived neurotrophic factor restores long-term potentiation in polysialic acid-neural cell adhesion molecule-deficient hippocampus. *Proc Natl Acad Sci U S A* 97:4315–4320. [CrossRef Medline](#)
- Nitabach MN, Wu Y, Sheeba V, Lemon WC, Strumbos J, Zelensky PK, White BH, Holmes TC (2006) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. *J Neurosci* 26:479–489. [CrossRef Medline](#)
- Overstreet-Wadiche LS, Bromberg DA, Bensen AL, Westbrook GL (2006) Seizures accelerate functional integration of adult-generated granule cells. *J Neurosci* 26:4095–4103. [CrossRef Medline](#)
- Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci* 17:3727–3738. [Medline](#)
- Plotkin MD, Snyder EY, Hebert SC, Delpire E (1997) Expression of the Na-K-2Cl cotransporter is developmentally regulated in postnatal rat brains: a possible mechanism underlying GABA's excitatory role in immature brain. *J Neurobiol* 33:781–795. [CrossRef Medline](#)
- Ramamoorthi K, Fropf R, Belfort GM, Fitzmaurice HL, McKinney RM, Neve RL, Otto T, Lin Y (2011) Npas4 regulates a transcriptional program in CA3 required for contextual memory formation. *Science* 334:1669–1675. [CrossRef Medline](#)
- Ren D, Navarro B, Xu H, Yue L, Shi Q, Clapham DE (2001) A prokaryotic voltage-gated sodium channel. *Science* 294:2372–2375. [CrossRef Medline](#)
- Ribak CE, Tran PH, Spigelman I, Okazaki MM, Nadler JV (2000) Status epilepticus-induced hilar basal dendrites on rodent granule cells contribute to recurrent excitatory circuitry. *J Comp Neurol* 428:240–253. [CrossRef Medline](#)
- Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K (1999) The K⁺/Cl[−] co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* 397:251–255. [CrossRef Medline](#)
- Schmidt-Hieber C, Jonas P, Bischofberger J (2004) Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. *Nature* 429:184–187. [CrossRef Medline](#)
- Seki T, Arai Y (1993) Highly polysialylated neural cell adhesion molecule (NCAM-H) is expressed by newly generated granule cells in the dentate gyrus of the adult rat. *J Neurosci* 13:2351–2358. [Medline](#)
- Shapiro LA, Ribak CE (2006) Newly born dentate granule neurons after pilocarpine-induced epilepsy have hilar basal dendrites with immature synapses. *Epilepsy Res* 69:53–66. [CrossRef Medline](#)
- Thind KK, Ribak CE, Buckmaster PS (2008) Synaptic input to dentate granule cell basal dendrites in a rat model of temporal lobe epilepsy. *J Comp Neurol* 509:190–202. [CrossRef Medline](#)
- Tsien JZ, Chen DF, Gerber D, Tom C, Mercer EH, Anderson DJ, Mayford M, Kandel ER, Tonegawa S (1996) Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* 87:1317–1326. [CrossRef Medline](#)
- Turrigiano GG, Nelson SB (2004) Homeostatic plasticity in the developing nervous system. *Nat Rev Neurosci* 5:97–107. [CrossRef Medline](#)
- van Praag H, Kempermann G, Gage FH (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci* 2:266–270. [CrossRef Medline](#)
- Wang C, Shimizu-Okabe C, Watanabe K, Okabe A, Matsuzaki H, Ogawa T, Mori N, Fukuda A, Sato K (2002) Developmental changes in KCC1, KCC2, and NKCC1 mRNA expressions in the rat brain. *Brain Res Dev Brain Res* 139:59–66. [CrossRef Medline](#)
- Wiedenmann B, Franke WW (1985) Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of presynaptic vesicles. *Cell* 41:1017–1028. [CrossRef Medline](#)
- Yue L, Navarro B, Ren D, Ramos A, Clapham DE (2002) The cation selectivity filter of the bacterial sodium channel, NaChBac. *J Gen Physiol* 120:845–853. [CrossRef Medline](#)