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AXONAL PLASTICITY OF AGE-DEFINED DENTATE GRANULE CELLS IN A RAT MODEL OF MESIAL TEMPORAL LOBE EPILEPSY

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Abstract

Dentate granule cell (DGC) mossy fiber sprouting (MFS) in mesial temporal lobe epilepsy (mTLE) is thought to underlie the creation of aberrant circuitry which promotes the generation or spread of spontaneous seizure activity. Understanding the extent to which populations of DGCs participate in this circuitry could help determine how it develops and potentially identify therapeutic targets for regulating aberrant network activity. In this study, we investigated how DGC birthdate influences participation in MFS and other aspects of axonal plasticity using the rat pilocarpine-induced status epilepticus (SE) model of mTLE. We injected a retrovirus (RV) carrying a synaptophysin-yellow fluorescent protein (syp-YFP) fusion construct to birthdate DGCs and brightly label their axon terminals, and compared DGCs born during the neonatal period with those generated in adulthood. We found that both neonatal and adult-born DGC populations participate, to a similar extent, in SE-induced MFS within the dentate gyrus inner molecular layer (IML). SE did not alter hilar MF bouton density compared to sham-treated controls, but adult-born DGC bouton density was greater in the IML than in the hilus after SE. Interestingly, we also observed MF axonal reorganization in area CA2 in epileptic rats, and these changes arose from DGCs generated both neonatally and in adulthood. These data indicate that both neonatal and adult-generated DGCs contribute to axonal reorganization in the rat pilocarpine mTLE model, and indicate a more complex relationship between DGC age and participation in seizure-related plasticity than was previously thought.

Keywords

Mossy fiber sprouting; Seizure; Neurogenesis; Axonal reorganization; Neural stem cell

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Introduction

Dentate granule cell (DGC) axonal reorganization is a common feature of dentate gyrus histopathology in patients with mesial temporal lobe epilepsy (mTLE) that is recapitulated in many animal models of the disease (Sutula & Dudek 2007). Schleibel and colleagues first discovered the presence of aberrant terminals of DGC axons, known as mossy fibers, in the inner molecular layer (IML) of mTLE patients in 1974 (Scheibel et al 1974). In rodent models of mTLE, mossy fiber sprouting (MFS) into the IML creates a functional excitatory feedback loop, primarily forming synapses onto DGC apical dendrites, with a minority of sprouted synapses in the IML onto interneurons (Buckmaster et al 2002, Scharfman et al 2003). MFS has been linked with recurrent excitation of the DGC network (Scharfman et al 2003, Tauck & Nadler 1985, Winokur et al 2004, Zhang et al 2012) and positively correlated with the number of spontaneous seizures (Hester & Danzer 2013, Xu et al 2004). However, the role of MFS in epileptogenesis remains controversial as some studies find that it is not required for spontaneous seizure activity (Buckmaster 2014, Buckmaster et al 2009). Importantly, the focus of most research on DGC axonal reorganization in mTLE has been on IML MFS, but this plasticity also occurs within hippocampal area CA3 and potentially also in the dentate hilus.

The mechanisms that drive axonal reorganization of DGCs in mTLE models are not fully understood. IML sprouting is associated with seizure-induced changes such as cell death, altered extracellular matrix protein expression, and loss of axon guidance cues (Cavazos et al 1991, Cavazos & Sutula 1990, Holtmaat et al 2003, Pollard et al 1994). Plasticity of MFs in CA3 can occur in response to physiological stimuli such as exercise, in addition to seizures, and has been linked to loss of synaptic contacts in CA3 in a mTLE model (Danzer et al 2010, McAuliffe et al 2011, Schwarzer et al 1995, Toscano-Silva et al 2010). While nothing is known about seizure-related plasticity at the MF-CA2 synapse, recent data indicate that this synapse can be modulated by exercise and inflammation (Llorens-Martin et al 2015). In addition to external stimuli, there are cell-autonomous factors, such as mTOR signaling, that influence axonal reorganization within individual DGCs (Buckmaster et al 2009).

Not every DGC contributes to MFS (Buckmaster & Dudek 1999), and it is not known what influences the likelihood to contribute. DGC birthdate is one potential factor because of the well-documented role of adult-born DGCs in seizure-related plasticity (Jessberger et al 2007, Kron et al 2010, Walter et al 2007). Previous work suggested that DGCs developing during or after an epileptogenic insult are responsible for most, if not all, MFS in the IML (Kron et al 2010). However, the retrovirally-delivered green fluorescent protein (RV-GFP) label used in these experiments did not allow for reliable resolution of small MF boutons in the IML and hilus. Moreover, extensive dendritic labeling made it difficult to distinguish GFP-labeled axons in the IML. Moreover, a potential relationship between DGC birthdate and axonal plasticity within the hilus and hippocampal pyramidal cell regions has not previously been investigated.

In this study, we compared MF structural plasticity of DGCs born in the neonatal period, and thus mature at the time of injury, with those generated in adulthood after injury in the rat pilocarpine model of mTLE. To more specifically label the synaptic boutons of birthdated DGCs, we used a construct carrying the synaptophysin (syp) gene fused to yellow fluorescent protein (YFP) to direct YFP to synaptic terminals (Umemori et al 2004), and this construct was packaged in a RV vector to restrict infection to actively dividing cells. With this tool, we found that both neonatal and adult-born populations of DGCs participate in MFS in the rat pilocarpine mTLE model. We also showed that the DG-CA2 “alternative trisynaptic circuit” is altered after SE. Our findings suggest that both pre-existing, mature DGCs and those generated after SE contribute to various aspects of seizure-induced plasticity.

Methods

Virus production

We generated the syp-YFP RV using a vesicular stomatitis virus G-protein (VSV-G) pseudotyped Moloney murine leukemia virus backbone in which the expression of syp-YFP is driven by the human synapsin-1 promoter. The syp-YFP fusion gene was amplified from the pCMV-syp-YFP plasmid (Umemori et al 2004) via PCR using primers 5'-cgagctcaaggatccaattctgcagtcg-3' and 5'-ctgattatgatcacgcgtcgcggcc-3'. To express the syp-YFP under the control of the human synapsin-1 (syn1) promoter (a generous gift from Ed Callaway), the amplified and isolated syp-YFP fragment was subcloned into the RV vector pCAG-mCherry-WPRE (woodchuck post-transcriptional regulatory element) by replacing the mCherry fragment between pCAG and WPRE (**Figure 1A**). The accuracy of the cloned syp-YFP fragment in the construct was verified by DNA sequencing and the expression of syp-YFP was tested by transfecting HEK 293 cells using Lipofectamine™ (Invitrogen, CA) as it is weakly expressed in HEK cells (and strongly in neurons). The cells were cultured at 37°C for 3 days prior to analysis. We also used a GFP-expressing RV as described previously (Kron et al 2010).

High-titer, replication incompetent pseudotyped RV was produced as previously described (Kron et al 2010) by co-transfection of CAG-GFP-WPRE or syn1-syp-YFP-WPRE and VSV-G plasmids into the GP₂-293 packaging cell line (Clontech, CA). Cells were plated in 10 ml DMEM supplemented with 10% FBS the day prior to transfection. The co-transfection was performed using calcium phosphate precipitation. The transfected cells were incubated at 37°C for 6 hours, the medium was replaced and cells were allowed to grow for 65 hours. The supernatant containing RVs were harvested and filtered through a 0.45-μm pore size filter (Gelman Sciences, MI). The filtered supernatant was concentrated by centrifugation in a Sorvall model *RC 5C PLUS* at 50,000×g at 4°C for 90 min. The RV-containing pellet was resuspended in 1X PBS, aliquoted, and was stored at -80°C until use. Final RV titers for RV-GFP ranged between 2-5 X 10⁸ cfu/ml as measured by GFP colony formation on NIH 3T3 cells, and were similar for the RV-syp-YFP virus based upon in vivo transduction efficiency (the syn1 promoter does not drive expression in NIH 3T3 cells).

Animals

Animal procedures were performed using protocols approved by the University Committee on Use and Care of Animals of the University of Michigan. Animals were purchased from Charles River and kept under a constant 12 hour light/dark cycle with access to food and water *ad libitum*. Epileptic animals (n = 32) and sham controls (n = 11) were generated as described previously (Kron et al 2010). Briefly, eight week-old male Sprague Dawley rats were pretreated with atropine methylbromide (5 mg/kg i.p.; Sigma-Aldrich, St. Louis, MO) 20 minutes prior to induction of SE with pilocarpine hydrochloride (340 mg/kg i.p.; Sigma-Aldrich, St. Louis, MO) for epileptic animals, or an equivalent volume of 0.9% saline for sham animals. After 90 minutes of SE, seizures were terminated with diazepam (10 mg/kg i.p.; Hospira Inc, Lake Forest, IL). Sham controls were treated with diazepam two hours after the saline injection.

Intrahippocampal RV injections

Animals were injected with Syp-YFP RV, or in some cases GFP RV, bilaterally into the dorsal dentate gyrus at either postnatal day 7 (P7) or postnatal day 60 (P60) as described previously (Kron et al 2010). Briefly, male P7 rat pups were anesthetized on ice and placed on an ice cold neonatal rat stereotax adaptor (Stoelting, Wood Dale, IL) in a Kopf (Tujunga, CA) stereotaxic frame. Bilateral burr holes were drilled in the skull and 1 μ l of RV was injected, using a Hamilton syringe and microinjection pump, at 0.1 μ l/min into each hemisphere with the following coordinates (in mm from bregma and mm below the skull): caudal 2.0, lateral 1.5, deep 2.7. P60 male rats were anesthetized with a ketamine/xylazine mixture and placed in a Kopf stereotaxic frame. Bilateral burr holes were drilled in the skull and 2.5 μ l of RV was injected as in neonatal rats but with the following coordinates (in mm from bregma and mm below the skull): caudal 3.9, lateral 2.3, deep 4.2.

Immunohistochemistry

At 1, 2, 4 or 8-10 weeks post SE or sham treatment, rats were deeply anesthetized with pentobarbital and transcardially perfused with 0.9% saline and 4% paraformaldehyde (PFA). Brains were removed and post-fixed overnight in 4% PFA. Sixty μ m sections were cut in the coronal plane using a vibrating blade microtome (VT1000S, Leica Microsystems Inc, Buffalo Grove, IL). Representative sections (4-6 sections, 720 μ m apart) through the rostro-caudal axis of the brain were processed with standard fluorescent immunohistochemical techniques using the following primary and secondary antibodies: mouse anti-bassoon (1:1000, Enzo Life Sciences), rabbit or chicken anti-GFP (1:2000 for both, Invitrogen and Aves), mouse anti-striatal enriched phosphatase (STEP) (1:1000, Cell Signalling), rabbit anti-zinc transporter-3 (Znt3) (1:3000, Synaptic Systems), or mouse anti-regulator of G-protein signaling 14 (RGS14) (1:100, NeuroMab). Fluorescent signals were achieved using Alexa Fluor secondary antibodies: goat anti-mouse 594, goat anti-mouse 647, goat anti-rabbit 488 or 594, and goat anti-chicken 488.

Microscopy

Images were acquired using a Zeiss LSM 510-META Laser Scanning Confocal Microscope or Leica Upright SP5X Confocal Microscope. For confirmation of co-localization and

analysis of bouton size and density, images were acquired with a 63× objective at 2× optical zoom as 1 μm stacks through the z-plane with the pinhole set at 1 Airy unit and 0.5 μm step size. For analysis of amount of axon sprouting into the IML and pyramidal cell regions, sections were imaged at 2048 resolution with a 10× objective at 1 or 2× optical zoom, as 40-60 μm stacks through the z-plane with the pinhole set at 1 Airy unit and a 6 μm step size.

Image analysis and statistics

Image files were imported into ImageJ for analysis. Statistical analyses were performed using GraphPad Prism 6.0 software. Co-localization of YFP-positive bouton-like structures with bassoon was first established using 1 μm optical slice images, prior to further image processing. To calculate IML and hilar bouton density, axon segments were first traced through image stacks and straightened. Images were then made binary and the analyze particle function was used to count all boutons with at least 10% circularity and an area larger than 0.25 μm². The number of boutons was then divided by the length of axon segment to determine bouton density. The average density was determined for each animal, and group means were compared with a one-way ANOVA and Sidak's multiple comparisons test. All error bars represent standard error.

Relative amounts of sprouting into the IML by birthdated DGCs was determined from z-projections by identifying regions within tissue sections containing IML sprouting where labeled axons were present but labeled apical dendrites were not. Regions in the IML were outlined using the Region of Interest (ROI) function in ImageJ. The same sized ROI was then copied and placed over the hilus directly below the GCL. Using the same ROI for both areas of each section allowed for direct comparison of the percentage of YFP labeling within the ROI. Once ROIs were determined for all sections within the image, the image was made binary and the number of labeled and unlabeled pixels was determined using the histogram function. From these data, the percentage of labeled pixels was determined for each ROI. The percent of labeling in the IML ROI was divided by the percent of labeling in the hilar ROI to determine the sprouting ratio for that section. The sprouting ratios for all sections from a given animal were averaged to determine a single sprouting ratio for each animal. Group means were statistically compared using a student's t-test.

Length of CA2 was determined for each section containing syp-YFP-labeled boutons by using the segmented line function in ImageJ and tracing the cell body layer of STEP-labeled cells. Tissue from a subset of animals was stained for both RGS-14 and STEP and the patterns were similar for both sham and SE-treated animals, so only data from STEP-labeled tissue were quantified. The borders of CA2 were defined by a sharp reduction in STEP label intensity. Sections for which the borders of CA2 could not be clearly delineated were not included in the analysis. The amount of syp-YFP innervation in the CA2 region was measured with the segmented line function by starting from the CA3/CA2 border and tracing to the furthest syp-YFP labeled structure within the STEP-labeled area. The length of the syp-YFP-containing region in CA2 was divided by the total length of CA2 within an individual slice to determine percent of CA2 that is innervated by labeled axons.

Results

Synaptophysin-YFP retrovirus labels DGC axon terminals

Previous work by our group and others has involved using RV carrying a cytoplasmic GFP reporter as a means of birthdating and labeling subsets of DGCs to study their morphology after they mature (Jessberger et al 2007, Kron et al 2010, van Praag et al 2002). This tool is excellent for examining changes in soma location, dendritic morphology, and giant MF boutons, but is limited for studying changes in finer axon terminal structures. Given our interest in seizure-induced plasticity of MF axon terminals, we developed a RV that preferentially labels presynaptic terminal structures to better visualize seizure-induced changes in axonal projections and synaptic remodeling.

Synaptophysin is a synaptic vesicle-associated protein that is abundant in presynaptic terminals (Li & Murthy 2001). The syp-YFP fusion protein is comprised of full-length synaptophysin directly conjugated to full-length YFP, such that YFP expression is highly enriched in synaptic boutons (**Figure 1A, B**). Delivering this construct in a RV vector allows us to simultaneously birthdate DGCs and intensely label the MF terminals to a much greater extent than with the cytoplasmic GFP RV (**Figure 1B-D**). RV overexpression of syp-YFP often leads to diffuse, albeit weaker, labeling in the soma and dendrites (**Figure 1D₂ yellow arrows**) in addition to strong labeling of putative MF boutons. While the difference in label intensity between MF boutons and non-bouton-labeled structures provides a straightforward means of distinguishing them, we also used double-label fluorescence immunohistochemistry (IHC) to identify co-labeling of syp-YFP+ MF boutons with the synaptic protein bassoon, which is expressed exclusively in presynaptic terminals (tom Dieck et al 1998) (**Figure 1E**). Most tissues showed well-delineated bassoon immunoreactivity, and all syp-YFP-labeled putative boutons (identified on the basis of their size and shape) co-expressed bassoon. These findings suggest that RV-syp-YFP is a powerful tool for both birthdating DGCs and tracking their axonal projections.

Both adult- and neonatal-born DGCs contribute to MFS in the IML

Our previous work using a GFP-expressing RV showed that neonatal-born DGCs that were 7 weeks old when animals underwent SE rarely exhibited morphological hallmarks of aberrant seizure-induced plasticity. In contrast, DGCs born after SE exhibited substantial aberrant plasticity, including the presence of persistent hilar basal dendrites (HBDs), ectopically located somas or MFS (Kron et al 2010). To better assess seizure-induced axonal changes, we used syp-YFP RV to label neonatal- or adult-generated DGC cohorts and examined YFP immunoreactivity in hippocampal sections after labeled DGC progenitors had at least 8 weeks to mature. Rats received syp-YFP RV at P7 or P60, underwent pilocarpine-induced SE at P56 and were euthanized 8 weeks later. These two time points were chosen for RV injection to label DGCs at two different stages of development during epileptogenesis: pre-existing, mature DGCs and those generated 4 days after SE. The syp-YFP RV allowed us to clearly distinguish between axons and dendrites in the IML and to identify low levels of MFS. Using this approach, we found that both neonatal and adult-born DGC populations contributed to MFS in the IML at 8 weeks after SE, while the controls as expected showed no punctate terminal labeling in the IML (**Figure 2**).

Neonatal-born DGCs participate in MFS earlier after SE than adult-born DGCs

Sprouted MF terminals begin to appear in the IML within days after SE in rodent models and continue to increase in number until they densely innervate the IML (Cavazos et al 1991, Mello et al 1993). We therefore performed a time course experiment in which tissues were collected at 2 or 4 weeks after SE to determine when the two populations of cells begin to contribute to MFS. We found that sprouted MFs from neonatal-born DGCs, but not adult-born DGCs, were present in the IML by 2 weeks post SE (**Figure 3A**). At 4 weeks after SE, sprouted MFs from both populations appeared in the IML (**Figure 3B**), and the amount of MFS increased by 8 weeks post SE (**Figure 3C**).

To further support the finding that MFS from neonatal-born DGCs, which are mature at the time of SE, begins sooner after SE than for adult-born DGCs, we examined syp-YFP labeling of neonatal-born DGCs at 1 week after SE. We observed clear evidence of YFP-positive MF terminals in the IML at this time point in 2 of 4 animals (**Supplemental Figure 1**). Dividing DGC progenitors are unlikely to have sufficient time to become post-mitotic and differentiate to the point of axon elaboration within a week after SE. Thus, these data support the idea that YFP+ IML MF terminals in P7-injected rats arose from DGCs that were mature at the time of SE, rather than from P7 labeling of NSCs that continued to generate DGCs into adulthood.

To investigate the relationship between DGC birthdate and amount of IML sprouting, we determined a sprouting ratio for each animal by measuring the percentage of syp-YFP+ bouton labeling in the IML vs. hilus of discrete regions within the dentate gyrus (excluding the apex and outer edges of the GCL). There was no significant difference in the mean sprouting ratio between neonatal- and adult-generated DGCs (0.3794 and 0.3370, respectively) (**Figure 4**; $p = 0.65$, student's t-test).

Features of IML and hilar MF boutons are similar between adult- and neonatal-born DGCs after SE

The dentate hilus undergoes substantial structural changes during epileptogenesis that may influence local MF synapses. These include rapid loss of interneurons and mossy cells, and a progressive increase in aberrant DGC dendrites, either from HBDs or hilar-ectopic DGCs (Buckmaster & Dudek 1997, Parent et al 2006, Parent et al 1997, Scharfman et al 2000, Spigelman et al 1998). To determine whether mossy fiber terminals of adult- or neonatal-born DGCs in the hilus are altered after SE and compare them to IML MFs, we examined bouton density of individual YFP+ axons in the IML and hilus from pilocarpine- and sham-treated animals. We identified axon segments that could be easily distinguished from other structures (**Fig. 5A-C**) and determined the average number of boutons per 10 microns of axon for each animal. We found that axon segments of adult-born DGCs had a greater bouton density in the IML than in the hilus (**Figure 5D**; $p = 0.031$, one-way ANOVA with Sidak's multiple comparisons test). Bouton densities of axon segments in the IML of neonatal-born DGCs after SE were not significantly different from those in the hilus ($p = 0.51$), and we found no statistically significant difference in bouton density of DGC axons across the 4 populations in the hilus ($p = 0.99$ for P7 vs. P60 birthdate; $p = 0.37$ and $p = 0.94$ for P7 and P60 SE vs. sham, respectively; **Figure 5D**).

Plasticity of pyramidal cell innervation by MFs

DGCs synapse onto CA3 pyramidal neurons as part of the classic trisynaptic circuit in the hippocampus. The giant MF boutons form synapses primarily onto the apical dendrites of pyramidal cells in stratum lucidum. Under normal conditions, a small proportion of MF boutons also synapse onto basal dendrites in stratum oriens (SO) (Blaabjerg & Zimmer 2007). In some mTLE models, MFs sprout additional collaterals into more caudal regions of SO (Schwarzer et al 1995). We observed syp-YFP labeled axons in CA3 SO from DGCs in all four treatment groups (data not shown). In sham-treated animals, axon terminals from both neonatally generated and adult-born DGCs were present in SO of rostral sections. However, we did not observe any apparent differences in amount of SO labeling across any of the groups.

An “alternative trisynaptic circuit” was recently identified in which small boutons of DGCs functionally innervate CA2 pyramidal neurons (Kohara et al 2014). We investigated whether this innervation changes after SE and if so, whether there is a difference between neonatal- and adult-born DGCs. Using antibodies against the CA2-specific marker STEP, we observed considerable damage to CA2 neurons in many animals that had undergone SE (**Figure 6A**). We found that the CA2 region was significantly smaller in animals that underwent SE, compared to sham-treated animals (**Figure 6B**; $p = 0.003$, student's t-test). As a result, we normalized each measurement to the length of CA2 within individual sections to determine the percentage by length of CA2 that was innervated by syp-YFP-labeled axons. We found that labeled axons from both neonatal- and adult-born DGCs traverse a greater percentage of CA2 after SE when compared to sham-treated animals (**Figure 6C, D**; $p = 0.01$, one-way ANOVA with Sidak's multiple comparisons test), but no difference was present between the two birthdated populations within SE ($p = 0.94$) or control ($p = 0.99$) groups.

Discussion

The goal of this study was to determine the relative contributions of age-defined cohorts of DGCs to aberrant axonal reorganization in a rat mTLE model. Using syp-YFP RV to birthdate neonatal- and adult-born DGCs and label their axon terminals, we found that cells that are mature at the time of SE and cells that are born after SE contribute similarly to seizure-related axonal plasticity. We also report a novel type of seizure-induced plasticity not previously described, which involves changes to mossy fiber innervation of CA2 pyramidal cells.

Initially, we were surprised to find sprouted MF axons from neonatal-born DGCs in the IML after SE. Previous work from our laboratory found that DGCs that were born neonatally and were 7 weeks old when animals underwent SE were resistant to seizure-related plasticity, whereas many DGCs born after SE exhibited HBDs, ectopic somas, or MFS (Kron et al 2010). However, those results were obtained using a cytoplasmic GFP RV, which has strong somatic and dendritic labeling, but much weaker labeling of axons. With the syp-YFP RV, we identified labeled axons in the IML from both birthdated populations, neonatal-born and adult-born, after SE and confirmed the presence of synaptic boutons by double labeling with bassoon.

We considered the possibility that a small number of radial glia-like (RG-like) neural stem cells may have been infected when RV was injected in neonatal animals. RG-like neural stem cells are relatively quiescent and so could retain the label and divide after SE to produce adult-born DGCs, even after P7 RV injection, which might be responsible for the observed IML syp-YFP labeling. The time course data indicate, however, that the labeled fibers do indeed arise from DGCs that were born neonatally and had matured by the time of SE because MFS arises from this population within one week after SE, while MFS could not be detected from the adult-born population until four weeks post-SE.

It is interesting that the axons of adult-born DGCs examined at 2 weeks post SE are not yet found in the IML, considering the fact that they are already present in the hilus and the neonatal-born DGCs have begun to sprout axons into the IML by this time. This suggests that MFs of adult-born DGCs are not simply responding to axon guidance cues induced after SE, and that additional factors are required for them to sprout into the IML. One such factor might be excitatory synaptic input. Excitatory synaptic activity, via long-term potentiation (LTP) induction in the perforant path, can induce MFS in the absence of SE (Adams et al 1997). Perhaps differential excitatory drive could account for differences in timing of sprouting or even explain why only subsets of cells participate in sprouting (Buckmaster 2014). To label adult-born DGCs, the RV is injected four to five days after SE. Therefore, these cells are 9-10 days old at 2 weeks post SE. At this stage in normal rodents, adult-born DGCs have short processes and do not receive excitatory synaptic input (Overstreet-Wadiche et al 2006a, Zhao et al 2006). Although adult-born DGCs in mouse develop more rapidly after SE (Overstreet-Wadiche et al 2006b), 10-day-old cells are still unlikely to be receiving significant amounts of excitatory synaptic input. Thus, the requirement for excitatory innervation would explain temporal and inter-cell variability in sprouting and this mechanism may also account for the observation that MFS is an ongoing process, occurring throughout the disease course (Buckmaster 2014, Lew & Buckmaster 2011). Nonetheless, other factors are likely involved given that altering intrinsic signaling pathways can temporarily suppress MFS, as discussed below.

After finding that both neonatal- and adult-generated DGC populations sprout axons into the IML after SE, we sought to determine whether the degree of sprouting differed between these cohorts. To determine the relative amount of MFS, which varies between animals partly due to RV infection efficiency, we calculated a sprouting ratio for each animal. This ratio varied across animals, but did not differ significantly between the two birthdated populations. Thus, the net contribution to MFS appears similar for both neonatal- and adult-born DGCs.

We did find a significant difference in bouton density between IML and hilar axon segments of adult-born DGCs. Without a measurement of total axon length in IML vs hilus, however, it is difficult to speculate on the net impact of this increased bouton density. Several comprehensive studies examining the structure of DGC axon arbors from epileptic and intact tissue indicate that the total axon length of DGCs increases in epileptic animals (Buckmaster & Dudek 1999, Claiborne et al 1986, Sutula et al 1998). One study, using the kainic acid SE model of mTLE, also found a significant increase in IML bouton density when compared to hilar bouton density, as well as a small, but significant increase in hilar

bouton density when compared with control animals (Sutula et al 1998). None of the aforementioned studies examined DGC birthdate, and it is unclear why only adult-born DGCs exhibit significantly increased IML bouton density and whether this translates into relatively increased recurrent excitation for this subpopulation of DGCs.

The net impact of MFS in the IML on epileptogenesis is still unclear. Recent work has shown that seizure-induced MFS is blocked by continuous treatment with rapamycin (Buckmaster et al 2009, Zeng et al 2009). Interestingly, rapamycin treatment does not reliably affect seizure frequency, and MFS in the IML recovers once drug treatment is stopped (Buckmaster et al 2009, Buckmaster & Lew 2011, Lew & Buckmaster 2011). These data indicate that MFS is not necessary for the development of spontaneous seizure activity in rodent mTLE models, and that there is no critical window during epileptogenesis for the development of MFS. Importantly, however, DGC MFS in the IML is not the only feature of seizure-related plasticity that is affected by continuous rapamycin treatment. Rapamycin acts to block mechanistic target of rapamycin (mTOR) signaling, which is involved in many cellular processes, including new protein synthesis (Sandsmark et al 2007). Continuous rapamycin treatment also blocks inhibitory axon sprouting, which is hypothesized to be an important homeostatic mechanism to reduce network excitability (Buckmaster & Wen 2011). In addition, the effects of this treatment on axonal plasticity in the dentate hilus, CA3 and CA2, or on other aspects of seizure-related plasticity have not been fully examined. Therefore, the effect of rapamycin treatment on seizure activity should not be regarded as equivalent to the effect blocking DGC axon plasticity on seizure activity.

In addition to axonal reorganization within the dentate gyrus, we examined seizure-related plasticity of DGC axons in pyramidal cell layers CA3 and CA2. While others have found sprouting of MF axons into SO of CA3, we were unable to consistently detect axons of birthdated cells – either neonatal or adult-born – in this region. This finding is likely due to variability in viral infection efficiency and location of labeled DGCs within the granule cell layer, because the path of DGC axons is influenced by their location within the different blades of the layer (Claiborne et al 1986).

We did, however, observe axon terminals in CA2 in all animals. The functionality of this projection has only recently been described (Kohara et al 2014) and its role in the propagation of seizure activity is still unknown. To our knowledge, this is the first report of seizure-induced plasticity at the MF-CA2 synapse. We found that MFs of both birthdated populations of DGCs innervate a greater proportion of CA2 in epileptic than in control tissue. Somewhat surprisingly, this plasticity may be driven, at least in part, by cell death of CA2 pyramidal neurons. Seizure-induced pyramidal cell death within CA3 and CA1 is widely acknowledged, but CA2 pyramidal neurons are thought to be relatively spared after SE (Margerison & Corsellis 1966). However, we observed a significant reduction in the size of CA2, consistent with pyramidal cell loss, after SE. While this is not the first report of damage to the CA2 subfield following pilocarpine-induced SE (Fujikawa 1996), it is the first using CA2-specific antibodies to assess this region.

Llorens-Martin and colleagues investigated the impact of prolonged inflammation on the structure of MF boutons at the CA2 synapse and found a significant shift toward smaller

boutons in animals exposed to chronic inflammation (Llorens-Martin et al 2015). We saw no change in average bouton area or in distribution of large boutons in animals that experienced pilocarpine- induced SE for either neonatal- or adult-born DGCs (data not shown). Given that inflammation is a well-established component of SE, we were somewhat surprised to find no change in CA2 bouton size. However, the discrepancy may be due to a difference in species (rat vs mouse) or different experimental time courses. Llorens-Martin et al. examined CA2 bouton size immediately after 8 weeks of persistent inflammation induced by continuous lipopolysaccharide infusion (Llorens-Martin et al 2015), whereas we waited until 8 weeks after SE to measure CA2 bouton size.

We have shown that both adult- and neonatal-born DGCs contribute similarly to aberrant axonal reorganization after SE. However, this finding does not necessarily indicate that the axonal reorganization of the two populations has an equivalent impact on the abnormal network formation and hyperexcitability that contributes to chronic epilepsy. Further work is needed to establish why specific DGC subsets participate in MFS, whether the extent of axonal plasticity varies for individual cells, and what cell-intrinsic and extrinsic factors control MFS. Additionally, whether specific populations of DGCs selectively receive recurrent mossy fiber innervation remains a critical question. One of these populations may be hilar ectopic DGCs, which have been shown to receive MF inputs (Dashtipour et al, 2001; Pierce et al, 2005). We were not able to resolve whether birthdated, syp-YFP-labeled MF terminals synapse specifically onto hilar ectopic granule cells as it is difficult to specifically label the cytoplasm or plasma membrane of these cells. Future studies combining the use of a cytoplasmic RV reporter with the syp-YFP retrovirus could address this question. Lastly, the effect of birthdated populations of DGCs on the development of aberrant network structure needs to be considered in the context of other aspects of seizure-induced plasticity, which are known to be unique to adult-born DGCs, including ectopic location and persistent hilar basal dendrites. Novel methods, such as the RV axon terminal reporter described in this study, should help to clarify these issues, eventually unravel the mechanisms underlying mTLE, and lead to anti-epileptogenic therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations (not standard in the field)

mTLE	mesial temporal lobe epilepsy
DGC	Dentate granule cell
MFS	Mossy fiber sprouting

GCL	Granule cell layer
IML	inner molecular layer

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Highlights

- A retroviral synaptic reporter birthdates neurons and labels their axon terminals
- Neonatal- and adult-born DGCs contribute to seizure-induced mossy fiber sprouting
- Mossy fiber remodeling occurs in CA2 in experimental temporal lobe epilepsy

Figure 1.

The syp-YFP RV allows better resolution of small MF boutons in the hilus and IML of the hippocampal dentate gyrus as compared to GFP RV. **A.** Diagram of the RV-syp-YFP construct with syp-YFP driven by the human synapsin-1 promoter (Syn1). LTR = long terminal repeats of the RV backbone. Dashed lines outline the granule cell layer (GCL). **B.** Low magnification (5×) confocal image obtained from an animal injected with syp-YFP-RV at P60, 4 days after pilocarpine treatment, and euthanized 8 weeks later. Axon terminals can be seen throughout the hilus, in CA3, and even in the IML (arrow). Scale bar = 200 μm. **C,**

D. Representative lower (**C**; 10× objective, scale bar = 150 μm) and higher (**D**; 20× objective with 2× optical zoom, scale bar = 25 μm) magnification confocal images from rats that experienced SE and injections of eGFP RV (**C₁**, **D₁**) or syp-YFP RV (**C₂**, **D₂**) to label DGC progenitors. Rats were euthanized 8 weeks later. MFS (white arrows in **C₂**, **D₂**) and abundant hilar boutons are detected from DGCs that express syp-YFP (**C₂**, **D₂**), while the most prominent hilar structures from DGCs that express eGFP (**C₁**, **D₁**) are HBDs (yellow arrowheads in **D₁**) and glia (yellow asterisk in **D₁**) with minimal axonal labeling. The syp-YFP RV does label some somas and dendrites (yellow arrows in **D₂**). Blue is Hoescht and green is GFP (**C₁**, **D₁**) or YFP (**C₂**, **D₂**). **E.** Confocal z-stack images obtained with a 63× objective and 2× optical zoom of a hilar axon segment from a syp-YFP expressing DGC. **E₁** YFP alone (green). **E₂** Bassoon (red), a presynaptic terminal marker. **E₃** Merged images showing co-localization of YFP and bassoon in the terminals. Scale bar = 10 μm.

Figure 2.

Both neonatal- and adult-born DGCs contribute to MFS in the IML. **A.** Confocal images of neonatal-born DGCs injected with syp-YFP RV at P7 and expressing syp-YFP at 8 weeks after **A₁**) sham treatment or **A₂**) pilocarpine-induced SE. **B.** Confocal images of adult-born DGCs injected with syp-YFP RV at P60 and expressing syp-YFP at 8 weeks after **B₁**) sham treatment or **B₂**) pilocarpine-induced SE. Arrows (in **A₂**, **B₂**) indicate YFP-labeled axon terminals in the IML. Scale bar = 200 μ m.

Figure 3.

Neonatal-born DGCs begin to sprout axons into the IML sooner after SE than adult-born DGCs. **A-C.** Confocal images showing neonatal-born (**A₁**, **B₁**, **C₁**) and adult-born (**A₂**, **B₂**, **C₂**) DGCs at **A)** 2 weeks, **B)** 4 weeks, or **C)** 8 weeks post SE. Arrows indicate axon terminals in the IML. Note the absence of labeling at 2 weeks after SE for adult-born DGCs (**A₂**). Scale bar = 50 μ m.

Figure 4.

Neonatal-born and adult-born DGCs contribute to a similar degree to MFS in the IML. **A.** MFS in the IML from neonatal-born DGCs in rats receiving syp-YFP injection at P7, pilocarpine at P56 and surviving for 8 weeks after SE. **B.** MFS in the IML from adult-born DGCs in rats receiving pilocarpine at P56, syp-YFP injection at P60 and surviving for 8 weeks after SE. White outlines in both panels indicate ROI segments. For each image, the number of labeled pixels was determined for the ROI from the IML and ROI from the hilus. The value for the IML was divided by the value from the hilus to determine MFS ratio for that section, which corrects for variability in the degree of labeling. Scale bar = 20 μ m. **C.** Histogram of mean MFS ratio for neonatal-born and adult-born DGCs. The means of the two populations were not significantly different ($p = 0.66$, student's t-test).

Figure 5.

Hilar bouton density is not influenced by SE or DGC birthdate, but adult-born DGCs have increased bouton density in the IML compared to the hilus after SE. **A-C.** Representative images of axon segments from each respective group. All images are z-projections from confocal stacks acquired with a 63× objective at 2× optical zoom. Scale bar = 10 μm. **D.** Histograms of mean bouton densities. The increase in density for IML boutons compared to hilar boutons of adult-born DGCs in SE tissue is statistically significant ($p = 0.008$, one-way ANOVA, Sidak's multiple comparisons test).

Figure 6.

Seizures are associated with CA2 damage, and MF axon terminals from both neonatal- and adult-born DGCs extend further into CA2 after SE. **A.** Representative images from animals that underwent sham treatment (**A₁**) or pilocarpine-induced SE (**A₂**) and were euthanized 8 weeks later, immunolabeled for the CA2 pyramidal cell-specific marker STEP (magenta), YFP (green), and Hoechst (blue). Brackets outline the length of CA2. Scale bar = 200 μm. **B.** Graph showing the total length of CA2 in sham (576.2 μm) and SE (364.4 μm) groups (data from sham and SE animals for this measurement were not separated according to age at RV injection). CA2 size was significantly reduced in pilocarpine-treated rats ($p=0.003$, student's t-test). **C.** Representative images from neonatal- (**C₁**) and adult-born (**C₂**) sham, and neonatal- (**C₃**) and adult-born (**C₄**) SE groups of syp-YFP-labeled MF terminals in CA2 (green is YFP, magenta is STEP, and blue is Hoechst). Arrows indicate the most distal location (from the CA2/CA3 border) of syp-YFP labeling in CA2. Scale bar = 50 μm. **D.** Graph showing the percentage, by length, of CA2 innervated by syp-YFP-labeled terminals. The differences were significant between SE and sham animals for both neonatal born and adult-born DGCs ($p = 0.01$ one-way ANOVA, Sidak' multiple comparisons test). No differences were found between neonatal- and adult-born DGCs within sham ($p = 0.99$) or SE ($p = 0.94$) groups.