Mice with conditional NeuroD1 knockout display reduced aberrant hippocampal neurogenesis but no change in epileptic seizures

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Keywords: Temporal lobe epilepsy, Seizure, Pilocarpine, NeuroD1, Neurogenesis, Therapy

Abbreviations: Spontaneous Recurring Seizures (SRS), Temporal Lobe Epilepsy (TLE), Granule Cell Layer (GCL), Subgranular Zone (SGZ), Dentate Gyrus (DG), YFP (Yellow Fluorescent Protein), Kainic Acid (KA), Doublecortin (Dcx), Conditional knock-out (cKO), wildtype (WT), NeuroD1 (ND1)

Acknowledgements: We thank Zane Lybrand, Farrah Tafacory, and Ling Zhang for technical assistance and Jose Cabrera for graphics support. This work was supported by grants from National Institute of Health (NIH) (R01NS081203, R01NS089770, R01NS093992 and K02AG041815 to J.H.), Department of Defense W81XWH-15-1-0399 to J.H., American Heart Association 15GRNT25750034 to J.H., a grant from the Texas Institute for Brain Injury and Repair to J.H. and an NIH pre-doctoral training grant 5T32GM083831-05 to R.B.

Abstract

Adult neurogenesis is significantly increased in the hippocampus of rodent models of temporal lobe epilepsy (TLE). These adult-generated neurons have recently been shown to play a contributing role in the development of spontaneous recurrent seizures (SRS). In order to eventually target pro-epileptic adult neurogenesis in the clinical setting, it will be important to identify molecular players involved in the control of aberrant neurogenesis after seizures. Here, we focused on NeuroD1 (ND1), a member of the bHLH family of transcription factors previously shown to play an essential role in the differentiation and maturation of adult-generated neurons in the hippocampus. Wild-type mice treated with pilocarpine to induce status epilepticus (SE) showed a significant up-regulation of NeuroD1+ immature neuroblasts located in both the granule cell layer (GCL), and ectopically localized to the hilar region of the hippocampus. As expected, conditional knockout (cKO) of NeuroD1 in Nestin-expressing stem/progenitors and their progeny led to a reduction in the number of NeuroD1+ adult-generated neurons after pilocarpine treatment compared to WT littermates. Surprisingly, there was no change in SRS in NeuroD1 cKO mice, suggesting that NeuroD1 cKO fails to reduce aberrant neurogenesis below the threshold needed to impact SRS. Consistent with this conclusion, the total number of adult-generated neurons in the pilocarpine model, especially the total number of Prox1+ hilar ectopic granule cells were unchanged after NeuroD1 cKO, suggesting strategies to reduce SRS will need to achieve a greater removal of aberrant adult-generated neurons.

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1 Introduction

2 Epilepsy is a set of neurological disorders characterized by spontaneous recurrent
3 seizures (SRS). Mesial Temporal Lobe Epilepsy (mTLE) is one of the most common adult
4 epilepsies, but is often refractory to currently available therapeutics (Jessberger S. & Parent J.
5 2015). Animal models of mTLE have demonstrated several important cellular changes in the
6 hippocampal formation and surrounding structures. These include astrogliosis, mossy fiber
7 sprouting, hilar basal dendrites, ectopic migration of newborn neurons, neuronal cell death,
8 granule cell layer dispersion, and an increase in adult neurogenesis (Jessberger S. *et al.* 2005;
9 Shapiro L.A. *et al.* 2005; Binder D.K. & Steinhauser C. 2006; Noebels J.L. *et al.* 2012;
10 Jessberger S. & Parent J. 2015). Neurons born after epileptic seizures have been demonstrated to
11 become aberrant, often mis-migrating and forming inappropriate connections with the
12 surrounding hippocampal network (Parent J.M. *et al.* 1997; Scharfman H.E. 2002). This has led
13 to the hypothesis that these aberrantly integrated adult-generated neurons contribute to the SRS
14 seen in mTLE (Noebels J.L. *et al.* 2012; Bielefeld P. *et al.* 2013; Cho K. *et al.* 2015). Use of
15 genetic ablation or pharmacological approaches to suppress adult neurogenesis resulted in a 40-
16 70% reduction in SRS frequency in rodent models of epilepsy, supporting this hypothesis (Jung
17 K.H. *et al.* 2004; Jung K.H. *et al.* 2006; Cho K. *et al.* 2015). In addition, ablation of neurogenesis
18 normalized many of the epilepsy-associated cognitive deficits indicating that neurogenesis plays
19 a significant contributing role in the development of epilepsy-related comorbidities (Cho K. *et al.*
20 2015). In order to translate these findings to the clinic, it would be ideal to identify molecules
21 that specifically promote aberrant adult-generated neurons as they may represent molecular
22 targets to reduce chronic seizures.

To better understand which molecular regulators of neurogenesis contribute to epilepsy, we have decided to study NeuroD1, a bHLH transcription factor expressed in immature neuroblasts in the hippocampus (Pleasure S.J. *et al.* 2000; Guillemot F. 2007; Gao Z. *et al.* 2009). Previously, we showed NeuroD1 is essential for the survival and maturation of adult-generated neurons in the hippocampus under physiological conditions (Gao Z. *et al.* 2009). Furthermore, kainic acid (KA)-induced seizures increased the number of NeuroD1+ immature neuroblasts in the dentate and hilus, while conditional deletion of NeuroD1 in that model reduced the number of YFP+ recombined neuroblasts, suggesting it may play a role in chronic seizure development(Cho K. *et al.* 2015). Here, we employ the pilocarpine model of epilepsy to investigate the role NeuroD1 plays in aberrant neurogenesis and SRS. Using this approach, deletion of NeuroD1 in mice prior to status epilepticus (SE) led to a significant reduction in the total number of NeuroD1+ neuroblasts, however, this did not affect the overall seizure frequency. These data reinforce the idea that aberrant neurogenesis needs to be reduced below a threshold to impact chronic epilepsy.

Materials and Methods

Animals

All the experiments were performed in compliance with the animal care guidelines issued by the National Institutes of Health and by the Institutional Animal Use and Care Committee (IACUC) at University of Texas Southwestern Medical Center. All mice were bred and housed in the animal facility with a 12-h light, 12-h dark cycle with no more than five mice per cage, food (2916 Global irradiated diet, Teklad Labs), and water ad libitum. To generate the mice used in this study, we crossed homozygous Nestin-CreER^{T2}; NeuroD1^{loxP/+} mice with homozygous

R26R-YFP; NeuroD1^{loxP/+} mice (Goebbels S. *et al.* 2005; Lagace D.C. *et al.* 2007) to generate male and female Nestin-CreER^{T2}/R26R-YFP; NeuroD1^{+/+} (NeuroD1 WT) and Nestin-CreER^{T2}/R26R-YFP; NeuroD1^{loxP/loxP} (NeuroD1 cKO) mice. Mice were genotyped by PCR using genomic DNA and primers for NeuroD1 (5' GTT TTT GTG AGT TGG GAG TG 3', 5' TGA CAG AGC CCA GAT GTA 3'), NestinCreER^{T2} (5' GGT CGA TGC AAC GAG TGA TGA GG 3', 5' GCT AAG TGC CTT CTC TAC ACC TGC G 3'), and R26R-YFP (5' AAA GTC GCT CTG AGT TGT TAT 3', 5' GCG AAG AGT TTG TCC TCA ACC 3', 5' GGA GCG GGA GAA ATG GAT ATG 3'). NeuroD1 WT and cKO mice were backcrossed to C57BL/6NHsd mice obtained from Envigo Laboratories (Cat no: 4403F/M) for at least 4-6 generations prior to beginning studies. Male and female NeuroD1 WT and cKO mice at approximately 5.5 weeks of age were administered tamoxifen (TAM) intraperitoneally (i.p.) at 150 mg/kg per day for 5 days prior to SE. TAM was dissolved in 10% EtOH/90% sunflower oil. In Fig. 1 experiments, we used female C57BL/6NHsd (Cat no: 4403F) purchased from Envigo. SNP analysis testing performed for NeuroD1 WT and cKO and C57BL/6NHsd mice showed an average 99.33% genetic similarity between these two lines.

Chemoconvulsant model of TLE

Male and female NeuroD1 WT and cKO mice at approximately 6 weeks of age were administered scopolamine methyl nitrate (i.p.; 2 mg/kg; Sigma-Aldrich S2250) and terbutaline hemisulfate salt (i.p.; 2 mg/kg; Sigma-Aldrich T2528) to block the peripheral effects of pilocarpine and dilate the respiratory tract, respectively. Thirty minutes later, pilocarpine hydrochloride (i.p.; Sigma-Aldrich P6503) at 220 mg/kg for males and 260 mg/kg for females was injected, and mice were placed in an incubator maintained at 31°C (ThermoCare). Acute seizures were behaviorally monitored using a modified Racine's scale (Racine R.J. 1972) (stage

1 1, mouth and facial movement; stage 2, head nodding; stage 3, forelimb clonus; stage 4, rearing
2 with forelimb clonus; stage 5, rearing and falling with forelimb clonus). Once SE began (defined
3 by continuous tonic-clonic convulsive seizures), mice were placed at room temperature for 3 h
4 and returned to the incubator after seizure activity was reduced using diazepam (10 mg/kg;
5 Sigma-Aldrich D0899). Only mice showing SE for a total of 3 hours were included in EEG
6 recording studies. After SE, mice were administered 5% dextrose solution (i.p.; 1 ml) and saline
7 (i.p.; 1 ml) to facilitate their recovery. Mice were weighed each day during the recovery period
8 and if found to have lost ≥ 2 g from the previous recorded weight were given a single i.p. dose of
9 1ml 5% dextrose, and moistened chow. At 3 days after SE, mice were returned to their home
10 cage. Male and female NeuroD1 WT and cKO mice included in the sham seizure group were
11 given (TAM) intraperitoneally (i.p.) at 150 mg/kg per day for 5 days prior to sham treatment. All
12 sham animals were administered scopolamine methyl nitrate (i.p.; 2 mg/kg; Sigma-Aldrich
13 S2250) and terbutaline hemisulfate salt (i.p.; 2 mg/kg; Sigma-Aldrich T2528) 30 minutes prior to
14 an i.p. vehicle (saline) injection. In some cases, male and female NeuroD1 WT and cKO mice
15 were given access to Metoclopramide (Henry Schein Animal Health, Cat no. 055411) treated
16 water (1mg/kg) for 5 days prior to SE to help alleviate gastrointestinal symptoms (constipation)
17 associated with use of pilocarpine in this mouse line. EnvigoC57BL/6NHsd female mice were
18 treated the same as above, but did not receive TAM injections, and received a dose of 185 mg/kg
19 pilocarpine to induce SE. For reference we have made available data on the total number of male
20 and female animals used in our experiments, the total number of each that entered SE and
21 survived for a total of 3 hours, and the total number of that made it to EEG recording are
22 represented in Table 1.

23 *Video/EEG monitoring*

Video/EEG recording was performed between 5–7 weeks after pilocarpine injection. One week before EEG recording, mice were stereotactically implanted with cortical surface electrodes connected to wireless EEG transmitters placed subcutaneously under the skin on the back (TA11ETAF10, Data Sciences International, St. Paul, MN). Mice were anesthetized using 2-3% isoflurane gas mixed in a 1L/min mixture of 70% nitrous oxide and 30% oxygen. Two cortical electrodes were placed at the coordinates from Bregma AP: +0.1 ML: +0.1 (Reference; R) and AP: -0.2 ML: +0.22 (Left parietal cortex; LPC). Mice received the analgesic buprenorphine (subcutaneously; 0.05 mg/kg) as necessary following surgery. Animals underwent continuous monitoring by video/EEG for a total of two weeks. Video-EEG data was reviewed and quantified by a user blinded to the experimental groups using NeuroScore Software (Version 3.0.7703-0, Data Sciences International). Behavioral seizures were defined by repetitive epileptiform spiking activity ($\geq 3\text{Hz}$) that persisted for ($\geq 10\text{s}$) and was confirmed using video recordings. Seizure activity was marked at the beginning and end of each event to account for seizure duration and the number of seizures for each mouse was recorded. After EEG recording was completed, mice were sacrificed and brains harvested for immunohistochemistry.

Immunohistochemistry

Mice were anesthetized using 250mg/kg Avertin injected i.p. and perfused transcardially with cold 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were removed and post-fixed in 4% PFA overnight, then cryoprotected in 30% sucrose in 0.1 M PBS. Brains were bisected and half-brains were coronally sectioned 30 μm thick on a freezing microtome (Leica, SM 2000R). Immunohistochemistry was performed with either tissue mounted on charged slides or free-floating tissue sections. Slides underwent antigen retrieval using 0.01 M citric acid, pH 6.0 at 100 °C for 15 min, followed by 12 min in 1x TBS at room temperature. Staining with free-

1 floating tissue sections was the same except for the antigen retrieval step that was omitted. For
2 Tyramide Plus signal amplification, we removed endogenous peroxidase activity by incubating
3 sections with 0.3% H₂O₂ for 30 min at room temperature. Nonspecific binding was blocked with
4 3% normal donkey serum and 0.3% Triton-X-100 or 3% normal donkey serum and 1% Triton X-
5 100 in 1x TBS for 1 h at room temperature. Primary antibodies used in this study were as
6 follows: goat anti-NeuroD1 (1:500, Santa Cruz Biotechnology sc-1084), chicken anti-GFP
7 (1:1,000 for free floating sections, 1:8,000 for Tyramide Plus Amplification; Aves Lab GFP-
8 1020), goat anti-Dcx (1:1,000, Santa Cruz Biotechnology sc-8066), guinea pig anti-Dcx
9 (1:1,000, Millipore AB2253), and mouse anti-NeuN (1:1,000, Millipore MAB377). For double
10 labeling, primary antibodies were simultaneously incubated and further processed for each
11 antibody. Antibodies combined for double immunostains were as follows: GFP/NeuroD1;
12 GFP/Dcx; GFP/Prox1; and NeuroD1/Dcx. For GFP, Dcx, Prox1, and NeuN, a fluorescent-tagged
13 secondary antibody was used (1:100-1:500, Donkey anti-Cy2/Cy3/Cy5 Jackson
14 ImmunoResearch). For GFP and NeuroD1 (slide mounted), primary antibody incubation was
15 followed with an appropriate biotin-tagged secondary antibody (1:200, Jackson
16 ImmunoResearch) for 1 h at room temperature, followed by ABC (Vector Laboratories PK-
17 6100) for 1 h, and Tyramide-Plus signal amplification (1:50, PerkinElmer NEL701001KT) for 1-
18 3 min. For Dcx, after biotin-tagged secondary antibody followed by ABC labeling was
19 completed, sections were visualized with a metal-enhanced DAB substrate kit (Thermo Scientific
20 34065). Sections visualized with DAB were mounted and dehydrated (70, 80, 90, 95, and 100%
21 EtOH - 3 min each; and 50% EtOH/xylene, and 100% xylene- 3 min each) before coverslipping.
22 Fluorescence stained sections were mounted in a 2.5% PVA-DABCO Media (PVA Sigma-
23 Aldrich D2522, DABCO: Sigma-Aldrich D2522).

Microscopic analysis and quantification

Quantification of cell number was performed by a user blinded to the experimental groups. In fluorescence labeled sections of the hippocampus, quantification was performed using either an upright microscope (BX60; Olympus), or a confocal microscope (LSM700/LSM710; Carl Zeiss Microscopy). In DAB labeled sections of the hippocampus, quantification was performed using a MicroBrightfield scope (BX51; Olympus) equipped with StereoInvestigator software (Version 11.03). Subgranular and hilar zones were defined as the area within and beyond the diameter of one granule cell from the margin of granule cell layer, respectively. Immunoreactive cells were quantified in every twelfth 30- μ m coronal section throughout the dentate gyrus. For double- or triple-stained sections, confocal images in Z planes were scanned for quantification. The numbers counted from each section were added and multiplied by 24 to estimate the total number of cells in one mouse brain.

Statistics

All of the data are expressed at mean $\pm$ S.E.M. Experimental groups were assigned by simple randomization. No statistical methods were used to pre-determine the sample size in each group, however the sample sizes are similar to those reported in previous publications (Parent J.M. *et al.* 1997; Pun R. *et al.* 2012; Cho K. *et al.* 2015). Data that passed selection criteria were collected blind. Prism software was used to perform statistical analysis (Version 6.0, Graphpad Software, Inc.) Statistical differences were analyzed using two-tailed Student's t-test for the data with equal variances, or Student's t-test with Satterwaite's correction for the data with unequal variance. **Figure 4C was analyzed using ANOVA analysis.** Values of $P < 0.05$ were considered statistically significant.

Results

Pilocarpine-induced SE increases the number of NeuroD1+ cells in the hippocampus

To determine the role of NeuroD1+ cells in chronic epilepsy, we used the cholinergic agonist pilocarpine to induce SE. This model has been shown to faithfully recapitulate much of the pathology seen in human patients with mTLE, and has the benefit of leading to SRS in mice with only a single i.p. injection of pilocarpine (Cavalheiro E.A. *et al.* 1991; Covolan L. & Mello L.E. 2000; Sarkisian M.A. 2001; Curia G. *et al.* 2008). Female EnvigoC57BL/6NHsd mice at approximately 6 weeks of age were given a single injection of pilocarpine (185mg/kg) to induce SE, which was allowed to continue for 3 hours before seizures were terminated using diazepam. Mice were euthanized 3 weeks later to determine the total number NeuroD1+ cells found in both the GCL and hilar regions of the hippocampus (Fig 1A). Pilocarpine-treated animals showed a significant increase in the total number of NeuroD1+ cells in both the GCL and hilus as compared to control animals (Fig 1 B,C). In addition, the vast majority (>99%) of NeuroD1+ cells in both the GCL and hilus were found to co-express doublecortin (Dcx) (Fig 1 D, E), a marker of immature neuroblasts (Kempermann G. *et al.* 2015). These results were consistent with our previous work using a KA-induced mouse model of epilepsy (Cho K. *et al.* 2015). Therefore, we decided to further investigate the role of NeuroD1 in the cellular and functional outcomes of epileptogenesis with a model showing more robust SRS.

Deletion of NeuroD1 reduces the number of adult-generated neurons in the hippocampus

In order to delete NeuroD1 from the adult neural progenitor cell population found in the hippocampus, we used Nestin-CreER^{T2}/R26R-YFP mice to conditionally delete NeuroD1 in the adult nestin-expressing stem/progenitor cell population (Lagace D.C. *et al.* 2007). Male and

female NeuroD1 WT and cKO mice were given 5 consecutive days of tamoxifen injections to induce Cre-mediated recombination followed by pilocarpine-induced SE (220 mg/kg for males and 260 mg/kg for females), and then sacrificed 3 weeks later to confirm NeuroD1 deletion (Fig 2A). Quantification revealed that the total number of NeuroD1+ cells in the cKO was significantly reduced in the GCL at 3 weeks post-pilo, but unexpectedly not the hilus (Fig 2 B,C). Therefore, we decided to further examine the total number of YFP+ labeled cells expressing NeuroD1, Dcx, and Prox1. Surprisingly, we did not observe a reduction of YFP/NeuroD1+, YFP/Dcx+, or YFP/Prox1+ cells in the GCL or hilus of the cKO compared to the NeuroD1 WT littermates, although there was a reducing trend (Fig 2D-I). This finding could potentially be explained by inefficient recombination of the NeuroD1 allele in our cKO mice for several reasons which we will explore further in our discussion section. Taken together, although the reduction of NeuroD1 in YFP+ cells is lower than expected, the significant reduction of total NeuroD1+ cells prompted us to examine the role of NeuroD1 in epilepsy using the NeuroD1 cKO mice.

Deletion of NeuroD1 in adult-generated neurons does not reduce SRS

To examine the role of NeuroD1 in epilepsy, we treated male and female NeuroD1 WT and cKO mice with 5 days of tamoxifen, followed by pilocarpine to induce epilepsy. These animals were then implanted with wireless EEG recording devices at 4 weeks post-pilo (Fig 3 A,B). Two cortical epidural screws were placed, one located over the hippocampus (Left parietal cortex; LPC) for recording generalized seizure activity, and the other over the olfactory bulb (Reference; R) to serve as reference activity (Fig 3 B). Starting at five weeks post-pilocarpine, freely moving animals were video/EEG recorded for a period of two continuous weeks (Fig 3A).

1 The total number of generalized seizures was quantified and confirmed using video. Removal of
2 NeuroD1 from adult-generated neurons prior to SE did not significantly alter either the total
3 number of seizures or the total duration of each seizure event (Fig 3 C,D). Together these results
4 suggest that adult neural stem/progenitor-specific knockout of NeuroD1 does not significantly
5 impact SRS.

7 *NeuroD1 deletion does not alter the number of aberrant adult-generated neurons*

8 To reconcile our data that NeuroD1 deletion reduces the total number NeuroD1+ adult-
9 generated neurons in the GCL and the surprising finding that NeuroD1 cKO mice had similar
10 levels of SRS compared to WT, we hypothesized that the level of aberrant neurogenesis was still
11 above the threshold to reduce SRS. Thus, we performed histological analysis on mice seven
12 weeks after pilocarpine treatment to determine the total number of immature Dcx+ neurons
13 present in the GCL and hilus, and the total number of Prox1+ mature granule neurons in the hilus
14 during the chronic period of epilepsy (Fig 4 A,D). Sections were stained with the immature
15 neuroblast marker Dcx (Fig 4 B). There was no difference in the total Dcx+ cells within the GCL
16 in NeuroD1 cKO mice compared to WT (Fig 4 C). This result is in contrast to sham-treated (non-
17 epileptic) animals sacrificed at the same time point which showed a significant reduction in the
18 total number of Dcx+ cells localized to the GCL (Fig 4 B,C,E). Interestingly, deletion of
19 NeuroD1 did however reduce the total number of Dcx+ immature neuroblasts ectopically
20 localized to the hilus in epileptic animals (Fig 4 E,F). However, the total number of Prox1+
21 mature ectopic granule cells was similar between NeuroD1 WT and cKO mice (Fig. 4G). These
22 results suggest that while NeuroD1 deletion can remove a population of total NeuroD1+ cells in
23 the GCL three weeks after pilocarpine treatment, this does not translate to a reduction of aberrant

Dcx+ immature neurons at seven weeks after pilocarpine. These results provide evidence that suggests persistent levels of aberrant neurogenesis may contribute to SRS.

Discussion

Aberrant adult neurogenesis is one of the key hallmarks of mTLE and has been suggested to contribute significantly in the development of SRS (Parent J.M. *et al.* 1997; Parent J.M. *et al.* 1998; Parent J.M. *et al.* 2006; Walter C. *et al.* 2007b; Scharfman H.E. & McCloskey D.P. 2009; Myers C.E. *et al.* 2013; Cho K. *et al.* 2015). Therefore, it is critical to understand the role of key molecular players involved in controlling aberrant neurogenesis for the development of future therapeutics. NeuroD1 is a bHLH transcription factor that is transiently expressed in late-stage adult neural progenitors and neuroblasts, and is essential for the differentiation and survival of newborn neurons in the hippocampus (Gao Z. *et al.* 2009). In this study, we have shown that the number of NeuroD1+ immature neuroblasts increase in the hippocampus after SE, and are found in a large population of ectopic adult-generated neurons. Additionally, we demonstrate that deletion of NeuroD1 in the pilocarpine model is sufficient to reduce the total number of Dcx+ ectopic neuroblasts by 52%. However, deletion of NeuroD1 from adult-generated neurons did not have any effect on SRS frequency or duration. Several possible explanations exist for why this might be the case, which we will discuss in more detail below.

Previous work from our lab has shown that near complete ablation (>98%) of adult-generated neurons prior to seizure can reduce overall seizure frequency by approximately 40% (Cho K. *et al.* 2015). Additionally, antimitotic treatment to kill proliferating neural progenitors after acute seizures also reduced the frequency of subsequent spontaneous seizures (Jung K.H. *et al.* 2004; Jung K.H. *et al.* 2006). The approaches utilized in prior work all removed a large

1 population of aberrant adult-generated neurons, leaving open the question whether partial
2 ablation would still be effective at reducing seizure frequency. Supporting the idea that a small
3 number of residual aberrant adult-generated neurons may still contribute to epilepsy, previous
4 work has shown that excessive activation of the mTOR signaling pathway in as few as 9% of
5 postnatally generated hippocampal neurons is sufficient to cause spontaneous seizures (Pun R. *et*
6 *al.* 2012). Therefore, it is possible that in our current study we failed to reduce the population of
7 aberrant adult-generated neurons below the critical threshold needed to reduce SRS frequency.
8 Indeed, in our work, while we saw a 52% reduction in the number of Dcx+ ectopic neuroblasts in
9 our NeuroD1 cKO mice, there was no change in the number of Dcx+ immature neurons in the
10 GCL or Prox1+ ectopic granule cells after pilocarpine treatment. This could be due to a cohort of
11 migratory Prox1+ cells that no longer required NeuroD1 at the time of TAM administration,
12 while the Dcx+ cells present still required NeuroD1 for their survival and maturation. Regardless
13 of whether it is the presence of Dcx+ cells in the GCL or Prox1+ cells in the hilus, a population
14 of aberrant neurons persisted potentially leading to the lack of reduction in SRS seen in this
15 study.

16 One limitation of this present study, and another possible reason for why we did not
17 observe a reduction in SRS frequency is that NeuroD1 deletion prior to SE removed granule cell
18 progenitors present at the time of SE while failing to remove immature granule cells born weeks
19 before or after the insult. This point is noteworthy because both immature granule cells born up
20 to 5 weeks before and granule cells born after the insult may integrate abnormally and contribute
21 to epilepsy (Jessberger S. *et al.* 2007; Walter C. *et al.* 2007a; Kron M.M. *et al.* 2010; Pun R. *et*
22 *al.* 2012). In support of this hypothesis, recent work has shown that ablation of cells directly after
23 SE that were labeled 5 weeks prior can lead to a 50% reduction in SRS suggesting that targeting

1 a more comprehensive population of adult born neurons might be necessary in order to reduce
2 SRS (Hosford B.E. *et al.* 2016). In the future, it would be interesting to determine whether more
3 extensive administration of tamoxifen to delete NeuroD1+ cells before and after SE might lead to
4 more efficient removal of aberrant adult-generated neurons that may serve as excitatory “hubs”
5 within the hippocampal network. Additionally, emerging work also suggests functional
6 heterogeneity amongst the newly generated granule cells, which may contribute differing, and
7 perhaps even opposing, effects on epileptogenesis. Despite many reports describing that adult-
8 generated granule cells display physiological and morphological changes in epilepsy models
9 consistent with a pro-excitatory function (Esclapez M. *et al.* 1999; Ribak C.E. *et al.* 2000;
10 Morgan R.J. *et al.* 2008; Pun R. *et al.* 2012), there are also studies suggesting these neurons
11 exhibit decreased excitatory input (Williamson A. *et al.* 1999; Kobayashi M. & Buckmaster P.S.
12 2003; Jakubs K. *et al.* 2006). Moreover, reducing newly generated neurons before acute seizures
13 increases sensitivity to kainic acid, suggesting that newly generated cells may show both
14 increased and decreased excitability (Iyengar S.S. *et al.* 2015). Since our current work to delete
15 NeuroD1 cannot distinguish between different populations of adult neural stem/progenitor
16 subpopulations, future work using longer tamoxifen treatment may allow for more
17 comprehensive labeling and deletion of NeuroD1+ immature neuroblasts within the
18 stem/progenitor population.

19 Lastly, the possibility remains that efficient Cre-mediated recombination of the NeuroD1
20 allele in our cKO mice was not achieved. Consistent with this idea, there was no change in the
21 number of YFP/NeuroD1+, YFP/Dcx+, and YFP/Prox1+ cells in NeuroD1 cKO mice compared
22 to WT, although there was a reducing trend in the GCL. These numbers are in contrast to our
23 previous work showing a significant difference in YFP/Prox1 and YFP/Ki67/Dcx at 3 weeks

1 post seizure (Cho K. *et al.* 2015). Several potential explanations for this exist which we will go
2 into greater detail below. Inefficient recombination could potentially be due to the fundamental
3 differences between the two chemoconvulsant seizure models utilized (Houser C.R. & Esclapez
4 M. 1996; Reddy D.S. & Kuruba R. 2013; Levesque M. *et al.* 2015). In our previous work, we
5 used the drug kainic acid (KA) to induce acute seizures in our NeuroD1 WT or cKO mice,
6 whereas in our current work we utilized the pilocarpine method of chronic seizure formation.
7 Experimental evidence suggests differences between each of these models, and it is possible that
8 use of pilocarpine allows for greater survival of the YFP population of cells at 3 weeks,
9 especially those ectopically localized to the hilus. This could be due to the fact that pilocarpine
10 treatment results in chronic SRS at higher, more reproducible rates in treated animals, a process
11 linked to an increase in neurogenesis. In other words, if YFP labeled cells were stimulated to
12 divide by the action of SRS in the pilocarpine model, the total YFP population and its progeny
13 would increase in number possibly explaining the strong trend, but ultimate lack of statistical
14 significance. Further, the age at which our experimental mice received neurogenic ablation
15 (ablation at 5.5 weeks of age for 5 days in our current pilocarpine model vs. ablation at 4.5
16 weeks of age for 5 days in our previous kainic acid model) followed by chemoconvulsant
17 treatment differed from that of our previous work. As a result, the mice in each of these studies
18 could have had differing rates of neurogenesis at the time of chemoconvulsant treatment
19 resulting in the lack of efficiency seen in our current work (Cho K. *et al.* 2015). Additionally, the
20 length of time between tamoxifen administration and chemoconvulsant treatment differed
21 between this and our previous work (24 hrs later in our current pilocarpine model vs. 1 week
22 later in our previous kainic acid model). This could have potentially allowed for more extensive
23 deletion of the YFP labeled cell population in our previous study contributing to the significant

1 difference in YFP/NeuroD1+ cells in our Cho et al. work, but not our current work (Cho K. *et al.*
2 2015).

3 Mesial temporal lobe epilepsy remains a major clinical challenge lacking interventional
4 strategies due to an incomplete understanding of the underlying cellular and molecular
5 mediators. Here in this study, we present data regarding the role of NeuroD1 in adult-generated
6 neurons, and link its requirement in the development of chronic seizures. Our work supports the
7 idea that efficient labeling of adult-generated neurons in combination with targeting key
8 molecules is necessary to achieve complete ablation of pro-epileptic neurogenesis and reduce
9 SRS. Furthermore, additional regulators of each step of seizure-induced adult neurogenesis may
10 allow for more effective targeting of aberrant adult-generated neurons below a threshold to
11 impact SRS. We also suggest that there may be a small population of aberrant adult-generated
12 neurons that contribute to the overall recurring seizure activity. By highlighting these lessons
13 learned, we hope this knowledge will inform future strategies aimed at preventing epilepsy.

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Figure Legends

Figure 1: Acute seizures increase the number of NeuroD1-positive cells in adult dentate gyrus.

A) Experimental Timeline. Female C57BL/6NHsd Envigo animals were subjected to pilocarpine induced status epilepticus (SE) and then euthanized 3 weeks post pilocarpine. B) Animals were euthanized 3 weeks post pilocarpine treatment and brains were stained with NeuroD1 to determine the total cell number in the granule cell layer (GCL) and hilus. (n=6 sham, n=6 pilo) C) There was a significant increase in the NeuroD1+ cells in both the GCL ($p=0.0013$) and hilus ($p=0.0016$) in pilocarpine treated animals compared to sham treated animals. D,E) The majority of pilocarpine induced NeuroD1+ cells co-localized with the immature neuronal marker Dcx. (n=8 sham, n=8 pilo). Scale bar in B: 100 μ m, inset bar in B: 25 μ m, both valid for D.

Figure 2: NeuroD1 is required for pilocarpine-induced adult-generated neurons

A) Experimental Timeline. Male and female NeuroD1 WT or cKO animals were given 5 days of tamoxifen and then subjected to pilocarpine induce status epilepticus (SE). Mice were euthanized 3 weeks post pilocarpine. B,C) Sections were stained with NeuroD1 to assess the total number of remaining NeuroD1 cells after NeuroD1 cKO in the GCL and hilus ($p=0.0128$). D,E) Sections were stained for YFP and NeuroD1 to confirm reduction of NeuroD1+ amongst the YFP+ population. F-I) Sections were stained with YFP/Dcx to assess reduction of immature neuronal, and YFP/Prox1 to assess reduction of mature neuronal cells in the recombined population in the GCL and hilus. (n=10 WT, n=7 cKO). Scale bar in B: 100 μ m, inset bar in B: 25 μ m, both valid for D,F,H. NS=Not significant.

Figure 3: No change in SRS in animals with NeuroD1-deficient adult-generated neurons.

A) Experimental Timeline. Animals were implanted with wireless EEG recording devices at 4 weeks post pilocarpine (pilo). EEG recording began at 5 weeks post-pilo and continued for a total of 2 weeks. B) Representative images showing where the wireless EEG screws were implanted and how the recording electrodes were attached. R=Reference, LPC=Left parietal cortex C) Deletion of NeuroD1 did not have a significant effect on spontaneous recurrent seizure (SRS) frequency or duration. D) Representative EEG seizure traces from WT and cKO animals. (n=11 wt, n=10 cKO), NS=not significant.

Figure 4: NeuroD1 is not required for Prox1-positive ectopic granule neurons.

A, D) Experimental Timeline. Male and female NeuroD1 WT or cKO animals were given 5 days of tamoxifen injections and then subjected to sham- or pilocarpine treatment. Mice were euthanized 7 weeks post-sham or post-pilocarpine. B, E) Representative images from sham-treated or pilocarpine-treated WT and cKO animals stained with Dcx. C) Deletion of NeuroD1 significantly reduced the number of Dcx+ cells in the GCL ($p=0.0226$) in sham-treated mice (n=6 WT, n=8 cKO) but not in pilocarpine-treated mice (n=11 WT, n=10 cKO). F: Deletion of NeuroD1 significantly reduced the total number of Dcx+ cells ectopically localized in the hilus ($p=0.0362$) (n=11 WT, n=10 cKO). G: Deletion of NeuroD1 does not affect the total number of Prox1+ cells in the hilus in pilocarpine-treated mice (n=9 WT, n=10 cKO). Scale bar in B: 100um, inset bar in B: 25um, also valid for E. NS=Not significant. For C; Wildtype (sham) vs. Wildtype (pilo) $p=0.2010$, and wildtype (pilo) vs. cKO (pilo) $p=0.3578$.

Table 1: Animal numbers included in study

1 This table details the total number of male and female animals used in our SRS recording
2 experiments and is further broken down into the total number of each sex that entered SE and
3 survived for a total of 3 hours, as well as the total number that survived to the SRS recording
4 phase of the study. The * in the table indicates a number where there were originally 12 animals,
5 but after re-genotyping 1 animal was excluded from the recording phase due to being
6 heterozygous for the NeuroD1 allele.

7 Table 2: Summary of Experiments

8 This table details the experiments conducted throughout this work and provides an easy
9 reference to the strain of mice, experimental condition, survival, and results of analysis across all
10 figures.

11

Table 1

	Received Pilo	Entered SE and survived	Included in data
Males	87	42	12*
Females	50	21	10

Table 2

Figure	Mouse Line	Tamoxifen?	Treatment	Sacrified	Staining	Result
1	HC57BI6	None	Pilocarpine or Sham	3 wpp	NeuroD1	Significant increased NeuroD1+ cells in GCL and hilus
2	NestinCreERT2; Rosa YFP; NeuroD1 +/+ or -/-	5 dpp	Pilocarpine	3 wpp	NeuroD1	Significant decrease in total NeuroD1+ in GCL of cKO
2	NestinCreERT2; Rosa YFP; NeuroD1 +/+ or -/-	5 dpp	Pilocarpine	3 wpp	YFP/NeuroD1; YFP/Dcx; YFP/Prox1	No significant change in GCL or hilus
3	NestinCreERT2; Rosa YFP; NeuroD1 +/+ or -/-	5 dpp	Pilocarpine	7 wpp	None	No significant change in EEG frequency or duration
4	NestinCreERT2; Rosa YFP; NeuroD1 +/+ or -/-	5 dpp	Pilocarpine	7 wpp	Prox1	No significant change in total Prox1+ cells in hilus of pilocarpine treated animals
4	NestinCreERT2; Rosa YFP; NeuroD1 +/+ or -/-	5 dpp	Pilocarpine or Sham	7 wpp	Dcx	Significant reduction of total Dcx+ cells in GCL of sham treated animals and total Dcx+ cells in hilus of pilocarpine treated animals. No change in total Dcx+ cells in GCL of pilocarpine treated animals

Figure 1

Figure 2

Figure 3

Figure 4