Title: A critical period for neurogenesis loss and cognitive decline in patients with mesial temporal lobe epilepsy.

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ABSTRACT

Mesial temporal lobe epilepsy is a syndromic disorder presenting with seizures and cognitive comorbidities. The cellular mechanisms contributing to epilepsy progression are not well understood. Here we show that intelligence, verbal learning, and memory decline at a critical period when hippocampal neurogenesis becomes undetectable. Individual patient analysis revealed that the number of immature neurons, rather than total granule neurons, particularly benefit verbal learning. These results illustrate a functional role for adult human neurogenesis and reveal a therapeutic target accompanying cognitive decline.

Main Text

INTRODUCTION

Epilepsy is a prevalent neurological disorder defined by recurrent seizures and presents many comorbidities including neurocognitive dysfunction. Mesial temporal lobe epilepsy (MTLE) is the most common and refractory adult epilepsy, with 70% of patients becoming pharmacoresistant[1]. In comparison to other epilepsies, MTLE patients are more likely to exhibit disease progression including worsening seizure control, structural abnormalities, cognitive decline, and behavior impairment[2]. Cognitive phenotypes in MTLE can be categorized according to clinical criteria[3]. Approximately half of the patients possess intact cognition, while one quarter

display impairments in learning, memory and language, and another quarter display more widespread dysfunction[4]. When and which structural abnormalities contribute to progressive cognitive impairment are not well understood.

MTLE is characterized by focal seizures in the hippocampus, which performs learning, memory, and emotional processing functions[5]. The hippocampus also contains a rare niche in which adult neurogenesis persists throughout human life[6]. Neurogenesis is crucial in regulating cognition in rodent models of aging and dementia[7,8]. A mild decline in hippocampal neurogenesis also occurs in healthy people with age[6], which is further exacerbated in Mild Cognitive Impairment (MCI) and Alzheimer's disease (AD)[9,10]. Our recent study on human MTLE shows the most profound decline in neurogenesis among neurological diseases to date. Neurogenesis rapidly deteriorates over the course of the disease and was not detected after 20 years of MTLE disease duration (DD)[11]. It remains unknown how the loss of neurogenesis associates with cognitive impairment.

Previous studies in primarily English-speaking individuals have shown that MTLE patients with severe cognitive decline have longer DD[3,12]. Examining an understudied cohort of Spanish-speaking MTLE patients, we first identified which cognitive domains are affected by DD, and when they significantly decline. Longitudinal neuropsychological and histological analysis revealed which cognitive domains are directly associated with neurogenesis. Our results link the decline and eventual loss of neurogenesis to verbal learning and memory impairment during a critical period of epilepsy progression.

RESULTS

Cognitive performance declines with advancing disease duration.

Cognitive functioning was evaluated using the Neuropsychological Screening Battery for Hispanics (NeSBHIS)[13,14]. We first identified which cognitive domains from the NeSBHIS (Extended Data Table 1) were affected by DD. Education-normalized NeSBHIS z-scores and age plus education normalized z-scores for Grooved Pegboard fine motor test[13] were analyzed from MTLE patients (N = 33), average age (M = 38.85 years; SD = 11.37), DD (M = 26.81 years; SD = 12.86) and reported years of education (M = 7.79 years; SD = 3.52) (Extended Data Table 2). Spearman’s correlation revealed declining performances across multiple cognitive domains

(Extended Data Table 3). Hippocampal-dependent functions of verbal learning and memory[15] significantly declined on learning (Fig 1b), and short delay recall trials (Fig 1c) tests. The verbal long delay recall trial (Fig 1d) also declined with DD, trending toward statistical significance. Additionally, less hippocampal-dependent functions like reasoning and intelligence (Fig 1a), and visuospatial skills (Fig 1e) showed significant declines with DD. The remaining cognitive domains also showed nonsignificant, declining trends with DD (Fig 1f, Extended Data Fig 1). Summarily, this cohort of understudied Spanish-speaking MTLE patients present progressive cognitive decline with DD consistent with previous studies of English-speaking patients where longer epilepsy DD was associated with severe cognitive impairment[3].

A critical period of cognitive impairment in MTLE patients.

To identify the critical period when cognitive performance significantly decreases, we separated our patients into three distinct groups to have approximately similar n-value based on DD. Group 1 (G1, N = 9) was < 20 years DD, group 2 (G2, N = 14) was 21 to 30 years DD, and group 3 (G3, N = 10) contained > 31-years DD. We then compared whether the NeSBHIS z-scores differed significantly between groups (Extended Data Table 4). With respect to intelligence (Fig 2a) and verbal learning and memory (Fig 2b-d), G2 and G3 had significantly lower z-scores compared to G1, but scores did not decline significantly from G2 to G3. This result indicates a sudden drop in performance rather than a slight gradual decline that was observed in visuospatial skills (Fig 2e). The fine motor test for the dominant hand also displayed a significant drop in performance between G1 and G2 (Extended data Fig 2h). The remaining tests did not display significant differences between any DD groups (Fig 2 e, f, Extended Data Fig 2a-g, i). Therefore, the most significant declines in intelligence, verbal learning, and memory occur in MTLE patients before a critical period of 20 years; revealing a window for therapeutic interventions to reduce the impact of cognitive comorbidities.

Neurogenesis levels particularly benefit verbal learning and memory.

The critical period (Fig 2) coincides with the time when neurogenesis becomes undetectable in human MTLE[11]. We next determined the relationship between the loss of neurogenesis and cognitive performance in intelligence, verbal learning, and memory tests. We first benchmarked our previous observation of neurogenesis decline with DD to our current cohort

of Spanish-speaking patients only (This cohort contains a subset of patients from our previous study with 1 additional case). Neurogenesis was quantified as the number of Dcx+Prox1+ cells (Fig 3a, b) by performing histology in surgically resected tissue (N = 14). Consistent with our previous report[11], we observed an exponential decline of hippocampal adult neurogenesis with DD (Fig 3c). We then analyzed the number of mature granule neurons (Prox1+) with DD and observed a mild non-significant decline (Fig 3d). Next, we investigated the association of neurogenesis and mature granule neuron levels across various cognitive domains (Extended Data Table 5). We observed a significant positive association between neurogenesis and verbal learning (Fig 3g). A trending, positive correlation also occurred between neurogenesis and long delay recall (Fig 3k) trial. On the contrary, mature granule neurons levels showed no significant correlation with any of the verbal learning and memory tests (Fig 3 h, j, l). Therefore neurogenesis, rather than mature granule neuron levels particularly benefit verbal learning. Neither neurogenesis nor mature granule neurons levels displayed a significant correlation with any other examined cognitive domains (Extended Data Fig 3, 4, Fig 3e, f, i), except for a trending positive correlation between mature granule neuron numbers and the language phonemic fluency test (Extended Data Fig 4e). In conclusion, our study provides the first direct evidence for an association between the loss of neurogenesis and decline in verbal learning in humans.

DISCUSSION

In MTLE, when and which structural abnormalities contribute to the progression in cognitive impairment are not well understood. We observed declining cognitive performances with MTLE DD among intelligence, verbal learning and memory, and visuospatial skills in a Spanish-speaking population. Our results extends previous research on English-speaking populations, which found an association between longer DD and decline across multiple cognitive domains[3,12]. The significant decline in auditory verbal learning and memory we observed is consistent with strong hippocampal dependency for these tasks[15] and its known impairment in other neurological disorders with hippocampal dysfunction[16,17]. Other tasks not considered primarily dependent on the hippocampus, such as intelligence, were also influenced by DD and could be due to the generalization of seizures that affect other brain regions or result in broader neural network inefficiencies. Importantly, we identified that intelligence, and verbal learning and memory significantly decline at a critical DD of 20 years which coincides with the loss of detectable

neurogenesis[11]. Hence, data from Spanish-speaking MTLE patients indicate that cognitive decline can occur in phases with a very pronounced initial progression[2].

The role of neurogenesis in specific cognitive functions has been better studied in rodent models[5], however it remains unknown in humans. Recent studies indicate declining neurogenesis with MCI and AD[9,10], and a positive association between neuroblast number and Global cognition score[9]. In this study we find a direct correlation between the loss of immature neurons and cognitive decline in epilepsy patients, most remarkably declining at DD of >20 years. Our analysis of distinct cognitive domains was critical in understanding the specific neuropsychological functions influenced by adult human neurogenesis. We observed a strong association between verbal learning and neurogenesis. Specifically, the neurogenesis effect is most significant on the verbal learning trial (AVLT5), trends in long delay recall (AVLT8) and not in short delay recall (AVLT7). Meanwhile, visuospatial domains and visual memory display a more gradual temporal decline that appears independent of neurogenesis levels. These outcomes differ from rodent studies that strongly implicate neurogenesis in spatial memory[5]. Such species divergence could reflect the evolution of human language circuits or the current limitations in studying rodent vocalization.

The contribution of the dentate gyrus (DG) to verbal learning is well established. A strong association between DG volume and memory is revealed by Magnetic Resonance Imaging in MCI patients[16]. Further, while DG granule neuron loss has been found to correlate with memory impairment in epilepsy patients[18], we observed no such association in this study. These divergent outcomes may be due to differences in the extent of granule cell loss and the learning and memory tests performed. DG granule number and volume are both a function of neuronal cell loss and cell genesis, with one-third of the DG granule neurons undergoing turnover during human lifespan[6]. Therefore, the current neurogenesis findings provide a new understanding of the cellular dynamics that drive DG volume change during MTLE disease progression. Altered neurogenesis in MTLE patients may reflect many cellular mechanisms, including a decrease in neural stem cell number, proliferation, neuronal fate commitment, or neuronal survival[11,19]. Remarkably, a previous study in healthy individuals has also implied a selective role for exercise-induced neurogenesis on verbal learning using indirect measures of dentate gyrus cerebral blood volume[20]. Our direct evidence quantifying the amount of immature neurons in hippocampal resections from MTLE patients indicates neurogenesis benefits human verbal learning. Further research is needed to understand

whether therapeutic or lifestyle changes that promote neurogenesis and cognition in model systems can be as regenerative in humans.

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Author contributions: A.A. and M.A.B. conceived the project. A.A, L.C, R.H.C, D.S, C.Y.L, J.J.R, J.A.D.S, and M.A.B designed the experiments. A.A, L.C, K.R, V.W, and J.N performed the experiments. N.J, L.M.D, V.C, C.M, K.R and A.A compiled the clinical information. J.J.R, C.Y.L. D.L. and B.L performed the neurosurgeries. G.N, L.K, and C.H conducted clinical review and assisted with specimen collection. L.C and A.A analyzed and compiled the data. L.C, A.A, J.A.D.S and M.A.B wrote the manuscript. C.Y., J.A.D.S and M.A.B supervised the project.

Competing interests: The authors declare no competing interests.

Data and materials availability: All data are available in the manuscript or supplementary materials. Any information regarding the availability of raw data, materials and methods can be directed to M.A.B.

FIGURE AND LEGENDS

Fig:1

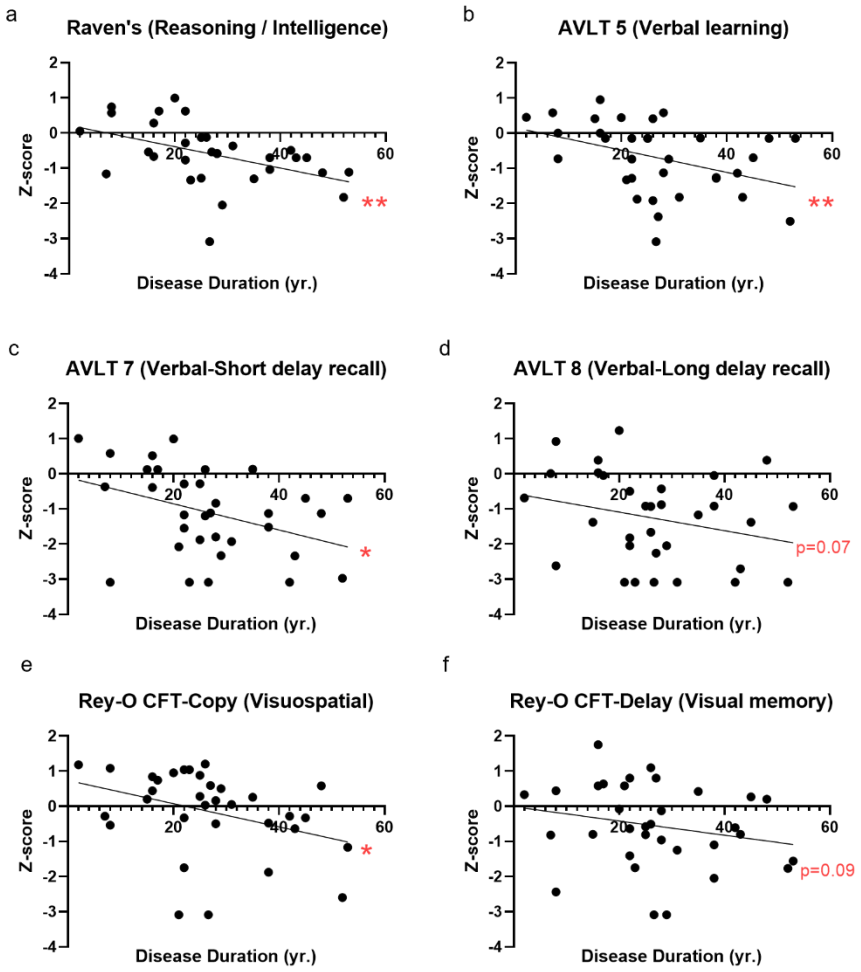

Figure 1. Cognitive performance declines with advancing disease duration. Association between DD and cognitive z-scores calculated by two-tailed Spearman's correlation for NeSBHIS tests: a) Raven's, b) AVLT 5, c) AVLT 7, d) AVLT 8, e) Rey-O-CFT Copy, f) Rey-O-CFT Delay, * $P \leq 0.05$, ** $P \leq 0.01$.

Fig 2

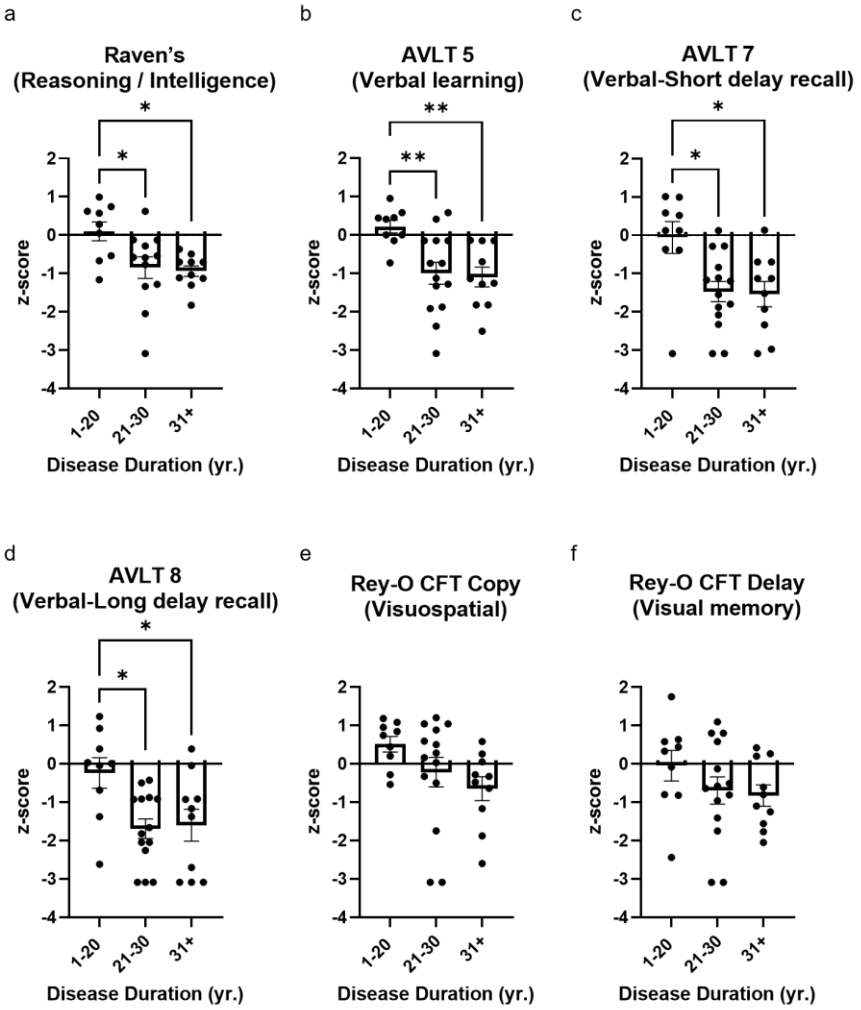

Figure 2. A Critical period of cognitive impairment in MTLE patients. Three DD groups were created; G1: 1-20 years DD, G2: 21-30 years DD, and G3: 31+ years DD. Cognitive z-scores between 3 groups were compared with a one-way ANOVA with post-hoc Tukey's analysis: a-d) Cognitive z scores were significantly higher for G1 compared to G2 and G3 for: a) Raven's, b) AVLT 5, c) AVLT 7, d) AVLT 8. e, f) Cognitive z scores were not significantly different between groups. e) Rey-O CFT Copy f) Rey-O CFT Delay. * $P \leq 0.05$, ** $P \leq 0.01$. All error bars represent S.E.M across individual cases.

Fig:3

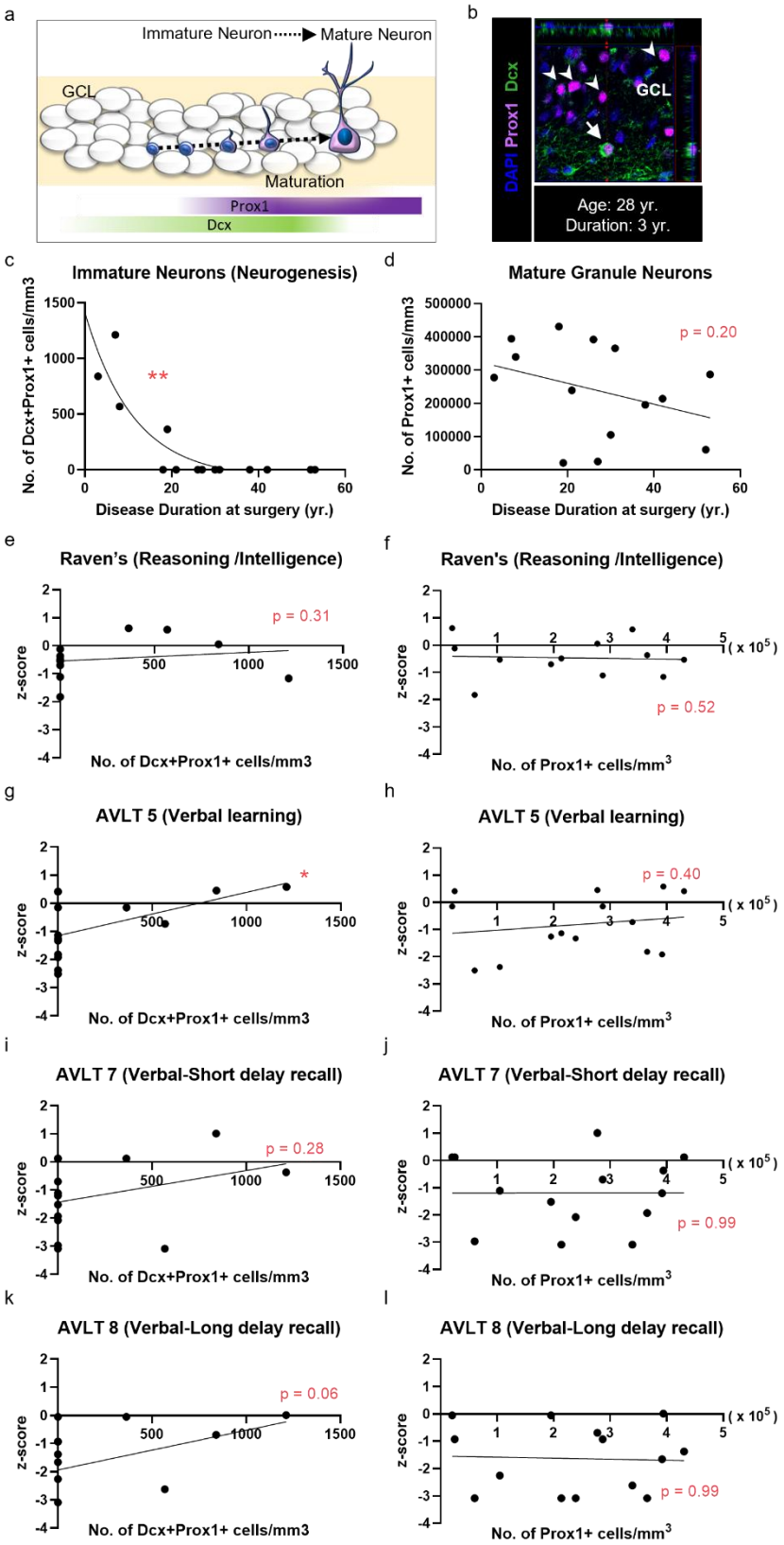
a
Immature Neuron
Mature Neuron
GCL
Maturation
Prox1
Dcx
b
DAPI Prox1 Dcx
GCL
Age: 28 yr.
Duration: 3 yr.
c
Immature Neurons (Neurogenesis)
No. of Dcx+Prox1+ cells/mm3
**
Disease Duration at surgery (yr.)
d
Mature Granule Neurons
No. of Prox1+ cells/mm3
p = 0.20
Disease Duration at surgery (yr.)
e
Raven's (Reasoning /Intelligence)
z-score
p = 0.31
No. of Dcx+Prox1+ cells/mm3
f
Raven's (Reasoning /Intelligence)
z-score
p = 0.52
No. of Prox1+ cells/mm3
g
AVLT 5 (Verbal learning)
z-score
*
No. of Dcx+Prox1+ cells/mm3
h
AVLT 5 (Verbal learning)
z-score
p = 0.40
No. of Prox1+ cells/mm3
i
AVLT 7 (Verbal-Short delay recall)
z-score
p = 0.28
No. of Dcx+Prox1+ cells/mm3
j
AVLT 7 (Verbal-Short delay recall)
z-score
p = 0.99
No. of Prox1+ cells/mm3
k
AVLT 8 (Verbal-Long delay recall)
z-score
p = 0.06
No. of Dcx+Prox1+ cells/mm3
l
AVLT 8 (Verbal-Long delay recall)
z-score
p = 0.99
No. of Prox1+ cells/mm3

Figure 3: Neurogenesis levels particularly benefit verbal learning and memory

a) Cartoon illustration of Dcx and Prox1 expression across stages of granule neuron maturation. b) Dcx+ (green) Prox1+ (purple) immature neurons (Arrow) and Dcx- Prox1+ mature granule neurons (Arrowhead) identified in the Granular Cell Layer (GCL) of adult human MTLE cases. c) Number of Dcx+ Prox1+ cells/mm^3 in the GCL with DD (yr.) at surgery. Two-tailed Spearman's Correlation ** $P \leq 0.01$. Spearman's $r = -0.7626$. d) Number of Prox1+ cells/mm^3 in the GCL with DD (yr.) at surgery. Two-tailed Spearman's Correlation $P = 0.2$ Spearman's $r = -0.3626$. e, g, i, k) Two-tailed Spearman's Correlation between the number of Dcx+ Prox1+ cells/mm^3 detected from the surgically resected hippocampus and presurgical cognitive z-scores for e) Raven's, g) AVLT 5, i) AVLT 7, k) AVLT 8, * $P \leq 0.05$. f, h, j, l) Two-tailed Spearman's Correlation between number of Prox1+ cells/mm^3 detected from the surgically resected hippocampus and presurgical cognitive z scores for f) Raven's, h) AVLT 5, j) AVLT 7, l) AVLT 8.

ONLINE METHODS

Cognitive assessment

Retrospective cognitive analysis of presurgical neuropsychological exams of Spanish-speaking patients (N=33) with intractable TLE was performed to explore the relationship between DD and TLE with cognitive functioning. The study was approved by University of Southern California Institutional Review Board approval number: HS-10-00162, and Rancho Los Amigos National Rehabilitation Center and the Rancho Research Institute's Institutional (RRI) Review Board, approval number: RRI IRB # 195 and the data use agreement with RRI and the University of Texas Southwestern Medical Center IRB Protocol approval number: STU-052018-086.

We have complied with all relevant ethical regulations. Written consents were obtained from the patients to study the clinical and demographic information. De-identified patient clinical details (age and DD of epilepsy at the time of Neuropsychological test, age of disease onset, sex, left vs right hemisphere of effected hippocampus, education years, ethnicity, nationality) are provided in Extended Data Table 2. Both male (N = 15) and female (N = 18) cases were included to examine sex as a biological variable in the study. The NeSBHIS was part of a comprehensive presurgical evaluation. Intellect, attention, processing speed, language, visuospatial, visual memory, verbal learning and memory, executive functioning and fine motor dexterity were measured to assess epilepsy comorbidities (Extended Data Table 1). To account for variability in education, which influences neuropsychological test performance[13], subtest raw scores were converted to z-scores using education-based normative data[14]. Grooved pegboard was used instead of the fine-motor Pin test from NeSBHIS, which was normalized using age and education based normative data as in Smith, et.al, 2020[13]. Upper and lower cut off values for z scores were set at +/- 3.09 to eliminate potential outliers due to very superior performance (z scores > +3.09) and clinically profound impairment (<3.09).

Human MTLE Hippocampal Tissue processing

Human hippocampal tissue was obtained from 14 out of 33 patients undergoing an *en bloc* resection of their hippocampus for the treatment of medically resistant MTLE, following strict ethical guidelines. Written consents for the surgical treatment of MTLE, tissue donation for the study as well as relevant clinical and demographic information collection (University of Southern California Institutional Review Board approval number: HS-17-00370) were obtained from all the

patients prior to the surgery. Both male (N = 7) and female (N = 7) cases were included to examine sex as a biological variable in the study. The surgery was performed by neurosurgeons at the Keck Medical Center or Los Angeles County Hospital, University of Southern California. The surgery was performed within a year of neuropsychology test for 9 out of 14 patients. Rest of the cases underwent surgery within 3 years of test (N = 2 after 1 year, N = 1 after 2 years, N = 2 after 3 years). DD at surgery is provided in Extended Data Table 2. Surgically resected hippocampal tissue was transported to the laboratory within 30 minutes to 1 hour of surgical removal in a tube containing HypoThermosol® FRS (BioLife Solutions, Bothell, WA) solution maintained at approximately 4˚C in an ice box.

Tissue Processing

Detailed tissue processing and immunostaining method is explained in our previous study identifying neurogenesis in human MTLE[11]. Briefly hippocampal tissue was cut into 400 µm thick slices in chilled (4˚C), oxygenated (95% O_2, 5% CO_2) N-methyl-D-glycamine - artificial cerebrospinal fluid (aCSF) solution (NMDG-aCSF; NMDG 93mM; KCl 2.5mM; NaH_2PO_4 1.2mM; $NaHCO_3$ 30mM; HEPES 20mM; Glucose 25mM; Sodium ascorbate 5mM; Thiourea 2mM; Sodium pyruvate 3mM; $MgSO_4$ 10mM; $CaCl_2$ 0.5mM) using a vibrating microtome (Leica VT1200S, Leica Biosystems, Germany). Vertical deflection of the blade was minimized with Vibrocheck technology (Leica Biosystems, Germany). Slicing parameters were set at speed 0.5-0.15 mm/s and vibration amplitude 1.5 mm. Hippocampal slices were first used for multi electrode array experiments explained in our previous study[11]. Slices were first allowed to recover in a chamber with warm (32˚C) aCSF (NaCl 124 mM; KCl 4 mM; $NaHCO_3$ 26 mM; Glucose 10 mM; $CaCl_2$ 2mM; $MgCl_2$ 2mM) solution (30˚C, 95% O_2, 5% CO_2) for 1 hour before multi-electrode array (MEA) recording. Continuous oxygenated, warm (32-34˚C) aCSF at a rate of 6 ml/min was circulated in the MEA ring well during tissue recording. A 5 min baseline recording was performed in aCSF followed by a 20-30 min recording in a modified aCSF solution containing high (8 mM) potassium, low (0.25 mM) magnesium and 100 µM 4-aminopyridine (4-AP) to induce inter-ictal like activity. Following the MEA recording, slices were immediately fixed using 4% PFA for immunohistochemistry.

Immunohistochemistry and cell quantification

Slices were pre-treated in a similar manner for methodological consistency between cases. Slices were fixed in 4% PFA - made freshly from 16% PFA stored at -20°C - for 30 minutes at room temperature and washed thoroughly with 1x phosphate buffered saline (PBS) overnight. The slices were dehydrated using 30% sucrose in PBS at 4 °C. After 1 week, once the slice sank it was embedded in O.C.T compound (Tissue-Tek), ensuring the DG region is flat. Slices were further sub-sectioned by cryostat (Leica CM 3050 S) into 30µm and mounted to super frost plus slides. The slides were dried overnight at room temperature and transferred to -20°C for long term storage. Before performing immunohistochemistry, slides were equilibrated to room temperature for 2 hours. Slides were further washed in PBS for 1 hour, followed by blocking and permeabilization in 3% Bovine serum albumin (BSA), 10% Donkey serum in 0.1% Triton-X in PBS for 2 hours at room temperature. The sections were incubated with primary antibodies for 18-20 hours at 4°C in 0.3 % BSA, 1% Donkey serum and 0.1% Triton X in PBS. Following a 1-hour equilibration to room temperature, slides were washed in 0.1% Triton X in PBS for 15 minutes 3 times. Secondary antibodies from Jackson Immuno research laboratories were diluted from a stock solution of 0.6mg/ml and incubated along with 1µg/ml of DAPI (Roche, Cat No: 10236276001) for 2 hours at room temperature in 0.3 % BSA, 1% Donkey serum and 0.1% Triton X. Sections were washed 3 times in 0.1% Triton X in PBS for 15 minutes and mounted with cover glass. The whole DG region was imaged at 20X using a Zeiss LSM 700 confocal microscope automated tile scan. All cell quantification was performed manually in Zeiss blue software. GCL boundaries were marked using the DAPI channel around the seemingly compact cell layer leaving a gap of two cell nuclear distance on both sides of the hilus and molecular layer. Each whole section was analyzed to quantify the number of mature granule neurons (Prox1+) and rare immature granule neurons (Dcx+ Prox1+). The remaining statistical analysis and plots were prepared using Prism8 from GraphPad Inc.

<u>List of antibodies:</u>

Primary Antibodies				
Antibodies	**Species**	**Dilution**	**Company**	**Catalog No:**
Doublecortin (Dcx)	Mouse	3 in 100	Santa Cruz	sc-390645
Prox1	Rabbit	1 in 200	Abcam	ab101851

Secondary Antibodies				
Antibodies	**Species**	**Dilution**	**Company**	**Catalog No:**
Anti-mouse Alexa Fluor 488	Donkey	1 in 200	Jackson Immunoresearch	715-546-151
Anti-rabbit Alexa Fluor 488	Donkey	1 in 200	Jackson Immunoresearch	711-546-152
Anti-mouse Cy3	Donkey	1 in 200	Jackson Immunoresearch	715-166-150
Anti-rabbit Cy3	Donkey	1 in 200	Jackson Immunoresearch	711-166-152
Anti-rabbit Alexa Fluor 647	Donkey	1 in 200	Jackson Immunoresearch	711-606-152

Statistical Analysis

All the statistical analysis was performed using GraphPad Prism 8. The statistical methods used for each figure is specifically noted in the figure legends. A two tailed Spearman's correlation was performed to calculate the strength of linear relationship between two variables (Fig 1 and 3). For figure 2 to identify the critical window of cognitive decline, the cohort was divided into three DD groups; < 20 years (G1, N = 9), 21 to 30 years (G2, N =14), and > 31-years (G3, N =10), allowing ANOVA analyses, followed by a Tukey's multiple comparison post-hoc test. For Fig 3c, a comparison of fits between linear regression, logarithmic, and exponential one phase decay was performed. The parameters for goodness of fit were better (R^2 value closer to 1, lower RMSE) for exponential compared to logarithmic and linear regressions.

	R^2	RMSE
Linear	0.5192	271.5
Exponential one phase decay	0.7903	179.3
Logarithmic	0.7200	199.82