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Original Article

Expression of neurogenesis genes in human temporal lobe epilepsy with hippocampal sclerosis

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Abstract: Both evoked and spontaneous seizures have been reported to increase neurogenesis in animal models. Less is known about whether neurogenesis and markers thereof are aberrantly expressed in human temporal lobe epilepsy (TLE) with hippocampal sclerosis. In the present study we measured protein levels of multiple neurogenesis marker genes using Western blotting. Tissue homogenates from sclerotic hippocampus surgically resected from patients with pharmacoresistant TLE (n = 7) were compared to hippocampal samples from a group of age- and gender-matched autopsy controls (n = 6). Expression of the mature neuron marker NeuN was significantly lower in TLE samples compared to controls. In contrast, levels of neurogenesis-associated genes including TUC-4, doublecortin, NeuroD and Numb, were all similarly expressed in TLE and control hippocampus samples. The present study suggests hippocampal expression levels of proteins associated with neurogenesis are not notably different in human TLE, implying the sclerotic hippocampus may retain neurogenic potential.

Keywords: Bromodeoxyuridine, epileptogenesis, neurogenesis, mesial temporal sclerosis, seizure

Introduction

Neurogenesis occurs in the adult brain of mammals, including human and non-human primates, within the subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone [1-3]. Within the hippocampal SGZ, progenitors mature into granule neurons, migrate into the granule cell layer and send their axons to the hilus and CA3 subfield [4]. Rates of neurogenesis, nevertheless, decline with age in mammals, including humans [5-8].

In addition to its role in certain cognitive tasks [9-10], neurogenesis may serve important reparative functions. Increased neurogenesis is detected in chronic neurodegenerative diseases, including Alzheimer's [11-12]. Acute brain injuries such as focal cerebral ischemia also increase neurogenesis, and this may contribute to functional recovery [13-15].

There is ample evidence that seizures increase hippocampal neurogenesis [16-19]. While seizure-induced new neurons can integrate into hippocampal circuits [20], others die by an apoptosis-like mechanism [21]. The functional significance of seizure-induced neurogenesis is not properly understood but seizure-generated new neurons may exert a pro-epileptogenic effect [22-23]. Decreased as well as increased neurogenesis has also been reported in the weeks following *status epilepticus* and in chronically epileptic animals [17, 24-28].

Studies of human hippocampus have reported enhanced neurogenesis in patients with TLE based mainly on immunohistochemical evidence of increased proliferation and early neuronal differentiation markers including Ki-67, doublecortin (DCX) and polysialylated neural cell adhesion molecule (PSA-NCAM) [29-31]. Other studies, however, found either no evidence of increased neurogenesis or detected it only in pediatric patients [6, 32-33]. The neurogenic potential of the sclerotic hippocampus in hu-

Table 1. Clinical data

Variable	Controls	TLE patients
Group size (*n*)	6	7
Age	29 ± 7 *y* (range 13 – 53 *y*)	40 ± 7 *y* (range 15 – 57 *y*)
Gender	Male, 5; Female, 1	Male, 2; Female 5
Side of resection	n.a.	3 left, 4 right

Data are mean ± SEM for controls (*n* = 6) and TLE (*n* = 7). *y*, year; n.a., not applicable/available

man TLE is therefore uncertain.

In the present study we aimed to assess neurogenesis in control and TLE patients by measuring hippocampal expression of a variety of marker genes. We report that while levels of the mature neuron marker NeuN are lower, expression of multiple neurogenesis marker genes in sclerotic hippocampus from TLE patients is similar to human autopsy control.

Materials and methods

Human brain tissue

Collection of human TLE samples was approved by the Legacy Health System Institutional Review board and informed consent was obtained from all patients, as described previously [34-35]. Patient demographics are presented in **Table 1**. All patients were determined to have medically intractable TLE with hippocampal sclerosis. No patient had experienced *status epilepticus* during the year of their surgery and all patients were taking antiepileptic drugs prior to surgery. Surgical evaluation followed referral by an epileptologist and included video-EEG recording and neuroimaging (magnetic resonance imaging). Hippocampal tissue obtained at surgery was flash-frozen in liquid nitrogen and stored at −70°C until use. Samples used in the present study (*n* = 7) were originally part of a larger (*n* = 10) cohort [35-36]. Insufficient sample precluded analysis of three (E3, E4 and E5). Thus, in the present study we analyzed samples from patients E1, E2, E6, E7, E8, E9 and E10.

Human control tissue samples (*n* = 6) were obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, Maryland, USA. The samples were also fresh-frozen, and had been donated from people who died of causes, reported elsewhere [36], not related to known neurological disease. Average autopsy delay was 8 ± 1 h (range 5 – 12 h).

Each hippocampal specimen was originally sectioned on a cryostat (12 μm) to prepare slides for immunohistochemistry, with the remaining bloc prepared for protein analysis [35]. Inter-specimen variability in quantity and orientation precluded microdissecting the tissue to obtain specific subfields.

Simulated post mortem delay

To evaluate whether autopsy delay in our controls had any striking effects on protein stability, a postmortem interval was simulated using mouse brain tissue. Procedures were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland, under license from the Department of Health, Dublin, Ireland. Mice (C57Bl/6, 8-9 weeks old (20-22 g) *n* = 3 per group) were killed and left at room temperature for either 4 or 8 h. In a third group, the hippocampus was extracted immediately after death to represent a scenario similar to surgical extraction. Hippocampus was then processed for western blotting as described below.

Western blotting

Western blotting was performed as previously described with modifications [34-36]. Tissue was first homogenized in a Tris-EDTA lysis buffer containing protease inhibitors Aprotinin, Leupeptin, Pepstatin and phenylmethylsulfonyl fluoride, to obtain whole cell protein lysates. Protein concentration was calculated using Bradford reagent and 50 μg samples boiled in a gel-loading buffer and run on 12-15% SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, PA,

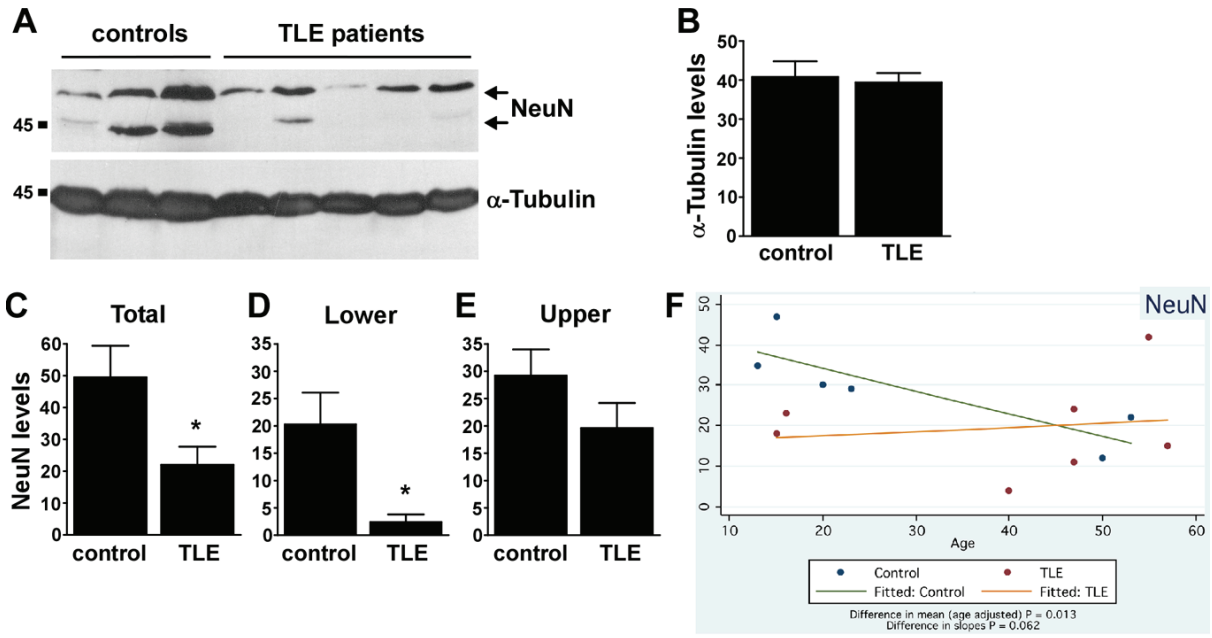

Figure 1. Lower expression of the mature neuron marker NeuN in TLE hippocampus. **A**. Representative western blots (n = 1 per lane) showing (top panel) NeuN in TLE patient hippocampus compared to autopsy controls and (bottom panel) α-Tubulin. Protein weight markers (in kD) depicted to left. Samples on the gels were from controls C1-C3 and patients E1, E2, E6, E7 and E8 (see Methods). **B-E**. Graphs showing semi-quantification of western blot data (n = 6-7 per group). Graph in *B* shows densitometry for α-Tubulin, *C*, NeuN (densities of both bands combined), *D*, the lower band of NeuN and E, the upper band of NeuN. * $p < 0.05$ vs. control. *y* axis data are in arbitrary units. **F**. Graph showing correlation between age and expression of the upper band of NeuN in control and TLE patient hippocampus. Note the decline in levels as a function of age in controls.

USA) and then incubated with antibodies against NeuN (MAB377, 1:500, mouse monoclonal), TUC-4 (AB5454, 1:2000, rabbit polyclonal) and PSA-NCAM (MAB5324, 1:1500, mouse monoclonal), all from Chemicon, Temecula, CA, USA. Anti-Numb (07-147, 1:500, rabbit polyclonal) was from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Antibodies against NeuroD (G-20 and N-19, both 1:100, goat polyclonal) and DCX (C-18, 1:250, goat polyclonal) were from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Membranes were washed and next incubated with HRP-conjugated secondary antibodies followed by chemiluminescence detection (NEN Life Science Products, Boston, MA, USA) and exposure to BioMax film (Kodak Scientific Imaging Systems, Rochester, New York, USA).

Densitometry and statistical analysis

For a given protein, samples from C1-C3 were run with E1, E2, E6-E8 on the first gel and C4-C6 with E9-E10 on the second gel. The resultant immunoblotted membranes encompassing all samples were then exposed at the same time to a single film. These were then scanned, digitized using Adobe® Photoshop® 6.0 and analyzed using gel-scanning integrated optical density (O.D.) software (AlphaEaseFC 4.0). Band density was semi-quantified using the object box function in AlphaEaseFC 4.0 after background subtraction. For statistical comparisons of densitometry measurements an unpaired *t*-test with Welch correction was used for two group comparisons and analysis of variance (ANOVA) for three group comparisons (GraphPad Instat v3.06, GraphPad Software, Inc, La Jolla, CA, USA). For correlative statistics, data were analyzed with StataSE release 10, using linear regression models with robust variance estimation. Separate terms were entered for control and epilepsy, and an indicator variable for epilepsy. Two hypotheses were tested: a) that the slope of the relationship between the parameter under test and age differed between

epilepsy and control and b) that adjusted for age, the mean value of the parameter under test differed between control and epilepsy. The former hypothesis was tested by a Wald test comparing slope between control and epilepsy, and the latter by testing the hypothesis that the coefficient for the indicator variable for epilepsy was zero. Data are presented as mean ± S.E.M and significance was accepted at $p < 0.05$.

Results

Lower expression of the mature neuron marker NeuN in human hippocampal sclerosis

Control and patient data are presented in **Table 1**. Western blot analysis of NeuN, a mature neuron marker [37], revealed a doublet running at its predicted weight of 46-50 kD (**Figure 1A**). Levels of the heavier band were typically higher than the lower weight band in both patient and control groups. Densitometric analysis determined total NeuN levels were significantly lower in TLE samples compared to controls (**Figure 1A, C**). When the doublet was analyzed as separate bands, expression of the lower weight band was significantly lower in TLE samples compared to controls (**Figure 1D**). In contrast, the heavier-weight band was ~ 30% lower in TLE samples but this did not reach statistical significance ($p = 0.18$) (**Figure 1E**). As a marker of protein loading we also probed membranes for α-tubulin. Expression of α-tubulin was not significantly different between control and TLE groups (**Figure 1B**).

Expression of neurogenesis genes in human hippocampal sclerosis

TUC-4 (turned on after division/Ulip-1/CRMP-4) is a gene expressed early in differentiation and a marker of immature, potentially new neurons [38-39]. TUC-4 was detected in control hippocampus at ~60kD. TUC-4 was also present in all TLE samples (**Figure 2A**). Densitometry determined there was no significant difference in expression of TUC-4 between control and TLE groups (**Figure 2B**). Interestingly, a heavier weight band running ~7-10 kD above the principal TUC-4 band was evident in 6 of 7 TLE samples but not the controls, which densitometry confirmed was significant ($p < 0.01$). The nature of this band is unknown but may represent a splice variant or post-translational modification such as ubiquitination.

Doublecortin (DCX) is a microtubule-associated protein found in immature migrating and differentiating neurons [40-41]. Expression of DCX was detected at ~43 kD in both control and TLE samples and there were no significant differences between the groups (**Figure 2A, B**).

NeuroD (Neurogenic differentiation factor 1) [42-43] is a 40 kD neurogenic basic helix-loop-helix protein required for differentiation of granule neurons in the hippocampus [44]. We detected NeuroD in control brain samples as a single

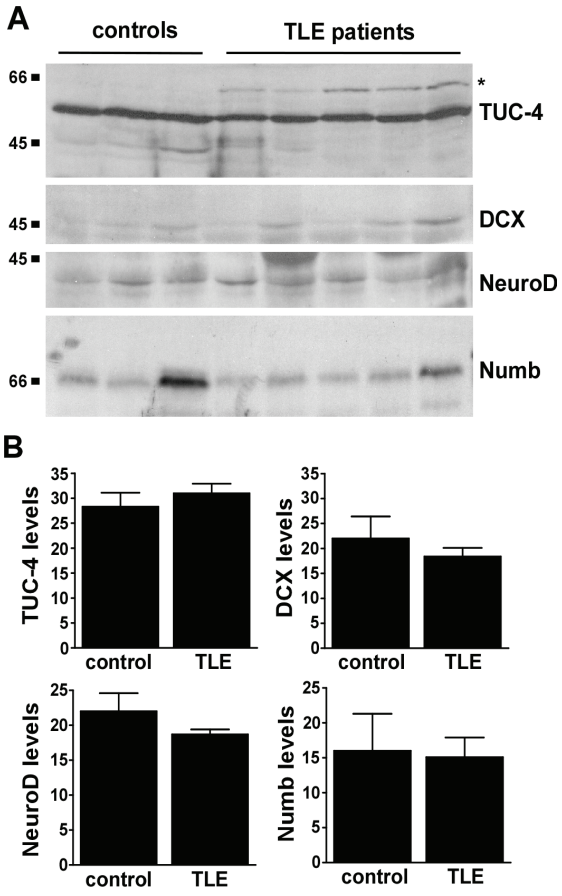

Figure 2. Expression of neurogenesis marker genes in human TLE hippocampus. **A**. Representative western blots ($n = 1$ per lane) of TUC-4, DCX, NeuroD and Numb in hippocampus from autopsy controls and TLE patients. Note, expression of each gene is similar between the groups. *denotes a TUC-4 immunoreactive band of unknown nature present only in TLE samples. Samples run on the gels were C1-C3 and E1, E2, E6, E7 and E8. **B**. Graphs showing semi-quantification of immunoblot data (in arbitrary units) (n = 6-7 per group).

band at its predicted weight (**Figure 2A**). Use of a second anti-NeuroD antibody also produced the same result. NeuroD was also present in TLE samples and densitometry determined there was no significant difference between control and TLE groups (**Figure 2A, B**).

Numb is implicated in progenitor cell maintenance, control of cell fate during development and radial glia junctions [45-47]. We detected Numb as a single band at the predicted molecular weight of ~70kD protein at low-levels in human control hippocampus (**Figure 2A**). Numb was also present in TLE samples and densitometry determined levels were not different to controls (**Figure 2A, B**).

Expression of a final neurogenesis marker, PSA-NCAM, which is a plasma membrane glycoprotein expressed by neuronal progenitors, differentiating neurons and astrocytes, could only be accurately measured in a subgroup of samples (n = 3 controls and n = 4 TLE samples). Again, no significant difference was found in expression between controls and TLE samples (data not shown).

Effect of subject age on expression of neurogenesis genes

Neurogenesis has been reported to display an age-dependent decline in humans and expression of neurogenesis genes, including DCX, may mirror this decrease [5-6]. To determine whether differences in subject age might have been a factor in our study we performed regression analyses of the relationship between subject age and expression of the various tested genes in controls and epilepsy patients. There were no age associations for DCX or Numb, and no evidence of differences in levels between epilepsy and control samples. For NeuroD, there was a tendency to decreased levels in controls with age, which fell short of significance (p = 0.084). Adjusted for age, the mean NeuroD level was lower in patients with epilepsy than in controls but this too fell short of significance (p = 0.089). TUC-4 values had a non-significant relationship with age in epilepsy patients, but increased with age in controls. The Wald test for equality of slopes was significant (F(1,9df)=7.7, p = 0.021). Adjusted for age, the mean level differed between epilepsy and control, with a higher mean value in those with epilepsy (t=2.6, p = 0.028). Last, we found that expression of the heavier weight NeuN band at ~55 kD fell significantly with age in controls (p = 0.015), but not in epilepsy patients (**Figure 1F**). The Wald test for equality of slopes, however, fell short of statistical significance (F(1,9 df)=4.5, p = 0.062). Of note, when adjusted for age, the mean level differed between epilepsy and control, with a lower mean value in those with epilepsy (p = 0.013). The lower-weight and NeuN combined bands showed no relationship with age, either in epilepsy or controls.

Effects of simulated postmortem interval on neurogenesis genes

To evaluate whether expression of the tested genes was influenced by autopsy delay, we performed a simulated post-mortem interval experiment in mice. Mice were killed and then hippocampus was extracted either immediately or following a 4 or 8 h delay at room temperature. We then performed western blot analysis of a selection of neurogenesis genes (**Figure 3**). Western blotting determined that levels of Numb, TUC-4 and DCX did not significantly change up to 8 h after being left at room temperature, although there was a slight trend to increased protein levels (Figure 3A, B).

Discussion

The results of the present study suggest various markers of immature, differentiating and migrating neurons are expressed in the sclerotic hippocampus of patients with TLE. While levels of the mature neuron marker NeuN were reduced (relative to control) supporting injury, markers of immature, differentiating and migrating neurons (including TUC-4, NeuroD, DCX and Numb) were similarly expressed. These data suggest neurogenesis is not necessarily aberrant in human TLE and that neurogenic capability is largely preserved in human mesial temporal sclerosis.

Hippocampal sclerosis is characterized by major neuron loss in CA1, CA3 and hilar regions, with relative sparing of the dentate granule neurons and CA2 subfield [48-49]. Whether the neurogenic potential is altered in hippocampal sclerosis is not certain and seizure-induced neurogenesis has been suggested to contribute to the epileptogenic process [22-23, 50]. There has been speculation that the pool of neural precursors in the hippocampus may be depleted in

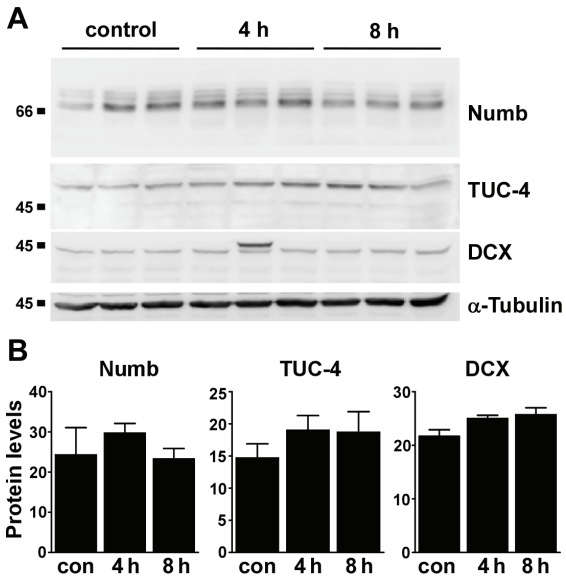

Figure 3. *Effect* of simulated autopsy delay on expression of neurogenesis genes. **A**. Representative western blots (n = 1 per lane) of Numb, TUC-4 and DCX in mouse hippocampus extracted immediately (control) or after a 4 or 8 h delay at room temperature. Note expression of each remained largely unaltered. **B**. Graphs showing semi-quantification of western blot data (n = 3 per group). Protein levels are given in arbitrary units.

human epileptic brain [32]. Also, data from animals has suggested that, in contrast to the increase seen after *status epilepticus*, neurogenesis is severely reduced in chronic epilepsy [25, 27].

The experiments here indicate the presence of multiple markers of early neuronal differentiation and development in sclerotic hippocampi from patients with TLE. Our study included a larger number of neurogenesis marker genes than has been undertaken in any previous single study [6, 29-31, 33]. While DCX and PSA-NCAM have previously been reported in human TLE [6, 29-31, 33], the present study included analysis of novel markers NeuroD, TUC-4 and Numb. TUC-4 and NeuroD expression appears to depend on the differentiation stage, with TUC -4 expressed during early differentiation versus terminal differentiation for NeuroD [4, 42, 51]. Numb is required for cortical neurogenesis and recent work has identified a role in maintaining the integrity of the neurogenic epithelium [46-47].

In the present study, hippocampal expression of the novel and previously reported neurogenesis genes was generally similar between TLE and control samples. It is therefore unlikely that neurogenesis is severely impaired in human TLE with hippocampal sclerosis. This contrasts with data from experimental animals that found severely depleted neurogenesis in modelled TLE [25, 27]. Instead, our data are in broad agreement with the human data of Fahrner *et al.,* who did not detect differences in various neurogenesis markers, including DCX [6]. Several studies of human hippocampus have reported, however, increased levels of certain proliferative markers and markers of early neuron differentiation, including DCX and PSA-NCAM [29-31]. Do our data conflict with these reports? Several factors must be considered, including what serves as an appropriate "loading" control. If the measure were of neurogenesis markers per quantity of mature neuronal markers then NeuN, rather than α-Tubulin, would be the appropriate standard. This could be argued to be the better way to "normalize" our data, in which case each of the neurogenesis markers would be at increased amounts. Of note, adjusting for age did show higher TUC-4 levels in epilepsy. Our data would therefore be consistent with the work showing increased neurogenesis in human TLE [29-31]. If instead we accept α-Tubulin as the better control then we must conclude our data support relatively normal, not increased, neurogenesis in human TLE with hippocampal sclerosis. Alternatively, we are left to conclude that the aspects of neurogenesis regulated by the several genes assessed in our study are not overly influenced by seizures or the presence of hippocampal sclerosis. Why should the various human findings differ? Our study relied on western blotting rather than immunohistochemical analysis, which enables semi-quantification of gene expression with high sensitivity but lacking spatial resolution. Differences in the degree of sclerosis between cohorts in the various studies, the surgical procedures, regions selected for analysis, and the autopsy control material are possible sources of variation. Nevertheless, we can be confident the levels of the tested genes in our control samples are representative since our data on TUC-4, DCX and NeuroD levels were similar to those published previously [12].

While expression of neuronal differentiation and migration markers was not different from autopsy controls, levels of NeuN were reduced. NeuN is a well-established marker of mature

neurons which was recently determined to be the product of the Fox-3 gene of RNA splicing factors [52]. Since our data suggest early differentiation markers are robustly expressed, and recognizing that NeuN can be co-expressed with neurogenesis markers such as PSA-NCAM and TUC-4 [4], we can speculate that many immature neurons born in the sclerotic hippocampus do not reach maturation. Indeed, many new cells born after seizures do not integrate into the granule cell layer and instead die. Molecular and pharmacologic studies suggest the cell death process is apoptosis [16, 21, 53]. Notably, there is biochemical evidence of apoptosis-associated signalling in hippocampus from TLE patients, although morphologic evidence of apoptosis is scarce [33, 36, 54-55]. While the apoptotic molecular signature seen in human TLE hippocampus has been largely assumed to reflect repeated seizures activating cell death pathways [56], we should consider that death of recently produced immature neurons may contribute.

We did not detect the reported age-dependent decrease in the neurogenesis marker DCX in human hippocampus [6, 8], nor indeed age-related decreases of other neurogenesis markers. This was surprising given the known decline in neurogenesis in aged brain [5, 7]. Whether this is due to the sensitivity of our measurements, sample *n*, the extent to which our hippocampal homogenates were or were not enriched with SGZ, an autopsy effect, or the subject's age range, is uncertain. Nevertheless, not all studies detect age-dependent declines in neurogenesis markers in normal or epileptic human brain [31]. Studies have also found the aged rodent brain to be normal with regard to capacity of seizure-induced neurogenesis [57]. Instead, our study found an age-related fall in expression of a NeuN species in controls. This finding is compatible with an age-dependent decline in expression of NeuN in normal human hippocampus which may relate to changing expression patterns or functions of this gene over time [52]. The association was not present in TLE samples where the NeuN band was instead expressed at lower levels regardless of subject age. This could reflect the majority of hippocampal damage occurring earlier in life following an initial precipitating insult [58].

Some caveats to the design and interpretation of the present study have already been mentioned. In addition, our patient numbers were small and our material lacked sufficient anatomic detail to determine the site of expression of the genes studied and, unlike the detailed localization of certain neurogenesis markers performed in other studies [6, 29-30, 32], we could not confirm the expressed genes localized to the SGZ. Finally, we did not examine certain markers reported by others such as Ki-67, or other markers of neurogenesis associated with the control of proliferation [59].

In conclusion, the present study profiled expression of a variety of neurogenesis-associated genes in human hippocampus. While other published evidence supports seizure-induced neurogenesis as having restorative as well as potentially epileptogenic functions, our findings suggest expression of genes associated with neurogenesis is not necessarily abnormal in human TLE with hippocampal sclerosis.

Acknowledgements

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