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An increase of hippocampal calretinin-immunoreactive neurons correlates with early febrile seizures in temporal lobe epilepsy

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Abstract Numerous studies indicate that initial precipitating injuries (IPI) such as febrile seizures during early childhood may play a pivotal role in the pathogenesis of temporal lobe epilepsy (TLE) and Ammon's horn sclerosis (AHS). Previous data demonstrate an increase of horizontally oriented neurons in molecular layers of hippocampal subfields, which are immunoreactive for calretinin (CR-ir) and resemble Cajal-Retzius-like cells. Cajal-Retzius cells are transiently expressed in the murine developing hippocampus and are critically involved in neuronal pattern formation. Here we investigated a potential relationship between the distribution of horizontally oriented calretinin-immunoreactive neurons and the clinical history of TLE patients with AHS. Horizontally oriented neurons in the molecular layer of the hippocampal formation have been visualized by antibodies against the calcium-binding proteins calretinin and calbindin D-28k. Cell counts derived from 27 epilepsy patients with AHS were compared with autopsy specimens from developing and adult normal human hippocampus (n = 26). During ontogeny, CR-ir cells showed a marked perinatal peak in the CA1 and dentate gyrus molecular layer (CA1-ML, DG-ML) followed by a gradual postnatal decline. In hippocampal specimens from TLE patients with AHS and seizure onset before the age of 4 years, significantly higher levels of CR-ir neurons in CA1-ML (P = 0.05) and DG-ML ($P < 0.05$) were encountered than in AHS patients without precipitating seizures or with an uneventful early medical history. However, all three groups had higher levels of CR-ir neurons compared to adult controls obtained at autopsy ($P < 0.01$). In addition, AHS specimens showed increased CR-ir neuropil staining throughout the DG-ML compared with the restricted distribution of CR-ir fibers within the superficial granule cell layer visible in controls. These findings suggest that a condsiderable number of TLE patients with AHS display signs of impaired hippocampal maturation and circuitry formation as indicated by increased numbers of Cajal-Retzius like cells. It remains to be elucidated, how these changes contribute to the pathogenesis of TLE.

Key words Ammon's horn sclerosis · Calcium-binding proteins · Cajal-Retzius cells · Development · Hippocampus

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Introduction

Ammon's horn sclerosis (AHS) is a common pathological finding in chronic, pharmaco-resistant temporal lobe epilepsy (TLE). The most striking morphological features of AHS include severe loss of principal neurons in the CA1, CA3 and CA4 subfields of the hippocampus [19, 47]. In addition, some populations of interneurons are also affected [14, 50], whereas other interneurons are well preserved in AHS [3, 8, 27, 38]. Surviving neurons show marked synaptic reorganization and axonal sprouting and this may lead to aberrant neuronal circuitries [4, 21, 27, 36, 42]. However, the exact sequence of pathological al-

terations during the pathogenesis of AHS cannot be systematically studied since hippocampal tissue is invariably obtained from chronically ill patients with a prolonged history of TLE. Therefore, it remains uncertain whether the typical neuropathological alterations in AHS represent a preexisting substrate for the development of TLE or are the consequence of repeated seizures [30].

Attempts to clarify this issue have been undertaken in animal models of TLE. Chronic activation of hippocampal afferent connections via electrodes implanted into the amygdala can lead to segmental cell loss and aberrant synaptic sprouting [11, 41]. In addition, animal models of chronic status epilepticus can reproduce some aspects of neuronal damage associated with human AHS [7, 10]. These data show that seizure activity may lead to structural alterations resembling AHS but do not exclude a preceding disturbance of hippocampal development in human TLE. There are some lines of evidence pointing to developmental abnormalities in TLE, including the known relationship between early risk factors and AHS [19, 29, 32, 35]. Such an association is of particular interest since the human hippocampus is not completely matured after birth [2]. In rat hippocampus, dentate granule cell neurogenesis persists postnatally [1, 24] and is increased by seizures [34]. However, granule cell dispersion in the dentate gyrus is a frequent finding in patients with chronic TLE [22], indicating that seizure-induced damage may alter normal hippocampal development and connectivity [34]. Another cell population that undergoes considerable regulation during ontogenesis is represented by calretinin-immunoreactive (CR-ir) Cajal-Retzius cells [26]. In rat hippocampus, peak numbers of this cell type are visible at early postnatal stages which subsequently decline towards low cell densities in the adult [40]. Interestingly, increased numbers of CR-ir horizontal cells resembling Cajal-Retzius neurons could be detected in the molecular layer of hippocampi with AHS compared to autopsy control tissue or hippocampi from patients with lesion-associated epilepsy [8]. Two hypotheses have been derived from this latter observation. CR-ir neurons may be generated de novo due to the epileptogenic process. This is, however, unlikely since no increase in CR-ir cells was found in the hippocampi from patients with lesion-associated epilepsy [8]. On the other hand, high levels of CR-ir Cajal-Retzius cells could persist from early postnatal stages. It was, therefore, of interest to analyze the density of CR-ir neurons during normal hippocampal development to confirm the ontogenetic regulation of this cell population in humans. In addition, we determined the impact of seizures in early childhood on CR-ir horizontal cells in patients with therapy-refractory TLE and AHS.

Materials and methods

Clinical data

Hippocampal specimens from 27 patients (age 32.9 ± 2.0 years) with chronic TLE (mean duration: 22.9 ± 2.1 years; mean age at the onset of seizures 10.0 ± 1.0 years) were included in this study. All patients had complex partial seizures (CPS) with a frequency between 1 and 120 events per month; 21 patients had secondary generalized seizures and 11 patients experienced simple partial seizures in addition to focal seizures. Recurrent episodes of status epilepticus were reported in 2 patients (A2 and A7, see Table 1). The epileptogenic focus was localized to the temporal lobe in all patients by noninvasive and invasive diagnostic procedures as described elsewhere [5]. In all patients, the removal of the hippocampus was clinically warranted to achieve seizure control. The following surgical procedures were used: lesionectomy with amygdalohippocampectomy (7 cases) [49] and selective amygdalohippocampectomy (20 cases). Fourteen resections were performed on the right temporal lobe and 13 on the left temporal lobe. Informed consent was obtained from all patients for additional histopathological evaluation. All procedures were approved by the University of Bonn ethics committee and conform fully to the standards set in the Declaration of Helsinki (last revised 1989). Seizure outcome data were obtained from clinical records and standard postoperative follow-up patient interviews. The incidence of seizures in the most recent 6-month period was recorded. Following epilepsy surgery, 3 patients were not seizure free (patients A2, A14 and A22) but 2 patients showed a > 75% reduction in seizure frequency. Isolated auras were recorded in 2 patients (patients A11 and A21, marked by asterisk in Table 1).

Processing of hippocampal specimens

For a routine neuropathological evaluation, the biopsy specimens were immersion-fixed in 4% buffered formalin at room temperature for 8 h–3 days and embedded in paraffin. Only specimens with a pathological diagnosis of classical AHS with severe neuronal cell loss in the CA1, CA3 and CA4 subfields and relative sparing of CA2 were selected for the study. None of the patients showed evidence of a focal lesion, e.g., neoplasm or neurodevelopmental malformation in the temporal lobe.

Six specimens from fetal human hippocampus (19th, 20th, 26th, 29th, 38th, 40th week of gestation, WOG), and 14 postnatally derived specimens ranging from the 6th day to 15 years of age were obtained at autopsy. In addition, 6 adult specimens from patients without neurological disorders were used as controls. The post-mortem intervals ranged from 12 h to 3 days. The tissues were immersion-fixed for at least 2 weeks in 4% formalin and samples were embedded in paraffin. Hematoxylin-eosin-stained sections were available for all specimens. None of these patients had clinical evidence for a neurological disease and the brains were normal as confirmed by a thorough neuropathological examination. The samples obtained from immature brains were carefully reviewed to exclude extensive brain swelling.

Immunohistochemical processing was performed with a polyclonal antibody against the calcium-binding protein calretinin (CR). The antibody has been shown to react reliably with formalin-fixed and paraffin-embedded human tissue [37]. In addition, monoclonal antibodies directed against human calbindin D-28k (Swant, Bellinzona, Switzerland) were used in some cases to additionally identify horizontally oriented, Cajal-Retzius like cells in the molecular layers (ML) of dentate gyrus (DG) and CA1 [26]. Paraffin sections were cut at 4 μm and stained under identical conditions using the capillary gap method and a slide holder with the capacity for 60 sections. The sections were heated on a 65° C hot plate for a few seconds until the paraffin had melted. After deparaffination with xylene and rinses in 100% and 95% ethanol, the slides were incubated in 2% hydrogen peroxide diluted in methanol for 15 min to block endogenous peroxidase activity. Subsequently, the sections were rinsed in 95% ethanol and transferred into phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Serva, Heidelberg, Germany). Preincubation with 2% goat serum (Vector Labs, Burlingame, Calif.) and 10% fetal calf serum (FCS; Seromed, Berlin, Germany) diluted in PBS was performed for 20 min at 42° C, followed by incubation with the primary polyclonal anti-CR antibody at room temperature for 24 h (1 : 5000 dilution). The sections were then incubated with biotiny-

Table 1 Clinical data of TLE patients with AHS used in this study. Patients were classified into three groups: group 1 (no IPI), no history of a precipitating injury during the first 4 years of life; group 2 (non-seizure IPI), history of a precipitating injury during the first 4 years of life without evidence for an early seizure; group 3 (seizure IPI), FS during the first 4 years of life. Age represents the age of the patients at surgery; onset, the age at which a first epileptic seizure was documented (average 9.9 ± 7.7 years), duration, duration of the epilepsy disorder (13.8 ± 6.2 years); Frequency, frequency of complex partial seizures per month determined over the last 3 months preceding presurgical evaluation under a full antiepileptic drug regimen; OP side, type of resection, side of resection; follow up, postoperative interval for which follow up data could be obtained (in months); seizure free, patients seizure-free during the follow-up period are designated with y, those without with n (*TLE* temporal lobe epilepsy, *IPI* initial precipitating injury, *FS* febrile seizure, *sAHx* selective amygdalohippocampectomy, *TLx + Hx* lesionectomy with amygdalohippocampectomy, *r/l* right/left temporal lobe, *CPS* complex partial seizures, *SPS* simple partial seizures, *sGS* secondary generalized seizures)

	Patient ID	Age	IPI	Onset	Duration	Frequency	Seizure	Op side	Follow-up	Seizure free
Group 1 No IPI	A1	22	–	11	11	5–7	CPS	sAHx, r	3	y
	A2	22	–	10	12	10	SPS, CPS	sAHx, r	3	> 75
	A3	31	–	14	17	1–10	SPS, CPS, sGS	sAHx, r	12	y
	A4	35	–	16	19	?	CPS	sAHx, l	6	y
	A5	32	–	7	25	4–5	SPS, CPS, sGS	sAHx, l	5	y
	A6	17	–	8	9	2–6	SPS, CPS, sGS	sAHx, l	6	y
	A7	35	–	14	21	2–10	CPS, sGS	sAHx, l	6	y
	A8	21	–	7	14	30–120	SPS, CPS, sGS	sAHx, l	3	y
	A9	43	–	28	15	10–20	CPS, sGS	TLx+Hx, r	12	y
Group 2 Non-seizure IPI	A11	42	Meningitis at 4 years	6	36	12	CPS, sGS	sAHx, r	6	y*
	A12	38	Meningitis at 8 months	13	25	?	CPS	sAHx, r	3	y
	A13	37	Perinatal hypoxia	6	31	4–8	CPS, sGS	sAHx, r	3	y
	A14	42	Perinatal hypoxia and hemiparesis	12	30	0–2	CPS, sGs	TLx+Hx, r	3	n
	A15	22	Meningoencephalitis at 9 months	9	13	6	SPS, CPS, sGS	TLx+Hx, l	3	y
	A16	34	Meningoencephalitis at 6 months	10	24	3	SPS, CPS, sGS	TLx+Hx, l	1	y
Group 3 Seizure IPI	A17	57	FS, complicated birth	3	54	8–10	CPS, sGS	sAHx, r	9	y
	A18	42	Multiple FS during first 2 years	6	36	2–5	CPS, sGS	sAHx, l	5	y
	A19	34	Multiple FS during first 2 years	17	17	4	SPS, CPS, sGS	sAHx, r	6	y
	A20	51	Complicated birth, multiple FS in first 2 years	14	37	1–6	CPS, sGS	sAHx, l	3	y
	A21	26	Multiple FS during first 2 years	6	20	5–8	SPS, CPS	TLx+Hx, l	2	y*
	A22	32	FS at 8 months	14	18	10–15	CPS, sGS	TLx+Hx, r	6	> 75
	A23	34	FS at 6 months, meningitis at 18 months	4	30	5–10	CPS, sGS	sAHx, r	3	y
	A24	20	FS during first 2 years	5	15	2	CPS, sGS	sAHx, r	6	y
	A25	48	Umbilical cord strangulation, FS at 8 months	6	42	2	SPS, CPS, sGS	sAHx, r	6	y
	A26	28	Meningitis with FS at 18 months	9	19	3–4	CPS, sGS	sAHx, l	3	y
	A27	16	Complicated birth, bronchitis with FS	8	8	6–20	CPS	TLx+Hx, r	12	y
	A28	28	Complicated birth, FS at 10 months	8	20	15	SPS, CPS, sGS	sAHx, l	3	y

lated secondary antibodies (goat anti-rabbit; Vector) diluted 1 : 200 in PBS containing 10% FCS at 42 °C for 60 min, followed by incubation with the avidin-biotin complex (Vector Labs) for 90 min at 42° C. The reactions were developed in a substrate solution of 0.05% diaminobenzidine (ICN, Cleveland, Ohio) and 0.01% hydrogen peroxide (Merck, Darmstadt, Germany) in 0.05 M TRIS-HCl, pH 7.4. The sections were washed, and lightly counterstained with hematoxylin, dehydrated in ethanol and mounted. Negative control sections were prepared by omitting the primary antibody or by substituting the primary antibody with equivalent dilutions of non-immune rabbit IgG (DAKO, Glostrup, Denmark).

Assessment of CR-ir cell densities

Cell densities of CR-ir neurons were determined in two hippocampal subfields of epilepsy patients and control brains. Two consecutive sections from each subject were analyzed at a magnification of × 1000 using a semi-automated morphometrical system (Contron, Zeiss, Oberkochen, Germany). Fifteen counting areas of 6350 μm^2 in size were randomly placed within the DG-ML and the CA1-ML and the number of labeled cells was recorded. Only immunolabeled neurons in which a soma and the nucleus could be identified were counted, and labeled cell bodies extending beyond the upper and the right border of the ocular grid were not included. The mean number of cell counts/mm^2, derived from two sets of 15 areas of the respective hippocampal subregion were calculated for each patient. This parameter yields quantitative information suitable for inter-group comparison [8].

Classification of patients with AHS

To assess the impact of precipitating events in early development on the number of CR-ir neurons, patients with AHS were classified according to the presence or absence of a medically significant in-

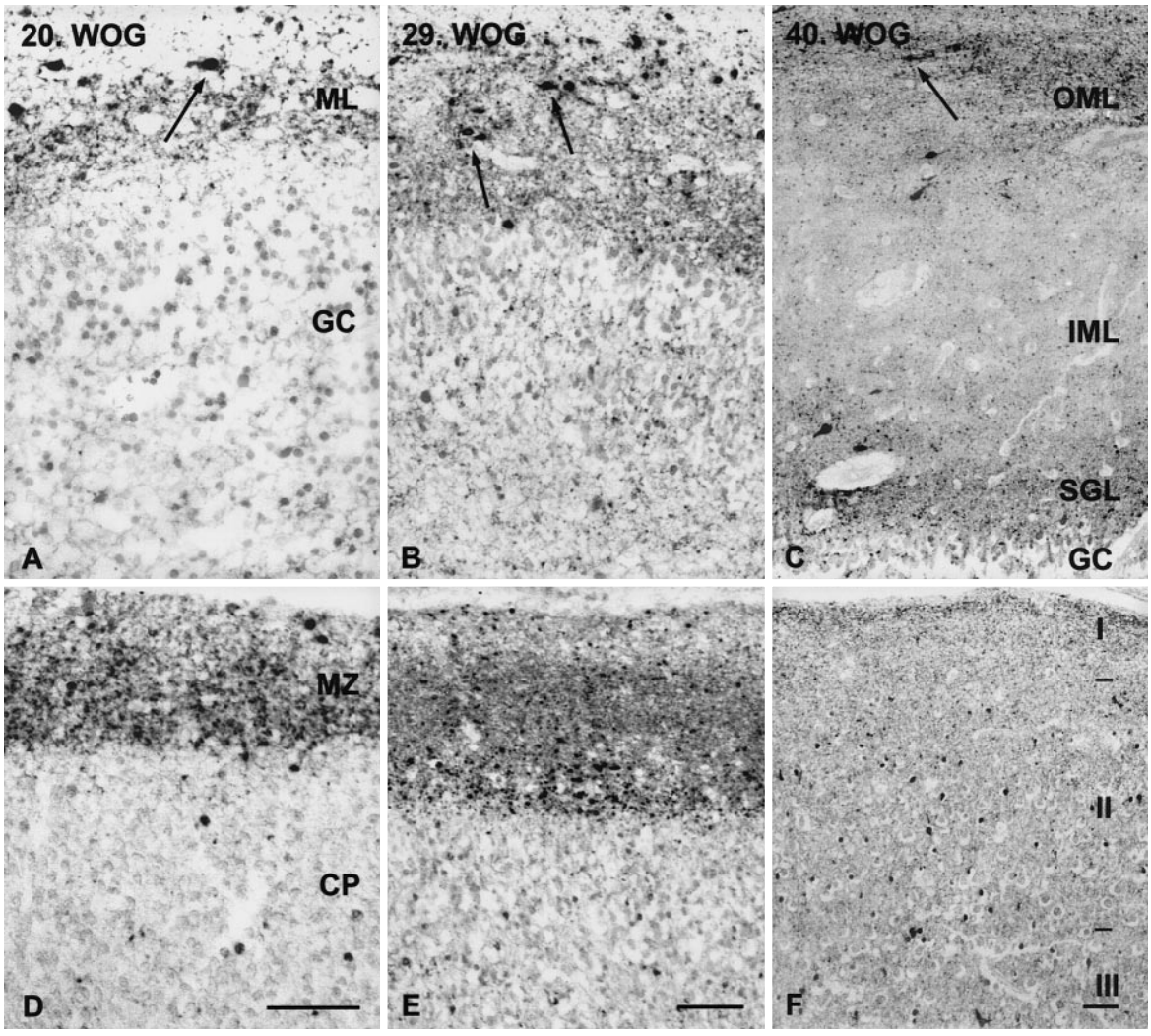


Fig. 1 Ontogenesis of CR-ir neurons in the human DG, (**A–C**) and temporal neocortex (**D–F**). **A** CR immunoreactivity in human DG at gestational week 20. Horizontally oriented CR-ir Cajal-Retzius cells *(arrow)* are found in the ML. **B** CR immunoreactivity in a hippocampal specimen at the 29th gestational week. The number of Cajal-Retzius cells, which were localized in the upper half of the ML *(arrows)*, has increased. **C** At the time of birth, the thickness of the DG-ML is significantly enlarged. However, Cajal-Retzius cells are still present within the upper portion of the ML *(arrow)*. **D** CR immunoreactivity in the adjacent temporal neocortex from the same patient shown in **A**. Note the dense labeling of CR-ir structures in the MZ which includes Cajal-Retzius cells. **E** CR immunoreactivity is still prominent in the marginal zone (same patient as in **B**). **F** At the time of birth, a significant labeling of CR-ir Cajal-Retzius cells is not longer visible. CR-ir cells in the neocortex resemble those already described for adult neocortex in rat, monkey and man [9, 25, 48] (*DG* dentate gyrus, *CR-ir* calretinin-immunoreactive, *ML* molecular layer, *OML* outer molecular layer, *IML* inner molecular layer, *SGL* supragranular layer, *GC* dentate gyrus granule cell layer, *MZ* marginal zone of the neocortex, *CP* cortical plate, *I-III* cortical layers I-III, *WOG* week of gestation). Paraffin sections, 4 μm thick. *Bars* **D–F** 50 μm

jury during the first 4 years of life. Such injury has been previously termed initial precipitating injury (IPI, [28]). Pertinent information was extracted from the medical records and gathered during patient interviews. Patients were classified into one of the following groups. Group 1: uneventful early medical history (n = 9); these patients had no known history of medical illness or seizures prior to the onset of chronic TLE. Group 2, IPI: history of trauma, ischemia or meningitis/encephalitis before the age of 4 years (n = 6); these events were not associated with early seizures in these patients. The distinction from group 3 was made on the basis of the presence of an early seizure. Most patients required hospitalization due to their condition and showed neurological deficits secondary to the disease. These patients had no recognized seizures until the onset of TLE, which was as early as 6 years of age. Group 3, IPI: patients with febrile or other generalized occasional seizures before the age of 4 (n = 12). Most patients in this group had a generalized seizure before the age of 2 years associated with significant medical illness such as encephalitis, meningitis, birth trauma, perinatal hypoxia or other systemic childhood disease with fever. The distinguishing factor from group 2 was the presence of a generalized seizure before the manifestation of the TLE disorder.

Statistical analysis

The data obtained from the measurements of neuronal densities of CR-ir nerve cells were statistically analyzed using the SPSS statistical software package (SPSS Inc., Munich, Germany). Mean cell counts of the three groups (control, lesion and AHS) were compared using an ANOVA and Duncan's multiple T-tests for each hippocampal subfield. Since normally distributed samples cannot be assumed, we also carried out a Kruskall-Wallis H test and a Mann-Whitney U-Wilcoxson Rank Sum test. Correlations were analyzed

with a Spearman Rank correlation. Values were expressed as mean ± standard error of the mean (SEM) unless otherwise stated.

Results

Ontogenesis of CR-ir neurons in human hippocampus

CR immunohistochemistry was performed in control specimens of different pre- and postnatal age groups. A characteristic feature of CR immunoreactivity in the prenatal hippocampus was the labeling of numerous neurons in the ML of the Ammon's horn and DG (Fig. 1). This particular subpopulation of nerve cells, which displayed a horizontal orientation of mono- or bipolar processes, has been classified as Cajal-Retzius cells (Fig. 2) [6, 20, 26]. An increase of Cajal-Retzius cells became apparent in human specimens older than 20 weeks of gestation (WOG). These cells were most frequently located in the outer ML bordering the hippocampal fissure. Although the width of the ML increased with hippocampal maturation (see Fig. 1 A vs. B, C), CR-ir cell bodies remained in a superficial position and the cell density increased until the 36th WOG. Around birth, the amount of Cajal-Retzius cells decreased. The postnatal decline showed some variability. In our material, specimens from patients older than 10 months showed only occasional staining of CR-ir cells in the ML of the hippocampus. In contrast, CR-ir Cajal-Retzius cells located in the marginal zone of the temporo-mesial neocortex decreased already during prenatal development (Fig. 1 D–F). The labeling of CR-ir profiles in the supragranular layer of the DG, which is a prominent feature of CR immunoreactivity in the human adult hippocampus [31], appeared perinatally and increased with postnatal maturation (Fig. 3 A).

CR immunoreactivity in AHS

The location and clustering of CR-ir neurons observed in hippocampal specimens with AHS showed morphological similarities to those described for the perinatal hippocampus. Numerous CR- and calbindin D28k-ir neuronal cell bodies were visible in the DG-ML and the ML of Ammon's horn (Fig. 3 C). In addition, the labeling pattern of CR-ir fibers in the DG-ML was also dramatically increased in all TLE patients with AHS. The quantitative numbers of horizontally oriented CR-ir neurons as examined by cell density measurements of CA1-ML and DG-ML corresponded to the amount of CR-ir neurons obtained in the early postnatal age groups (horizontal lines in Fig. 4). Compared to the hippocampus from normal adult post-mortem controls or lesion-associated TLE specimens [8], the number of CR-ir neurons in AHS was significantly increased.

Correlation between the number of CR-ir neurons and the presence of early precipitating injuries in TLE patients

The clinical features of the three different patient groups are summarized in Table 1. There were no significant inter-group differences with respect to the duration of the

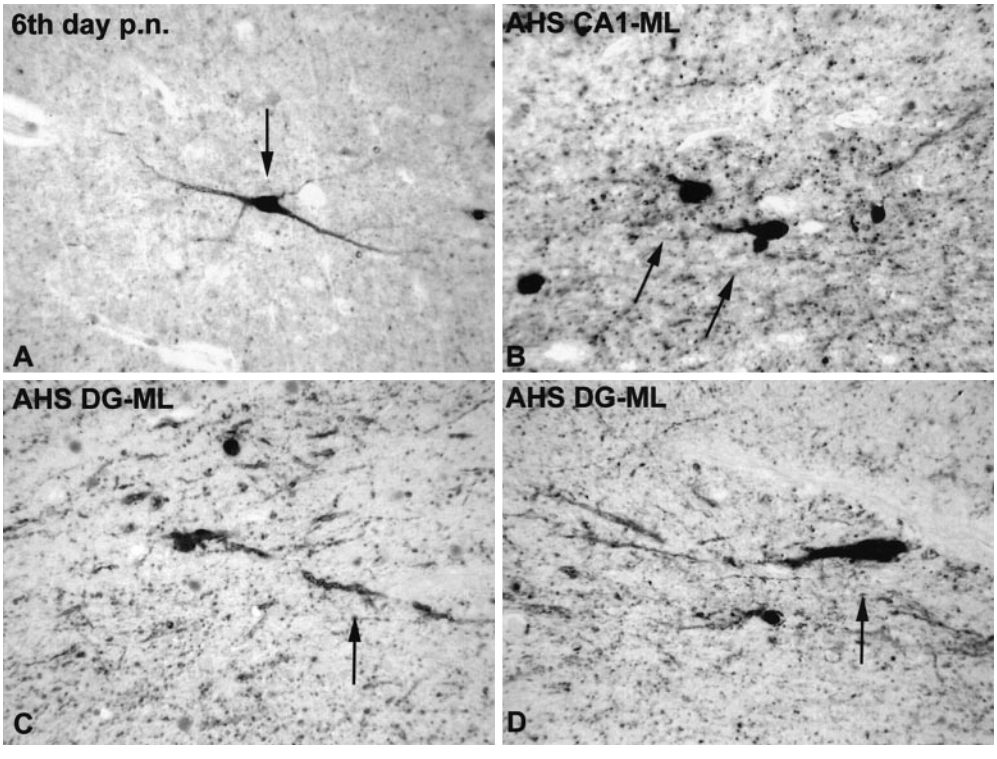


Fig. 2 CR-ir neurons in the developing human DG (**A**) and in AHS (**B–D**). **A** Horizontally oriented CR-ir neuron *(arrow)* depicted from the upper ML of the DG; 6-day-old human hippocampal specimen. **B** In patients with AHS, small clusters of horizontally oriented CR-ir neurons are frequently visible *(arrows)*. **C** This neuron was depicted from the DG upper ML. The *arrow* points towards a horizontally oriented process. **D** The micrograph demonstrates a horizontally oriented neuron with a unipolar dendritic morphology *(arrow)* (*p.n.* post-natally, *AHS* Ammon's horn sclerosis, *CA1-ML* CA1 molecular layer, *DG-ML* dentate gyrus molecular layer). **A–D** × 360

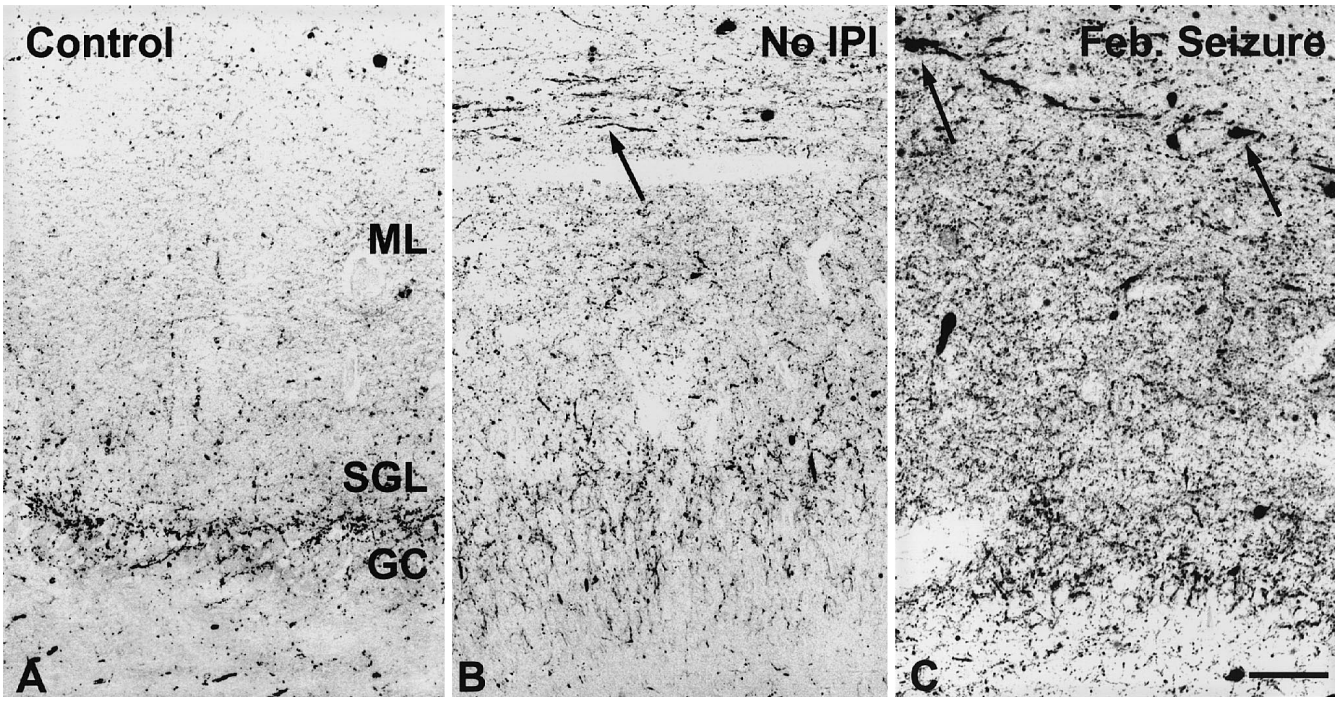
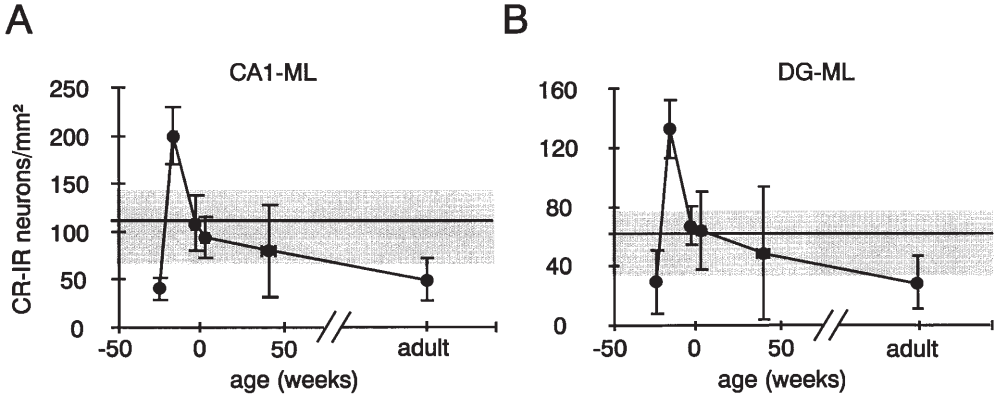


Fig. 3 A–C CR immunoreactivity in the adult hippocampus of control subjects and patients with AHS. **A** CR immunoreactivity in the dentate gyrus from a 60-year-old female *(Control)*. CR immunoreactivity is almost confined to hypothalamic afferents into the SGL [31]. **B** A 27-year-old TLE patient with AHS but no evidence for early precipitating injuries (*No IPI*; patient A6 in Table 1). Compared to controls, the SGL is less prominently stained and CR-ir processes were also visible in upper portions of the ML. Note occasional CR-ir horizontally oriented processes and cell bodies along the hippocampal fissure *(arrows)*. **C** A 42-year-old patient with severe AHS and early febrile seizures (*FEB. Seizure*; patient A18 in Table 1). The number of CR-ir neurons and fibers in the DG-ML is significantly increased. The *arrows* indicate horizontally oriented neuronal profiles similar to Cajal-Retzius cells shown in Fig. 1 A–C (*TLE* temporal lobe epilepsy, *HF* hippocampal fissure; *ML* molecular layer of the DG; *SGL* supragranular layer; *GC* granule cell layer of the DG). Paraffin sections, 4 μm thick. *Bar* 50 μm

seizure disorder or the frequency of complex partial seizures. The age of onset was lower in group 2 (9.3 ± 1.2 years) and in group 3 (8.2 ± 1.3 years) compared to group 1 (12.8 ± 2.2 years), but this difference did not reach statistical significance. Among patients with precipitating injuries (groups 2 and 3), the latency from occurence of the injury to onset of epilepsy was not significantly different.

CR-ir cell counts in the CA1-ML and DG-ML were higher in all patients with AHS compared to normal autopsy controls ($P < 0.01$). However, the extent and distribution of CR immunoreactivity differed between the three AHS categories. The most dramatic increase in CR-ir cell numbers was observed for group 3 specimens (IPI with

Fig. 4 Correlation between Cajal-Retzius cells in the developing human hippocampus and CR-ir neurons in AHS patients. During prenatal human hippocampal development, the number of CR-ir neurons in the CA1-ML (**A**) and the DG-ML (**B**) increase markedly. A peak of CR-ir neuronal cell numbers can be observed during the perinatal period followed by a decline towards the low adult level. The *horizontal lines* indicate the average numbers of CR-ir neurons found in patients with AHS ($n = 27$). *Lower* and *upper borders* of the *shaded area* indicate the 25th and 75th percentile, respectively. Note that the number of CR-ir neurons found in AHS correspond well to those seen in hippocampal autopsy specimens derived from the early postnatal period



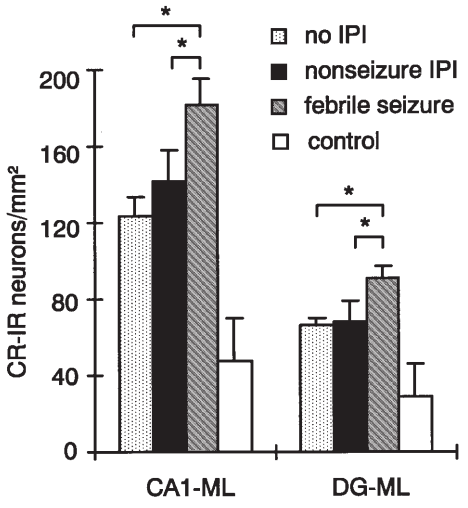


Fig. 5 Increased numbers of CR-ir neurons in AHS patients with febrile seizures. CR-ir neuronal cell densities in AHS group 1 (*dotted bars*, $n = 9$), AHS group 2 (*black bars*, $n = 6$) and AHS group 3 (*gray bars*, $n = 12$). The patients with febrile seizures (group 3) showed significantly higher numbers of CR-ir neurons in CA1-ML and the DG-ML compared to group 1 and group 2 (*asterisk* for $P < 0.05$). All AHS groups showed significant differences compared to adult controls obtained at autopsy (*white bars*, $n = 6$; $P < 0.01$) (*IPI* initial precipitating injury)

early seizures). This finding was statistically significant compared with group 2 (non-seizure IPI) and group 1 (without IPI) specimens using mANOVA (CA1-ML significance of $F < 0.01$, DG-ML < 0.05), Kruskall-Wallis H test (CA1-ML and DG-ML, $P < 0.05$) and Duncan's test for multiple group comparisons with the cut off level set at $P = 0.05$ (Fig. 5). However, multiple comparisons carried out with the non-parametric Mann-Whitney U-Wilcoxson Rank Sum test revealed significant differences only between group 3 and group 1 (2-tailed $P < 0.01$). To ascertain whether the age at occurrence of the first IPI was correlated with CR-ir neuronal cell numbers, a Spearman rank correlation for the patients of groups 2 and 3 was carried out separately. No correlation could be obtained for patients with non-seizure IPI (group 2). In contrast, the number of CR-ir neurons in CA1-ML was significantly higher in group 3 patients with early IPI ($P < 0.05$).

Discussion

The pathogenesis of AHS and its relationship to TLE has been the subject of a long-standing debate [30]. In particular, the key issue of whether AHS represents a pathogenetically relevant primary event or a secondary lesion is yet to be resolved. In some animal models of epilepsy, adult rats show severe segmental neuronal cell loss following chronic seizures, excitotoxic lesions or ischemia [46]. Although the developing rat and human hippocampus may be more prone to epileptogenesis (e.g. due to transient up-regulation of excitatory neurotransmitters), neuronal vulnerability at an early age is generally viewed as low [30, 32, 43, 46]. Nevertheless, numerous studies on human TLE have targeted risk factors early in ontogenesis as potential events contributing to the pathogenesis of AHS [12, 19, 28, 29, 35]. These studies have shown that severe loss of principal neurons in AHS correlates with convulsions in early childhood. However, the mechanisms by which early risk factors contribute to hippocampal hyperexcitability and neuronal cell loss in chronic TLE remains unknown.

In a previous study, we described an increase of CR-ir hippocampal interneurons in DG-ML and CA1-ML in TLE patients with AHS [8]. This increase was independent of the duration of TLE and the possibility of an overestimation of cell numbers due to tissue shrinkage in AHS was excluded. In contrast, this phenomenon could not be observed in a non-AHS patient group with lesion-associated TLE, despite similar clinical characteristics of these patients with respect to seizure frequency, age of onset and duration of epilepsy. The nature of CR-ir neurons within the adult hippocampal formation is not yet clearly defined. It has been argued that increased numbers of CR-ir neurons in biopsy specimens of the human hippocampus could result from an up-regulation of CR in a preexisting cell population. In addition, shorter fixation intervals, less post-mortem degradation, different fixation protocols, paraffin embedding or a bias in immunohistochemical staining intensities may also account for these changes. The striking differences between controls, lesion-associated and AHS biopsy specimens and the low density of neurons within the mature ML indicate that CR immunoreactivity depicts a cell population specifically increased in AHS but not present within the normal adult hippocampus. However, an up-regulation of this calcium-binding-protein within a preexisting cell population cannot be totally excluded. Furthermore, our data suggested that the increase of CR-ir neurons may represent an early event in the pathogenesis of AHS [8]. With respect to the location, morphology as well as the immunoreactivity for the calcium-binding proteins CR and calbindin D-28k [6, 26], the cell type described in the present study appears to correspond to persisting Cajal-Retzius-like cells. To further substantiate this hypothesis, we have correlated the pattern of CR-ir horizontal cells in hippocampal specimens from AHS patients with the time course of normal human hippocampal development. In developing rat hippocampus, a transient up-regulation of CR-ir Cajal-Retzius cells has been described with a postnatal peak expression [40, 44]. Indeed, the quantitative analysis of CR-ir neurons in the human CA1-ML and DG-ML performed in the present study showed a similar ontogenetic time course in human specimens, indicating that the hippocampus is not fully mature at the time of birth. Moreover, levels of CR-ir neurons in AHS corresponded well to those observed in early postnatal age groups. A second objective of our study was to correlate the number of CR-ir cells with the time point at which an IPI occurred. Such a correlation was obtained for the CA1-ML but not for the DG-ML. Our data further indicate that the preservation of CR-ir neurons may be an event occurring early in the course of the disease.

Cajal-Retzius cells are involved in the generation of laminar organization patterns of the cortex and the hippocampus [16, 26]. These effects may be mediated via the release of the extracellular matrix protein reelin. Mice with a mutation in the *reelin* gene show a severely altered organization of the cerebral cortex [13, 33]. In addition, Cajal-Retzius neurons have a different survival pattern depending on the onset of neuronal activity and connectivity [15]. Horizontally oriented neurons of rat DG-ML and CA1-ML receive both excitatory as well as inhibitory input [23, 45]. In human epileptic hippocampus, CR-ir neurons are contacted by inhibitory synapses and the density of CR-ir processes is significantly increased within the DG-ML [8]. These data raise the hypothesis that the persistence of Cajal-Retzius-like cells may also be associated with alterations of hippocampal circuitries and, thus, may contribute to epileptogenesis in the temporal lobe.

Whether the increase of CR-ir Cajal-Retzius-like cells in AHS patients occurs independently from febrile seizure and rather reflects another early developmental insult remains to be elucidated. Although we cannot exclude that group 1 or group 2 patients suffered from early seizures that were not clinically apparent, febrile convulsions may not be a prerequisite for increased levels of CR-ir Cajal-Retzius-like cells. Indeed, all AHS patients exhibited higher levels of Cajal-Retzius-like cells compared to control autopsy specimens from patients without neurological disorders or TLE patients with focal lesions, i.e. tumors or glioneuronal malformation [8]. Some factors with an impact on the fate of developmentally regulated neuronal cell types, i.e. Cajal-Retzius cells, have been identified. Reduced neuronal activity or disconnection from entorhinal input seem to represent key mechanisms for preventing the disappearence of Cajal-Retzius cells in vivo and in vitro [15]. Interestingly, severe neuronal cell loss can be observed within the entorhinal cortex of AHS patients as well as in rat models of TLE [17, 18]. On the other hand, early seizures may induce neurogenesis of dentate granule cells [34] or substantially alter the neurochemical profile of particular neuronal populations [39]. These parameters could also contribute to increased levels of CR-ir neurons.

In conclusion, our findings suggest that transiently occurring CR-ir Cajal-Retzius cells persist in the hippocampus of patients with AHS. Whether the persistence of these neurons in TLE patients with AHS is induced by early precipitating injuries, such as febrile seizures, or represents even earlier developmental abnormalities, affecting hippocampal neurogenesis, connectivity formation and hyperexcitability, remains to be resolved. However, the correlation between early febrile seizures and increased numbers of CR-ir neurons points to an early precipitating event and to a pathogenic role of this cell population in the sclerotic hippocampus.

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