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# Is neuronal death required for seizure-induced epileptogenesis in the immature brain?

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## Abstract

Do seizures cause neuronal death? At least in the immature hippocampus, this may not be the critical question for determining the mechanisms of epileptogenesis. Neuronal injury and death have clearly been shown to occur in most epilepsy models in the mature brain, and are widely considered a prerequisite to seizure-induced epilepsy. In contrast, little neuronal death occurs after even a severe and prolonged seizure prior to the third postnatal week. However, seizures early in life, for example prolonged experimental febrile seizures, can profoundly and permanently change the hippocampal circuit in a pro-epileptogenic direction. These seizure-induced alterations of limbic excitability may require transient structural injury, but are mainly due to functional changes in expression of gene coding for specific receptors and channels, leading to altered functional properties of hippocampal neurons. Thus, in some pro-epileptogenic models in the developing brain, neither the death of neurons nor death-induced abnormalities of surviving neurons may underlie the formation of an epileptic circuit. Rather, findings in the experimental prolonged febrile seizure model suggest that persistent functional alterations of gene expression ('neuroplasticity') in diverse hippocampal neuronal populations may promote pro-epileptogenic processes induced by these seizures. These findings also suggest that during development, relatively short, intense bursts of neuronal activity may disrupt 'normal' programmed maturational processes to result in permanent, selective alterations of gene expression, with profound functional consequences. Therefore, determining the cascade of changes in the programmed expression of pertinent genes, including their temporal and cell-specific spatial profiles, may provide important information for understanding the process of transformation of an evolving, maturing hippocampal network into one which is hyperexcitable.

## Introduction

The issue of neuronal Injury and death caused by seizures has haunted investigators in the field for several decades (Margerison and Corsellis, 1966; Babb and Brown, 1987; Armstrong, 1993). The striking cell loss in specific hippocampal regions and layers found in humans with temporal lobe epilepsy has suggested that seizures may cause epilepsy by killing neurons, leading to reduced inhibition or to the development of excitatory synapses and circuits (e.g., Sloviter, 1991; Dudek et al., 1994; Scharfman, 1999).

Indeed, a strong body of evidence using models of seizure-induced epilepsy has documented that prolonged limbic seizures (status epilepticus) as well as kindling (Cavazos et al. , 1994) result in hippocampal cell loss in a pattern reminiscent of that found in human temporal lobe epilepsy (e.g., Lothman and Collins, 1981 ; Nadler, 1981; Ben-Ari, 1985; Sperk et al., 1985;



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Obenaus et al., 1993; Buckmaster and Dudek, 1997). This cell loss has been found to be progressive (Sutula, 1991; Kalviainen et al., 1998), and associated with — in fact, probably required for (Schauwecker et al., 2000) — synaptic reorganization and increased excitability in the involved circuits (Dudek et al., 1994; Scharfman et al., 2000).

In analogy to the usefulness of mature animal models for elucidating the role of seizure-induced neuronal death in the epileptogenic process in human adults, limbic seizures have been induced during the first 2 weeks of life in the rat, using kainic acid and other convulsants (e.g., Albala et al., 1984; Nitecka et al., 1984; Holmes and Thompson, 1988; Veliskova et al., 1988; Holmes et al., 1998; McCabe et al., 2001). These experiments have attempted to provide clues about the immediate and long-term consequences of early-life seizures in the human, and in particular, about the relationship of seizures, such as those induced by neonatal asphyxia (Jensen et al., 1991), fever (Shinnar, 1998) or other triggers (Baram and Hatalski , 1998), to subsequent epilepsy. Specifically, whether seizures early in life cause cell death, and whether this death is required, sufficient, or even related, to subsequent epilepsy has been a topic of intense investigation (for discussions see Holmes and Ben-Ari, 1998; Sperber et al., 1999; Dube et al., 2000; Kubova et al., 2001; Bender and Baram, 2002).

Because seizures provoked by fever are the most common human developmental seizures which are associated with subsequent temporal lobe epilepsy and hippocampal cell loss, we developed and characterized a model of experimental prolonged febrile seizures in the immature rat. Here we discuss the issues of the occurrence of cell death after these seizures, of other structural alterations in the ‘wiring’ of the hippocampal network, and of the functional changes induced by these seizures, including enhanced susceptibility to further seizures. We consider the molecular and cellular processes that may underlie these changes. These findings indicate important differences between mechanisms of seizure-induced epileptogenicity in mature and still developing hippocampus, and suggest that the interaction of a bout of intense neuronal activity with programmed maturation of the hippocampal network may lead to long-lasting neuroplastic alterations which promote a hyperexcitable state without the requirement for neuronal death.

## The rationale for studying prolonged febrile seizures and temporal lobe epilepsy

Temporal lobe epilepsy (TLE) is the most prevalent type of human focal epilepsy, yet the processes leading to spontaneous seizures involving the hippocampus have not been fully determined. Specifically, the relationship of childhood febrile seizures to adult TLE has remained a focus of intense controversy (for brief recent reviews see Shinnar, 1998; Sloviter and Pedley, 1998; Lewis, 1999; Dube et al., 2000). Epidemiological evidence from prospective studies has convincingly shown that most febrile seizures carry a benign outcome: they do not lead to development of subsequent unprovoked seizures (epilepsy). However, retrospective analyses of adults with TLE have demonstrated a high prevalence (30% to > 60%) of a history of *prolonged* (longer than 10-15 min) febrile seizures during early childhood, suggesting an etiological role for these seizures in the development of TLE (e.g., Cendes et al., 1993; French et al., 1993). Specifically, neuronal damage induced by febrile seizures has been suggested as a mechanism for the development of mesial temporal sclerosis, the pathological hallmark of TLE (Bruton, 1988; Armstrong, 1993). In addition, recent imaging studies in children with prolonged febrile seizures have shown acute hippocampal swelling in some, indicating acute neuronal injury (VanLandingham et al., 1998). However, these data should not be taken to indicate a causal relationship, and alternative mechanisms may exist for the correlation of prolonged febrile seizures and TLE. For example, pre-existing (genetic or acquired, functional or structural) neuronal abnormalities may provide a predisposition to the occurrence of prolonged febrile seizures or for subsequent neuronal injury and TLE.

Given the high prevalence of febrile seizures (1 in 20-30 children), understanding their consequences for the developing brain is critical, because even a relatively small contribution by these seizures to the development of epilepsy will result in large numbers of affected individuals. However, febrile seizures cannot be induced, and the critical question of the causal relationship of prolonged febrile seizures and epilepsy cannot be studied in children. Therefore, an immature rat model for these seizures has been created. Because 'complex', typically prolonged, febrile seizures are those that have been associated with subsequent limbic epilepsy (TLE), the model aimed to reproduce this subtype of human febrile seizures as closely as possible. Seizures in the model are provoked by generating brain temperatures seen physiologically in ill infants, and seizure duration is regulated to reproduce relatively prolonged febrile seizures (~20 min), those considered complex by the International League Against Epilepsy, and associated retrospectively with the development of TLE. Several features of the model, which render it suitable for study of the mechanisms and consequences of these prolonged febrile seizures, have been characterized. These include an age-specificity, temperatures required for seizure induction, lack of mortality or acute morbidity and pharmacological profile, which are similar to those of human febrile seizures (Knudsen, 1996; Baram et al., 1997; Dube et al., 2000). In addition, the neuronal circuits involved in the seizures have been mapped (Chen et al., 1999; Dube et al., 2000; Hatalski et al., 2000).

Note that throughout this chapter, reference is made to the developmental stage when febrile seizures may be 'generated or provoked'. This, in the animal model, implies postnatal days 9–14 (Baram et al., 1997), and the seizures are induced on days 10–11. It is fully recognized that direct correlations of rat brain development to that in the human are imprecise. However, in general, evidence based on rates of neuronal birth and myelination (Dobbing and Sands, 1973, 1979), and saltatory growth stages (Gottlieb et al., 1977) suggest that the 5–7-days-old rat may be 'equivalent' to the human newborn. Rat brain development at 10–15 postnatal days, the age of maximal susceptibility to triggered seizures (including those induced by hyperthermia; Hjeresen and Diaz, 1988; Jensen et al., 1991; Baram and Hatalski, 1998) may thus best correspond to the stage of brain development during human infancy (Gottlieb et al., 1977).

This model of complex (prolonged) febrile seizures has been used to address the fundamental question of whether these seizures promote epilepsy by causing death of vulnerable hippocampal neuronal populations leading to mesial temporal sclerosis, neuroanatomical matrix of TLE. It should be noted that this specific pattern of neuronal loss is found in the majority of individuals with TLE (Armstrong, 1993; Kuzniecky et al., 1997), but whether neuronal death promotes epilepsy, or whether recurrent seizures result in neuronal death is unclear.

## Prolonged experimental febrile seizures result in permanent increase of hippocampal excitability, and in susceptibility to the development of further limbic seizures

Whether prolonged febrile seizures in the immature rat model lead to the development of spontaneous seizures was investigated using both in vivo and in vitro approaches (Dube et al., 2000). After induction of such seizures, animals were allowed to mature (three months), then underwent extensive hippocampal-EEG and behavioral monitoring. Both EEGs and behavioral measures failed to demonstrate spontaneous seizures. However, these animals showed a fourfold increase in susceptibility to kainic acid, an activator of the AMPA-type glutamate receptors. In essence, a dose that failed to provoke seizures in most adult rats which had not experienced prolonged febrile seizures in 'infancy' (normothermic controls and those who were subjected to hyperthermia but in which seizures had been blocked) led to severe seizures in all adult animals who had prolonged experimental febrile seizures early in life. This increased susceptibility to limbic convulsants was confirmed in vitro:

spontaneous epileptiform discharges were not observed in hippocampal–entorhinal cortex slices derived from either control or experimental groups. However, Schaffer collateral stimulation induced prolonged, self-sustaining, status-epilepticus-like discharges exclusively in slices from experimental rats. These data indicate that experimental prolonged febrile seizures do not cause spontaneous limbic seizures during adulthood. However, they reduce thresholds to chemical convulsants in vivo and to electrical stimulation in vitro, indicating persistent enhancement of hippocampal excitability that may facilitate the development of epilepsy.

## The mechanisms by which prolonged febrile seizures enhance excitability do not involve cell death

As mentioned earlier, a large body of literature involving *adult* animal models supports the notion that provoked seizures may lead to spontaneous seizures (epilepsy) by killing vulnerable neurons in hippocampus and amygdala, altering the balance of excitation/inhibition in the limbic network (Sloviter, 1994). Indeed, experimental models using pilocarpine (Mello et al., 1993; Obenaus et al., 1993) or kainic acid (Pollard et al., 1994; Buckmaster and Dudek, 1997) document that neuronal loss induced by these seizures *precedes* the emergence of spontaneous seizures. Therefore, the hypothesis was tested that experimental prolonged febrile seizures increased hippocampal excitability by causing the loss of vulnerable neuronal populations.

Using molecular methods for visualizing neuronal death, no excess of in situ end-labeled cells was found 1, 4, 8.5, 20 or 48 h after seizures lasting 20 or 60 min (Toth et al., 1998). In addition, stereological counts in amygdala (Toth et al., 1998) or in specific hippocampal sub-populations that are vulnerable to seizure-induced death in other models, failed to reveal loss of mossy cells or other seizure-sensitive neuronal populations (Bender and Baram, 1999, 2002). Thus, it was concluded that experimental prolonged febrile seizures do not lead to cell loss. This is in accord with data from other single prolonged developmental seizures such as those induced by hypoxia (Jensen and Baram, 2000) or by chemical provocation, during the second postnatal week (Sperber et al., 1992), and indicates that the mechanisms by which prolonged febrile seizures increase hippocampal excitability may not involve hippocampal cell death. In addition, the findings support the notion that these mechanisms may involve interaction with concurrent maturational processes in the developing hippocampus.

## Neurogenesis is not altered by prolonged experimental febrile seizures

Death of hippocampal CA3 pyramidal cells or of hilar neurons deprives the excitatory granule cells of their targets and leads to abnormal growth (‘sprouting’) of their axons, the mossy fibers. The excitatory synaptic connections formed by these axons are widely considered to contribute to the mechanism of enhanced hippocampal excitability and epileptogenesis (e.g., Pollard et al., 1994; Buckmaster and Dudek, 1997). A second trigger for abnormal granule cell excitatory innervation is seizure-induced alteration of their postnatal proliferation rate and total numbers (Parent et al., 1997; Parent and Lowenstein, 2002, this volume), as shown for several experimental seizure models in *adult* animals. However, no evidence for altered granule cell proliferation rate and only modest ‘sprouting’ of the granule cell axons was found after prolonged febrile seizures in the immature rat model (Baram et al., 2000; Bender et al., 2000). These findings reinforce the notion that these seizures affect the hippocampal network via processes which are distinct from those implicated in adult epileptogenesis, and which must interact with — and perhaps disrupt — developmental events coinciding with these seizures (Jensen and Baram, 2000).

## Prolonged 'febrile' seizures injure specific populations of hippocampal neurons

The data discussed above demonstrated a dichotomy between the functional and neuroanatomical changes induced by experimental prolonged febrile seizures. Namely, enhanced susceptibility to further seizures was observed long-term, but this was achieved without any evidence for acute or chronic cell death. Therefore, we evaluated the possibility that experimental prolonged febrile seizures induced neuronal injury that was sufficient to permanently alter the properties of these neurons, without leading to their death. A method considered sensitive to changes in cytoskeletal elements of neurons was chosen (Gallyas et al., 1990; Toth et al., 1998) to visualize the potential effects of hyperthermic seizures on neuronal structure. The overall approach involved a comparison of three experimental groups: normothermic and hyperthermic controls and animals subjected to prolonged experimental febrile seizures. For analysis of neuronal injury using the Gallyas 'dark'-neuron silver stain, animals were sacrificed 24 h, 1 week or 2 weeks after seizure induction. For cell counting, animals ($n$ = 12, four per experimental group) were sacrificed 4 weeks following the hyperthermic seizures.

As shown in Fig. 1, significant and prolonged alterations in the physicochemical properties of neurons in the pyramidal layer of the hippocampal CA1 and all the CA3 subfields were found. Specifically, starting within 24 h of seizures and persisting for at least 2 weeks, numerous pyramidal cells a well as less abundant hilar neurons exhibited pronounced avidity to silver stain (argyrophilia). The distribution of these argyrophilic neurons indicated the pattern of neuronal vulnerability to febrile seizures in this model, and shared significant similarities with the pattern of injury found with other limbic seizure types (and in human temporal lobe epilepsy). In the hippocampus, major involvement of CA3 and CA1 pyramidal cell layers and relative sparing of the granule cell layer and subiculum were consistent with vulnerability patterns in adult models of kainic acid-(Nadler et al., 1978; Sperk et al., 1983; Ben-Ari, 1985; Pollard et al., 1994) and pilocarpine-induced status epilepticus (Clifford et al., 1987; Mello et al., 1993; Liu et al., 1994).

However, unlike the chronic outcome of these seizures in adult rats, no evidence of neuronal cell loss was evident at any time after the hyperthermic seizures, and cell counts revealed no evidence of loss in specific vulnerable hippocampal cell populations (see above). Thus, these data, revealing striking but transient alterations of neuronal integrity in regions known to be affected by other limbic seizure paradigms, might provide a mechanism to reconcile conflicting reports regarding the effects of developmental limbic seizures on neuronal survival. Specifically, our findings suggest that similar neuronal populations share vulnerability to limbic seizures in both the immature and mature hippocampus, but immature neurons may undergo injury followed by recovery, whereas mature neurons progress from injury to death (Chang and Baram, 1994; Owens et al., 1997).

## Do argyrophilia, acid-fuchsin staining or in situ end-labeling (ISEL) of neurons indicate their death?

The data presented above demonstrate that the physicochemical properties of neurons may change dramatically, permitting increased avidity to silver stains (Gallyas et al., 1990; Van den Pol and Gallyas, 1990; Toth et al., 1998) without these changes being followed by neuronal degeneration. The *relative* nature of silver uptake by different classes of intact and injured neurons and subcellular organelles has been discussed (Gallyas et al., 1990). Indeed, avidity to silver staining can be induced by subjecting the brain to postmortem trauma, indicating that this process is independent from the process of cell death (Gallyas et al., 1992). This fact, together with earlier studies (Chang and Baram, 1994), raised the question as to the interpretation of neuroanatomical methods used to demonstrate 'cell death'. Put differently, acquisition of the avidity to silver in a variety of methods may not necessarily

mean neuronal death. The changes or injury which renders a cell argyrophilic may be reversible and not lead to cell loss (death). In analogy, the acid-fuchsin method, described originally for hypoglycemic cell death, may merely imply increased avidity to this dye, without 'fatal' injury to the cell (see discussion in Chang and Baram, 1994, and by Nehlig and Pereira de Vasconcelos, 1996; Motte et al., 1998; Pineau et al., 1999). Somewhat more controversial, the interpretation of TUNEL and ISEL, methods which rely on labeling of end-terminals of DNA, has come under question. Whereas breaking of DNA into pieces of roughly equal length ('laddering') has been described in the process of apoptotic cell death, this form of death may occur without overt DNA fragmentation. In addition, injury-induced repair may also yield enhanced numbers of DNA 'ends' with enzymatic labeling. Finally, in the immature brain, normal cell death occurs, and these dying cells may also be labeled. Because of the potential ambiguities of each of the methods described here regarding its specificity for cell death, a recent trend (Sankar et al., 1998; Toth et al., 1998; Kubova et al., 2001) has been the adoption of several methods in combination. In addition, several time points have often been analyzed in recent studies of seizure-induced neuronal death in the immature brain. Thus, Toth et al., 1998 (see above) found reversible silver staining with absence of in situ end-labeling or of neuronal dropout, and concluded that the prolonged hyperthermic seizures did not kill hippocampal neurons. Sankar et al. ( 1998) used several methods, including Fluoro-jade, to assess pilocarpine-induced cell death. In an elegant recent study, Kubova et al. (2001) resorted to electron microscopy to determine categorically that seizures killed specific populations in discrete thalamic nuclei.

## Changes in gene expression follow prolonged experimental febrile seizures, and may alter neuronal function to promote hippocampal excitability

The paragraphs above suggest that experimental prolonged febrile seizures induce pro-epileptogenic changes in the immature hippocampal network, and that neuronal death may not be required for this process. What then, might the responsible mechanisms be?

In addition to the absence of cell death, cell birth rate (and cell fate) has been found not to change after these seizures. Thus, recent research has focused on potential sequential changes in the expression of genes which may impact excitability in the developing hippocampal circuit. Because experimental (and clinical) febrile seizures occur uniquely in a distinct phase of development, emphasis has been placed on molecules that influence hippocampal excitability during this age.

We first tested the hypothesis that excitatory drive to the hippocampal circuit is enhanced persistently via long-term upregulation of the expression of the excitatory neuropeptide, corticotropin-releasing hormone (CRH), which is normally expressed in hippocampal interneurons (for review see Yan et al., 1998; Y. Chen et al., 2001). CRH immunoreactive interneurons are more numerous in the principal cell layers of the immature rat, compared with the adult, peaking during the second and third postnatal weeks (Y. Chen et al., 2001), and the pro-excitatory actions of the peptide on hippocampal neurons are also maximal during this developmental age (for review see Baram and Hatalski, 1998). Indeed, within hour of the seizures, robust increase in CRH-mRNA levels in the principal cell layers of the hippocampal formation was evident (Hatalski et al., 2000; Fig. 2). However, these changes persisted only for 24–48 h and were no longer evident at 1 week or 1 month after the seizures (Eghbal-Ahmadi, 2000), whereas the enhanced excitability in the limbic circuit persisted (Dube et al., 2000). It should be noted that the enhanced expression of CRH was not due simply to the stressful effects of the seizures, since it did not occur with other stressors such as cold (Hatalski et al., 2000). In addition, this enhanced gene expression required neuronal activity, since it was abolished when the seizures (but not the hyperthermia) were eliminated via pre-treatment with the short-acting barbiturate pentobarbital. Finally, lack of persistent changes in hippocampal CRH expression was not

due to the inability of this transcript to undergo long-term upregulation, since protracted enhancement (up to 12 months) of CRH-mRNA levels in the hippocampus was induced in the immature rat by other means (Brunson et al., 2001).

The permanent functional changes in the hippocampal network following prolonged experimental febrile seizures led to a search for persistent alteration in the expression of other candidate genes. One such candidate emerged from the in vitro electrophysiological studies (K. Chen et al., 2001; Thon et al., 2002). Whereas enhanced excitability of the hippocampal circuit was evident from both in vivo and in vitro data (Dube et al., 2000, and see above), single-cell electrophysiology (Chen et al., 1999) suggested that experimental prolonged febrile seizures led to significantly *increased* GABA release onto CA1 pyramidal cells. This change indicated *enhancement of inhibitory drive* (Walker and Kullmann, 1999), and failed to explain the *augmented excitability* in the hippocampal network after these seizures. However, more recent electrophysiology data (K. Chen et al., 2001) resolved this conundrum, demonstrating a remarkable and persistent change in an ion channel, which can convert the potentiated inhibition into excitation. Specifically, a channel activated by *hyper*polarization and leading to *de*polarization by permitting entry of $Na^+$ ions was altered, in a manner that rendered it more highly activated under physiological conditions. This channel (hyperpolarization-activated, cyclic AMP-regulated mixed-cation channel, HCN), producing the hyperpolarization activated ($I_H$) current, is composed of several, recently characterized subunit isoforms (Santoro et al., 1998, 2000; Siegelbaum, 2000).

Because the functional properties of the HCN channels are governed also by the subunit or isoform make up of the channel (Santoro et al., 1998, 2000; Siegelbaum, 2000), current experiments are aimed at determining whether prolonged experimental febrile seizures alter the relative expression of these subunit isoforms. The existence of these molecules in developing hippocampus has been demonstrated (Bender et al., 2001). Furthermore, the expression of each of the three hippocampal-expressed isoforms is highly age-dependent, demonstrating a distinct spatio-temporal expression profile in both interneuronal populations and pyramidal cells (Bender et al., 2001). Therefore, current experiments are testing the hypothesis that prolonged experimental febrile seizures may disrupt the normal evolution of maturational changes in HCN subunit expression, thus leading to permanent alteration in the expression of these subunits, and ultimately, to altered channel function.

## Conclusion

Experimental prolonged febrile seizures lead to enhanced excitability in the limbic circuit. However, this is not accompanied by neuronal death. Transient neuronal injury is induced by these seizures, manifested as enhanced avidity to silver stains. This structural alteration is associated with both transient and persistent functional changes that may derive from disruption of programmed, sequential and orderly expression of genes critical to normal hippocampal maturation. Thus, a single experimental febrile seizure may modify gene expression and resultant neuronal function persistently, leading to altered properties of the hippocampal network long-term.

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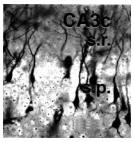


**Fig. 1.**

Injury to hippocampal neurons after a single 20-min episode of intense neuronal activity induced by hyperthermia (hyperthermic seizure, an experimental model of prolonged febrile seizures). Sections obtained from immature rats killed 24 h after a seizure. Silver-stained neurons (Gallyas' dark-neuron method; Gallyas et al., 1992) are evident in the CA3c pyramidal cell layer in this high-magnification photomicrograph. The distribution of the argyrophilic neurons involved also CA3a and b, CA1, some hilar interneurons, and discrete nuclei in amygdala and perirhinal cortex (Toth et al., 1998). Such neurons were not observed in animals subjected to hyperthermia alone, i.e., when the seizures were blocked. s.p. and s.r. are strata pyramidale and radiatum, respectively.

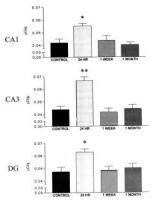


**Fig. 2.**

Time-course of CRH expression in the ammon's horn pyramidal layer (CA1 and CA3) as well as in the granule cell layer of the hippocampal formation after prolonged experimental febrile seizures. CRH expression in these layers is confined to basket and chandelier-type interneurons (Yan et al., 1998; Y. Chen et al., 2001).