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Cell death and survival mechanisms after single and repeated brief seizures

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The consequences to the brain of repeated brief seizures are germane to epilepsy researchers interested in mechanisms of cell death and the pathophysiology of epilepsy, and to clinicians observing declining cognitive performance and greater intractability in pharmacoresistant patients.

Repeated evoked brief seizures in some animal models can cause neuronal death. The mechanisms underlying this death may involve excitotoxic as well as programmed cell death via gene-dependent apoptotic signalling pathways. There is little evidence, however, that spontaneous seizures in epileptic animals cause cell death.

Are brief seizures harmful to the human brain? Many cross-sectional imaging and neuropathology studies suggest they are; patients with pharmacoresistant epilepsy display reduced brain tissue volume over time. Evidence of progressive damage from longitudinal studies is, however, less compelling.

Analysis of brain samples from pharmacoresistant patients has identified a biochemical signature suggestive of activated pro- as well as anti-apoptotic signalling pathways. Oxidative stress and mitochondrial DNA damage caused by brief seizures might also contribute some long-term effects.

In summary, concern remains that uncontrolled seizures are potentially harmful to the brain. Protecting neurons will be facilitated by improved understanding of cell death-regulatory pathways. This chapter summarises our knowledge of the pathways mediating cell death and survival after brief seizures.

INTRODUCTION

While it is broadly accepted that *status epilepticus* can directly cause neuronal death, whether single or repeated brief seizures cause neuron loss is controversial. This is an important issue with both scientific and clinical implications. Patients may be concerned with whether their seizures are capable of causing brain damage, and clinicians make treatment decisions based on an assumption that a few seizures are not really harmful. From a scientist's perspective, this issue is pertinent to the pathophysiology of epilepsy and the various mechanisms neurons employ to cope - or otherwise undergo cell death - in response to the repeated stress of frequent seizures. Research using animal models, and pathology and neuroimaging work in patients, show that single or repeated brief seizures under certain circumstances cause neuron loss, but also indicate that neuron loss is not

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an inevitable consequence of a seizure. Recent human studies show signalling pathways associated with apoptosis may be altered in patient brain, offering possible therapeutic opportunities to target seizure-induced neuronal death in different ways.

Hippocampal sclerosis is the most common pathologic finding in temporal lobe epilepsy (TLE). However, there are patients with TLE with no apparent hippocampal damage and people with hippocampal sclerosis without TLE. If hippocampal sclerosis causes TLE, then efforts to prevent this lesion's development are critical. If epileptic seizures cause neuron loss, however, therapeutic efforts to prevent all seizures from occurring become more important. This chapter describes cell death and survival mechanisms after single and repeated brief seizures in animal models and humans. What is the etiology of hippocampal and extra-hippocampal cell loss in intractable TLE? Is there ongoing cell loss in refractory epilepsy? The question of whether single epileptic seizures damage the brain has been the subject of several previous reviews, to which the reader is referred.[1–3] The focus of this chapter is to present the evidence for and against cell death after brief seizures and the molecular mechanisms which may underlie such an outcome. We omit discussion of other forms of neuronal damage (including reversible injury) which may also have significant behavioural or cognitive implications, and the influence of repair mechanisms such as neurogenesis. Discussion of these issues can be found elsewhere.[4]

Evidence from animal models that single or repeated evoked seizures cause neuron loss

Evidence that single or repeated brief seizures could cause neuronal death emerged from work in animals using electrical stimulation of various brain regions. While "kindling" paradigms are not ordinarily associated with permanent neuron loss,[4] papers published in the early 1990s, particularly by Sutula's laboratory, showed that kindling-induced seizures caused reductions in neuron numbers.[5] Cavazos *et al.,* showed repeated stimulation of the perforant path, olfactory bulb or amygdala resulted in progressive decreases in neuronal density in multiple subfields of the hippocampus, including the hilus, CA1 and CA3, and parts of the entorhinal cortex.[6] The somatosensory cortex was unaffected and changes were not attributable to tissue volume changes.[6]

Other studies using electrically-evoked seizures have reported similar findings.[7–8] Not only is neuron loss progressive, but it may increase with secondarily generalized tonic-clonic seizures.[8] Reduced hippocampal neuron densities have also been reported after electroshock seizures,[9] and in addition to hippocampal neuron loss, a subpopulation of amygdala neurons may also be vulnerable.[10–11] Finally, recent studies by Sloviter and colleagues showed that sustained electrical stimulation of the perforant pathway leading to the hippocampus, which did not cause convulsive seizures or *status epilepticus*, produced extensive neuronal death and hippocampal sclerosis.[12] Thus, repeated brief seizures or sub-convulsive stimulation of the hippocampus in certain models can reproduce patterns of neuron loss similar to those found in human hippocampal sclerosis. (Table 1)

Table 1. Summary of findings on neuron loss after single or repeated brief seizures in experimental models.

Pathologic outcome	*Findings*
Neuron loss after repeated evoked brief seizures	Observed in many but not all models
Neuron death detected after repeated evoked brief seizures	Observed in some models
Neuron loss after seizures in spontaneously epileptic animals	Inconclusive
Neuron loss after seizures in animals with acquired epilepsy	Not currently supported by the evidence
Apoptosis-associated signaling	Modulation of Bcl-2 family genes, caspases

Evidence against single and repeated evoked seizures causing neuron loss

Studies in kindling models have shown that brief single seizures do not necessarily lead to cell loss. Thus, Bertram & Lothman reported reduced neuronal density after kindling, but attributed this to tissue volume expansion.[13] The possible role of tissue volume changes and changes in neuronal morphology in reports of seizure-induced neuronal loss has been emphasized by numerous authors.[2, 14–15] Brandt *et al.,* also argued that neuronal density reductions after extended kindling were due to volume changes and not neuronal death.[16] Other groups also failed to detect neuronal death after kindling in rats,[17–18] and mice.[19] Thus, studies in which neuron counts were used as the principal measure of whether cell loss occurred are not in agreement as to whether brief seizures cause neuronal death (Table 1).

Detection of acute cell death after evoked single and repeated brief seizures

Direct evidence that brief seizures cause acute neuronal death has been provided by biochemical analyses. Bengzon *et al.,* showed that a single seizure evoked by electrical stimulation of the hippocampus could cause hippocampal neurons to die, as detected by silver staining and staining of cells for irreversible DNA fragmentation using TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling).[20] Notably, more stimulations caused proportionately more cells to die.[20] Using similar methods, other groups have also reported that repeated brief seizures cause hippocampal and extra-hippocampal cell death.[11, 21–23] These studies confirm that brief evoked seizures can cause neuronal death in animal models (Table 1).

Do spontaneous seizures in epileptic animals cause neuron loss?

While brief evoked seizures in nonepileptic animals are useful models they do not capture all aspects of the pathophysiology of spontaneous (i.e. epileptic) seizures. Is there evidence that spontaneous seizures in epileptic animals can cause neuron loss? This would be directly relevant to the etiology of progressive damage in human mesial temporal sclerosis. Two types of model have been studied in this context; animals that are spontaneously epileptic and animals which acquired epilepsy as the result of an initial precipitating injury. With the exception of certain genetically-altered mice with active neurodegeneration, neuron loss does not appear to occur in spontaneously epileptic animals. For example, the hippocampus of spontaneously epileptic EL mice, which experience multiple complex partial seizures with secondary generalization on a weekly basis, shows no obvious neuron loss.[24] Evidence of subfield-specific seizure-induced hippocampal neuron loss has been reported in spontaneously epileptic rats,[25] although no acute cell death after a seizure or a biochemical marker thereof was detected.[25]

Studies in animal models of acquired epilepsy also suggest spontaneous seizures do not cause further neuron loss. Pitkanen *et al.,* reported that a longer duration of epilepsy was not associated with lower numbers of neurons in epileptic rats.[26] Moreover, no acutely degenerating neurons were found in any of the chronically epileptic animals, despite some experiencing more than 10 seizures per day.[26] Other studies appear to corroborate these data; hippocampal damage may continue for some time following *status epilepticus*, but neuron loss does not progress once animals are epileptic.[27–29] (Table 1).

MOLECULAR MECHANISMS OF CELL DEATH FOLLOWING SINGLE AND REPEATED BRIEF SEIZURES

The molecular mechanisms underlying cell death following single and repeated brief seizures are not as well researched as they have been in models of *status epilepticus* (reviewed in refs. [30–32]). Glutamate-mediated excitotoxicity is the principal mechanism driving neuronal death after *status epilepticus,* whereby excessive

glutamate release leads to intracellular calcium overload, oxidative stress, organelle swelling and rupture of intracellular membranes, activation of proteases and necrosis.[33–34] Is glutamate-mediated toxicity the cause of neuron death after single or repeated brief seizures? We assume that it is, and necrosis has been detected after brief seizures,[11] but there have been no studies using appropriate pharmacological tools demonstrating that cell death can be prevented by glutamate receptor antagonists. Instead, there is biochemical and morphological evidence supporting cellular apoptosis occurring after brief seizures.[7, 20–21] Notably, administration of the *N*-methyl-D-aspartate glutamate receptor antagonist MK801 (which is neuroprotective in models of *status epilepticus*) did not prevent cell death after brief seizures.[20] The pathophysiologic changes caused by brief seizures are no doubt glutamate-driven and may feature perturbed intracellular calcium homeostasis,[35] but through other pathways. These might include non-NMDA receptor-gated calcium entry and disruption of endoplasmic reticulum or mitochondrial function. Thus, apoptosis, which may have overlapping mechanisms of activation with necrosis, contributes to cell death after single or repeated brief seizures.

Molecular control of apoptosis

Apoptosis is a form of programmed cell death used to dispose of unwanted or damaged cells in a controlled manner. Excess neurons are removed during brain development by apoptosis and apoptosis also occurs after the developing or mature brain is exposed to, or deprived of, certain substances. For example, ethanol exposure triggers widespread apoptosis in the developing rat brain,[36] and adrenalectomy triggers apoptosis of dentate granule neurons.[37]

Two main molecular pathways control apoptosis - extrinsic and intrinsic.[38–39] The extrinsic pathway is triggered by surface-expressed death receptors of the tumor necrosis factor (TNF) superfamily on binding their ligands (secreted cytokines such as TNFα). The intrinsic pathway is mitochondria-mediated, and activated by an array of intracellular stressors including DNA damage and perturbation of intracellular calcium homeostasis or organelle function.[40–41] This pathway is regulated by members of the Bcl-2 gene family at the point of initiation. Both pathways result in the downstream activation of a group of enzymes called caspases.

Caspases

The caspases are a family of cysteinyl aspartate-specific proteases expressed in healthy cells in an inactive zymogen form. Caspases share a common structure comprising an *N*-terminal pro-domain followed by a large ~20 kD subunit and smaller ~10 kD subunit. Caspases regulating apoptosis are typically organized into two functional groups: The upstream initiators, have long pro-domains. Activation of these requires protein-protein binding interactions between the pro-domain and scaffolding molecules activated in response to pro-apoptotic stimuli. For example, the pro-domain of caspase-8 binds to regions on signalling molecules recruited to the intracellular side of activated death receptors, whereas the pro-domain of caspase-9 associates with the apoptotic protease activating factor 1 (Apaf-1) forming the so-called apoptosome in association with released cytochrome *c* from mitochondria.[42] Activated initiator caspases then cleave and remove the short pro-domain of apoptosis effector (or executioner) caspases, thereby activating them.[42] Caspase-3 and other effector caspases such as caspases 6 and 7 then cleave numerous proteins within the cell, including structural proteins (a full listing can be found at http://bioinf.gen.tcd.ie/casbah/). Collectively, the caspase system results in hallmark morphological changes, DNA fragmentation (which can be detected by TUNEL), and eventual dispersal of the cell within membrane-enclosed apoptotic "bodies" to be phagocytosed by surrounding cells.

Bcl-2 family proteins

Bcl-2 family proteins function as critical regulators of apoptosis by controlling the release of intra-mitochondrial apoptogenic molecules via effects on outer mitochondrial membrane permeability. The Bcl-2 family comprises both pro- and anti-apoptotic members which share one or more Bcl-2 homology (BH) domains. Anti-apoptotic members include Bcl-2 and Bcl-xL, which possess four BH domains in common and a transmembrane

anchoring domain. The multi-domain pro-apoptotic members include Bax and Bak which only possess BH domains 1–3.[43]

BH3-only proteins are a sub-group of the pro-apoptotic Bcl-2 family. These function as upstream initiators of apoptosis by binding and either inactivating anti-apoptotic Bcl-2 family proteins or directly activating pro-apoptotic Bax/Bak. BH3-only proteins are highly heterogeneous. Some reside inactively in normal cells and require post translational modification to be active, while others require transcriptional upregulation by cell stress or damage. Bad, for example, is expressed in many cells (including neurons) but requires dephosphorylation and disengagement from a chaperone protein called 14-3-3 to be active. In contrast, the more potently pro-apoptotic members such as Puma, require transcriptional up-regulation, for example via DNA damage-sensing proteins such as p53.[43] Once activated, Bax/Bak trigger release of cytochrome *c* from mitochondria initiating the intrinsic apoptosis pathway, culminating in caspase-dependent or –independent cell death.[41] (Figure 1)

Other survival pathways

In addition to the anti-apoptotic arm of the Bcl-2 family, other anti-apoptotic molecules have been identified. These include protein kinase B (Akt) which is activated downstream of phosphatidylinositol 3 (PI3) kinase, which itself lies downstream of certain cytokine and surface-expressed growth and survival factor receptors.[44] Activated Akt can block apoptosis by phosphorylating and inhibiting Bad or the FoxO/Bim pathway.[45–46] The inhibitor of apoptosis protein (IAP) family functions mainly by direct inhibition of caspases and by targeting them for degradation by the proteasome.[47] (Figure 2)

Evidence of apoptosis-associated signalling pathways after brief seizures

Molecular evidence of apoptosis after brief seizures was first reported by Zhang *et al.* who detected an increase in *bax* mRNA, but unchanged *bcl-2,* expression in hilar neurons in the rat hippocampus following multiple kindling seizures.[21] Other studies have confirmed that kindling seizures cause hippocampal up-regulation of *bax* as well as an increase in Bax protein.[48] Down regulation of anti-apoptotic Bcl-2 family proteins also occurs after repeated brief seizures.[48] The extrinsic apoptosis pathway may also be activated by brief seizures since kindling increases brain levels of TNFα.[49] Increased caspase-like enzyme activity and *in situ* staining of activated caspase-3 has been found in hippocampus after kindling seizures.[22, 50]

Changes to Bcl-2 family protein expression have also been observed in models of electroshock-induced convulsions. Here, anti-apoptotic changes predominate, including down regulation of pro-apoptotic *bcl-xs* and Bim,[51–52] and up-regulation of anti-apoptotic Bcl-w.[53] This pattern supports protection, rather than cell death.

There are no data on Bcl-2 family protein expression or function in epileptic animals. There is, however, evidence of caspase activity within the hippocampus of epileptic animals,[54–55] which may reflect ongoing cell death. The location of the active caspase signal within dendritic fields also supports caspase-mediated restructuring of neurons or other processes. [56].

It should be emphasized that the studies to date have not proven the apoptosis-associated gene changes are responsible for cell death in these models. This requires protein-protein interactions to be demonstrated and functional studies assessing, for example, damage in mice lacking specific genes. Evidence that genes associated with apoptosis can regulate seizure-induced neuronal death has been provided, however, from models of *status epilepticus*. Mice lacking BH3-only proteins Bim and Puma are protected against *status epilepticus*-induced neuronal death.[57–59] and Bcl-2 and death receptor signalling protein complexes are formed in the hippocampus in these models.[52, 60–61]

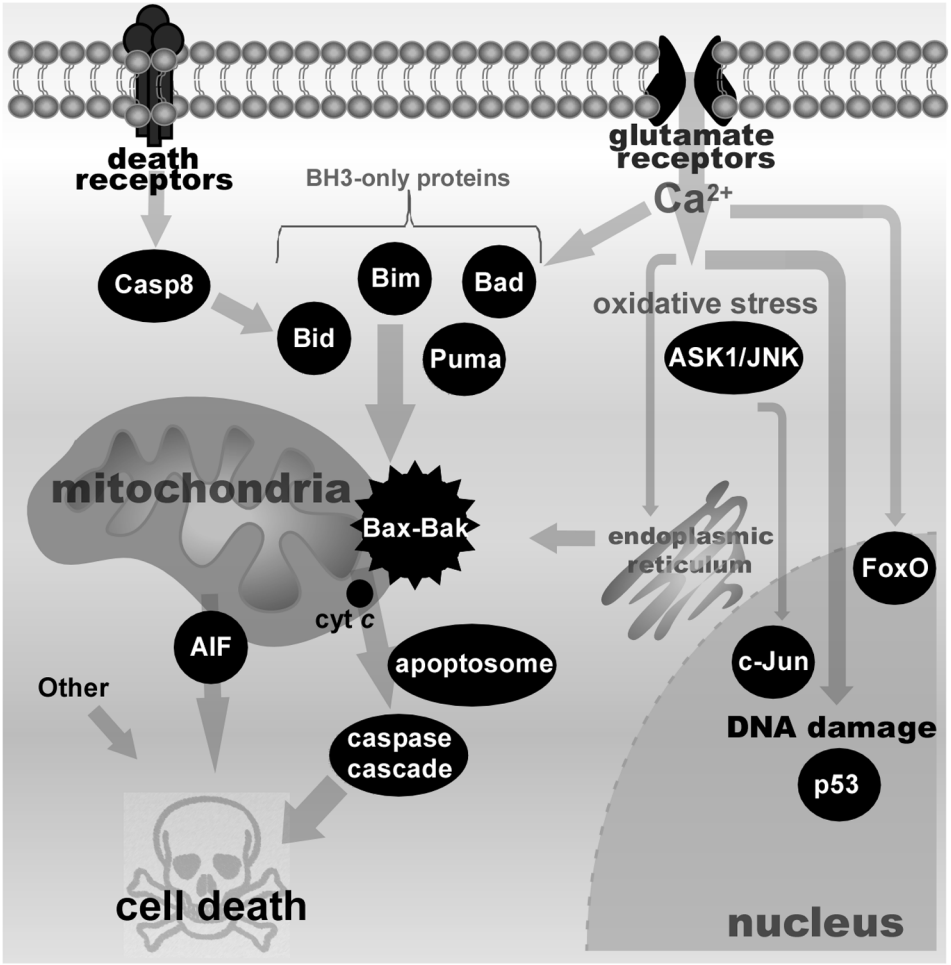

Figure 1. Pro-apoptotic signaling pathways. Diagram depicts major pro-apoptotic signaling pathways. BH3-only proteins function as upstream sentinels of cell stress. Bid is activated by caspase-8 which is itself activated downstream of death receptors. Bad is activated by calcium-dependent phosphatases, while Bim and Puma are up-regulated by FoxO or p53, respectively. BH3-only proteins trigger Bax-Bak activation and apoptogenic factors are released from mitochondria, including cytochrome *c* (cyt c) which activates the apoptosome and caspase cascade, and apoptosis-inducing factor (AIF). Ancillary pathways include induction of apoptosis via the ASK1/JNK/c-Jun pathway and other pathways such as those downstream of endoplasmic reticulum stress.

Animal studies - summary

Evoked brief seizures can cause neuronal death within the hippocampus and neocortex in animal models. The cell death has some of the biochemical and morphological features of apoptosis. In contrast, we do not have compelling evidence that spontaneous seizures in epileptic animals cause neuron loss. Both types of model show alterations in the expression of genes from the major families regulating apoptosis. Although we await evidence this contributes to neuronal death after brief seizures, it has been demonstrated in models of prolonged seizures.

HUMAN CLINICO-PATHOLOGIC STUDIES: IS THERE DAMAGE PROGRESSION IN INTRACTABLE TEMPORAL LOBE EPILEPSY?

Neuron loss, particularly within the hippocampus, is a widely observed pathologic hallmark of refractory TLE in humans. Whether chronic epileptic seizures in patients cause neuron loss, however, or instead that pathology arises independently from an initial precipitating injury or other (for example, genetic) factor(s),[62] remains debated. In the next part of this chapter, we summarize various clinico pathological and neuroimaging studies which have provided evidence for and against neuron loss as a result of repeated brief seizures in humans.

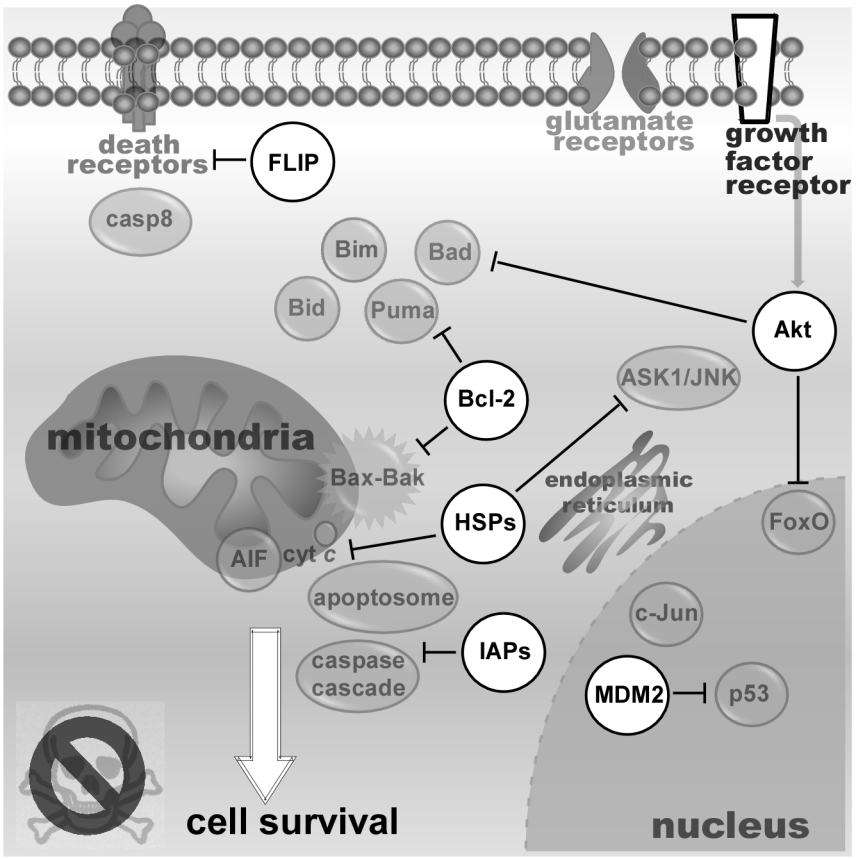

Figure 2. Anti-apoptotic signaling pathways. Diagram depicts key anti-apoptotic proteins which disrupt cell death at various points. FLIP (FLICE-like inhibitory protein) blocks the death receptor/caspase-8 pathway. Bcl-2 (plus the related Bcl-xL and Bcl-w) blocks pro-apoptotic Bcl-2 family proteins. Heat shock proteins (HSPs) inhibit mitochondrial apoptogenic proteins and the ASK1/JNK pathway. Akt downstream of various growth factor and other pathways inhibits the FoxO transcription factor and Bad. Inhibitor of apoptosis proteins (IAPs) mainly function by blocking caspases. Murine double minute 2 (MDM2) regulates p53 levels.

Hippocampal neuron loss in human temporal lobe epilepsy

There is a long history of studies identifying hippocampal neuron loss in TLE.[63–65] Most commonly, neuron loss is evident within the CA1 and endfolium or hilar region of the dentate gyrus.[66] Neuron loss and attendant gliosis are usually also evident in the CA3 subfield. While damage to the CA2 subfield and dentate gyrus tends to be less overt, neuron loss is evident in more severe cases.

A common source of evidence for seizures causing neuron loss in the human hippocampus is the association between longer seizure histories and greater neuron loss. For example, Mouritzen Dam, in a study of 20 patients with partial complex and generalized tonic-clonic seizures, found bilateral neuron loss that was most extensive in the end folium, CA3/4 and dentate granule cell layer.[67] The severity of neuron loss correlated with a longer duration of epilepsy, implying an effect of chronic seizures.[67] Similar conclusions were drawn from the large study by Mathern *et al.*, of neuron densities in 572 hippocampal specimens from TLE patients.[68] Neuron counts decreased with longer seizure histories independent of TLE pathology or aging, implying repeated seizures over many years (or factors linked to these) cause additional hippocampal neuron loss. However, the authors emphasized their data showed hippocampal sclerosis to be an acquired pathology generated mainly by an initial precipitating injury, with a relatively small contribution from chronic seizures.[68] Similar conclusions were reached when hippocampal damage was examined in patients with TLE as a result of a temporal lobe mass.[69] (Table 2)

Table 2. **Summary of clinical findings on neuron loss in human epilepsy.**

Pathology	Neuron loss in hilus, CA1, CA3 >> CA2, granule neurons; Cerebral cortex (layers II–III), cerebellum
Cross-sectional neuroimaging	Hippocampal volume loss proportional to duration of epilepsy
Longitudinal imaging	Mixed; evidence for and against progressive damage
Acute cell death markers	Temporal lobe TUNEL-stained cells in some but not all studies

Pathology in non-hippocampal regions

Extra hippocampal pathology within adjacent mesial limbic structures is present in a subset of patients with hippocampal sclerosis.[66] Affected structures include the amygdala, thalamus and neocortex. Nevertheless, a majority of patients with TLE do not develop cortical neuron loss. A recent study reported just 11 % of surgically-treated TLE cases also had neocortical neuron loss,[70] and no significant cortical neuron loss was reported in another study.[71] The explanation for extra hippocampal/neocortical neuron loss in a subpopulation of patients is not yet known. It may be the result of more frequent or generalized seizures, or it may be due to an earlier initial precipitating injury.[70] (Table 2).

Neuroimaging evidence of damage progression in intractable TLE

Hippocampus

Many cross-sectional neuroimaging studies have reported lower hippocampal volumes in patients with epilepsy compared to controls, and lower still in patients with drug-resistant epilepsy.[72–79, 80, 81–84] Studies have also shown that hippocampal volume loss correlates with the number of epileptic seizures.[74, 79] It should be noted, however, that not all studies explicitly state whether patients who experienced *status epilepticus* were excluded, and there are examples of cross-sectional studies which failed to find an association between epileptic seizures and hippocampal volume reduction.[85] Twin studies using imaging have also contributed evidence that hippocampal sclerosis is an acquired lesion. For example, volumetric and T2 imaging of monozygotic twins by Jackson *et al.* determined hippocampal sclerosis was present only in the twin with epilepsy.[86]

Longitudinal studies allow imaging of the same patients and controls over time, although they generally feature small cohorts and cover quite short periods of time.[87] Conclusions from such studies on whether epileptic seizures cause progressive volume decline are mixed. Neuroimaging over periods of less than 4 years has detected hippocampal volume decline in relation to the number of generalized,[88] and complex partial,[89] seizures. However, several reports failed to detect reductions in hippocampal volume in epilepsy patients that exceeded those in controls over the same period.[83–84, 90–91] Thus, current longitudinal studies have not resolved the question of progressive hippocampal atrophy in TLE (Table 2).

Imaging: non hippocampal regions

Neuroimaging studies have reported extra-hippocampal atrophy in patients with pharmacoresistant TLE. Regions affected include the entorhinal cortex and the amygdala ipsilateral to the seizure focus,[81, 92–94] as well as frontal poles, lateral temporal and occipital regions,[95] and contralateral regions.[94] Some cortical decreases were found to relate to the duration of epilepsy implying a role for repeated seizures in the changes.[95] Interestingly, extra hippocampal atrophy is more prominent in patients with left hemisphere TLE.[93]

We have few longitudinal imaging studies of non hippocampal atrophy on which to base conclusions. Progressive atrophy involving orbitofrontal, insular and angular regions has been reported in pharmacorefractory TLE patients.[94] Studies by Liu and colleagues, however, found that although patients with chronic epilepsy developed more neocortical volume loss compared to controls over a 3.5 year period, this related to age and medication history rather than an association with frequency of seizures.[96]

Summary – imaging evidence of seizure-induced neuron loss in human studies

Cross-sectional imaging studies support recurrent epileptic seizures as a cause of neuronal damage in the hippocampus of patients with TLE. There may also be seizure-induced neocortical neuron loss in some patients. Nevertheless, major hippocampal atrophy probably results from an initial precipitating injury rather than because of recurring epileptic seizures. Longitudinal neuroimaging offers a better method for determining the effects of recurrent seizures in epilepsy patients, but findings to date are mixed. Taken together, neuroimaging studies suggest structural damage is not an inevitable consequence of epileptic seizures in humans, in agreement with animal studies.

Histologic evidence of acute cell death in human temporal lobe epilepsy

Histological analyses of resected material have found evidence of acute cell death in patients with pharmacoresistant TLE. Henshall *et al.* detected TUNEL-positive cells in two of six neocortical resections from pharmacoresistant patients.[97] The same group, studying hippocampal sections, found TUNEL-positive cells in 9 out of 10 samples,[52, 98]; TUNEL-positive cells displayed features consistent with apoptosis.[98] However, the numbers were very low (ranging from zero to four per section) and did not differ statistically from controls.[52, 98] TUNEL-positive cells were also reported to be higher in TLE sections compared to controls in another study,[99] but were not found in three other reports.[100–102] (Table 2). These studies suggest there is at most very small-scale acute cell death in temporal lobe structures from pharmacoresistant epilepsy patients. Isolated, dying cells may, however, be rapidly removed after seizures and difficult to detect; no study has yet undertaken an assessment of complete hippocampal resections and counting has not been stereological.

MOLECULAR EVIDENCE OF APOPTOSIS-ASSOCIATED SIGNALLING IN HUMAN TEMPORAL LOBE EPILEPSY

The first studies to address whether programmed cell death/apoptosis signalling pathways were altered in the temporal lobe of patients experiencing frequent seizures emerged in the late 1990s. These descriptive reports noted increased Bcl-2 staining in astrocytes, although found Bcl-2 and Bcl-xL immunoreactivity in residual neurons of sclerotic hippocampi was similar to that in controls.[103] Glioneuronal hamartias, a form of cerebral dysgenesis, were strongly immunoreactive for Bcl-2.[103] Another early study noted that Bax immunoreactivity was stronger in TLE patients compared to control subjects and elderly drug-treated epileptics.[104]

Bcl-2 and caspase family genes

The first study to apply quantitative measures of apoptosis-associated gene expression was done by Simon and colleagues at the University of Pittsburgh.[97] They reported data from 19 resected TLE patient temporal neocortex samples and six age- and gender-matched autopsy controls. Using Western blot analysis, they showed higher levels of Bcl-2 and Bcl-xL in patient brain (Tables 3, 4). Immunohistochemistry showed that neurons were the main cell type expressing Bcl-2, while Bcl-xL stained mainly astrocytes.[97] The cleaved form of caspase-1 and caspase-3 were also detected in TLE samples but not in the controls.[97] The elevated Bcl-2 and Bcl-xL levels might be molecular adaptations to inhibit cell death in surviving cells, while the activated caspases might be contributing to progressing pathology. Indeed, animal data show over-expressing Bcl-2 or Bcl-xL is neuroprotective against excitotoxic insults,[105–106] while over expression of caspase-3 enhances neurodegeneration after ischemia.[107] Caspase-1 knockout mice are refractory to kainic acid-induced seizures[108] so the presence of cleaved caspase-1 in human TLE might have pro-epileptic consequences in addition to, or instead of, a cell death-regulatory function.

Table 3. **Expression of pro-apoptotic proteins in human TLE.**

Increased
Pro-caspases 2, 3, 6, 7, and 9 (hippocampus or neocortex)
Cleaved caspases 1, 3, 7, 8 and 9 (hippocampus or neocortex)
Bax* (hippocampus, neocortex)
p53 (hippocampus)
Tumor necrosis factor receptor 1** (hippocampus)
Nuclear caspase-activated DNase (hippocampus)
Apoptosis signal-regulating kinase 1 (hippocampus)
Decreased/inhibited
BH3-only subgroup protein Bim (hippocampus)
FoxO transcription factors (hippocampus)

* studies have also reported no changes to Bax
** TNFR1 may have non-cell death related functions.

Table 4. **Expression of anti-apoptotic proteins in TLE brain tissue.**

Increased

1. Bcl-2, Bcl-xL* and Bcl-w (hippocampus or neocortex)
2. Akt phosphorylation (hippocampus)
3. X-linked IAP binding to caspase-7 (hippocampus)

Decreased

1. MDM2 (p53 negative regulator)

* Bcl-xL was increased in neocortex but not in hippocampus

Findings from other cohorts

Pro-apoptotic Bax expression has been reported to be moderately elevated in TLE hippocampi,[101] although this was not found in another study.[102] Several laboratories, using cohorts ranging from 12–24 patients, have also detected higher Bcl-2 levels in neurons and also glia in resected TLE hippocampi.[101–102, 109] Levels of Bcl-w, another anti-apoptotic Bcl-2 family protein, are also elevated in resected TLE hippocampus.[53] Notably, Bcl-w expression in the hippocampus of mice is increased by exposure to repeated brief seizures.[53] This increase may protect hippocampus since over-expressing Bcl-w prevents excitotoxic (ischemic) injury *in vivo,* [110] while the absence of *bcl-w* increases neuron loss after *status epilepticus.*[53]

Another protective adaptation may be a reduction in levels of BH3-only protein Bim in TLE hippocampus.[52, 111] (Table 3) Again, animal models of brief seizures have recapitulated this pattern,[52] which is very likely protective since the hippocampus of mice lacking *bim* are protected against *status epilepticus.*[59] Other BH3-only proteins may also be important; mice lacking the BH3-only protein Puma develop less hippocampal damage after *status epilepticus.*[57–58]

Other caspases

Differences in the expression many caspases have been detected in human TLE brain samples. Caspases 2, 3, 6, 7 and 9 have all been reported to be over-expressed and their active forms found,[55, 102, 111–112] and immunohistochemistry has localized cleaved caspases within neuron-like cells in TLE brain.[112–113] These data

are evidence that caspase-mediated pro-apoptotic signalling occurs in human TLE. Caspases appear to localize within both the cell soma and dendrites,[55, 111, 113] supporting caspase-mediated cleavage of intracellular structural or synaptic proteins.[114]

Other pro- and anti-apoptotic proteins

Other putatively pro-apoptotic proteins have been reported to be increased in human TLE tissue. There are increased nuclear levels of the caspase-activated DNase, the enzyme responsible for the hallmark DNA laddering seen in apoptosis, in TLE samples.[113] Other pro-apoptotic proteins showing higher expression in TLE include apoptosis signal-regulating kinase-1 (ASK1),[112, 115] c-Jun,[116] death-associated protein kinase,[98] Fas and its signalling components,[102, 115] p53,[102, 117] and TNF receptor 1.[115]. (Table 3)

In addition to anti-apoptotic Bcl-2 family proteins, several other anti-apoptotic proteins are over- expressed in resected TLE tissue. This includes protein kinase B (Akt),[52] heat shock protein 70,[116] endoplasmic reticulum stress-activated proteins such as glucose-regulated proteins 78/94, [111–112] and the cellular inhibitor of apoptosis protein-2 (cIAP-2).[118] (Table 4)

Summary – molecular evidence of apoptosis in human TLE

Alterations to apoptosis-associated signalling pathways are widely found in TLE tissue. Human findings probably reflect seizure-induced stress and the resulting adjustments to the molecular repertoire between adaptations which prevent neuron loss and, occasionally, signalling which ultimately results in cell death. Higher levels of anti-apoptotic Bcl-2 family proteins and related molecules may raise the threshold required for a seizure to cause cell death thereby countering the influence of pro-apoptotic molecules such as caspases. This may explain why so little acute cell death occurs in patients experiencing frequent seizures. This interpretation is consistent with animal data showing thatchanges to levels of apoptosis-associated genes can prevent, or exacerbate, seizure-induced neuronal death.

MITOCHONDRIAL DNA DAMAGE IN EPILEPSY

Repeated seizures may result in other changes which enhance neuronal vulnerability over time. Mitochondrial function is critical for normal neuronal excitability but mitochondria are also the primary sites in the cell for production of reactive oxygen species (ROS). Seizures increase ROS production, and studies suggest this depletes cellular antioxidants, interferes with function of electron transport chain enzymes and causes DNA damage.[119] Indeed, mice over-expressing the mitochondrially-localized superoxide dismutase 2 are protected against seizure-induced neuronal death.[120] Mitochondrial DNA (mtDNA) damage,[121] and mtDNA copy number reductions,[122–123] have also been reported in hippocampal tissue from epileptic rats, which has been suggested to contribute to reduced electron transport chain activity. Over time, neurons may become more susceptible to seizures and to their deleterious consequences, such as cationic overload.[119] Other groups, however, have not detected a reduction in electron transport chain enzymes in epileptic animals,[124] or even found expression to be increased.[125] Evidence of mitochondrial dysfunction has been reported in hippocampal tissue from TLE patients.[126–127] Together, cumulative mtDNA damage and compromised mitochondrial function may enhance neuronal vulnerability to seizures and contribute to epileptogenesis.

CHAPTER SUMMARY AND FUTURE QUESTIONS

Brief seizures can cause neuronal death in animal models. There is emerging evidence that apoptosis-associated signalling pathways are activated by these seizures, but so far we only have proof these contribute to cell death in models of *status epilepticus*. There is little evidence that spontaneous seizures in epileptic animals cause acute cell death, but these animals nevertheless display alterations in apoptosis-associated pathways. In humans, there is evidence that recurrent seizures cause subtle or diffuse neuron loss in affected structures. Histopathologic

analyses have found a molecular signature of apoptosis-associated signalling in resected neocortical and hippocampal material from pharmocoresistant TLE patients.

Several questions remain to be answered. Is the frequency/clustering of seizures, or particular seizure types, for example secondarily generalized, more harmful? We await additional longitudinal neuroimaging studies specifically focused on comparing outcomes of different seizure types and severities. Future studies of pro- and anti-apoptotic signalling molecules should determine whether these occur in the same or different cells. Mouse models can make important contributions by allowing us to test which genes actually affect cell death following repeated brief seizures; in particular, they will allow us to test the influence of the particular genes in epileptic animals. This chapter has summarised the evidence for and against neuron loss after single and repeated brief seizures in animal models and human epilepsy and highlights the molecular pathways of apoptosis as a potential contributor to cell death and survival decisions.

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