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Neuronal degeneration is observed in multiple regions outside the hippocampus after lithium pilocarpine-induced status epilepticus in the immature rat

Erika A. Scholl¹, F. Edward Dudek², and Jeffrey J. Ekstrand¹

¹Department of Pediatrics, University of Utah, School of Medicine, Salt Lake City, Utah

²Department of Neurosurgery, University of Utah, School of Medicine, Salt Lake City, Utah

Abstract

Although hippocampal sclerosis is frequently identified as a possible epileptic focus in patients with temporal lobe epilepsy, neuronal loss has also been observed in additional structures, including areas outside the temporal lobe. The claim from several researchers using animal models of acquired epilepsy that the immature brain can develop epilepsy without evidence of hippocampal neuronal death raises the possibility that neuronal death in some of these other regions may also be important for epileptogenesis. The present study used the lithium pilocarpine model of acquired epilepsy in immature animals to assess which structures outside the hippocampus are injured acutely after status epilepticus. Sprague Dawley rat pups were implanted with surface EEG electrodes, and status epilepticus was induced at 20 days of age with lithium pilocarpine. After 72 h, brain tissue from 12 animals was examined with Fluoro-Jade B, a histochemical marker for degenerating neurons. All animals that had confirmed status epilepticus demonstrated Fluoro-Jade B staining in areas outside the hippocampus. The most prominent staining was seen in thalamus (mediodorsal, paratenial, reuniens, and ventral lateral geniculate nuclei), amygdala (ventral lateral, posteromedial, and basomedial nuclei), ventral premammillary nuclei of hypothalamus, and paralimbic cortices (perirhinal, entorhinal, and piriform) as well as parasubiculum and dorsal endopiriform nuclei. These results demonstrate that lithium pilocarpine-induced status epilepticus in the immature rat brain consistently results in neuronal injury in several distinct areas outside of the hippocampus. Many of these regions are similar to areas damaged in patients with temporal lobe epilepsy, thus suggesting a possible role in epileptogenesis.

Keywords

Fluoro-jade B; lithium pilocarpine; status epilepticus; immature brain

Early neuropathology studies of patients with temporal lobe epilepsy (TLE) demonstrated evidence of profound neuronal loss with associated sclerosis in the hippocampus,

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Correspondence to: Dr. Jeffrey J. Ekstrand, Dept. of Pediatrics, School of Medicine, University of Utah, 420 Chipeta Way, Suite 1700, Salt Lake City, Utah 84108, jeffrey.ekstrand@hsc.utah.edu.

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parahippocampal areas (e.g. entorhinal cortex), amygdala, and other areas including structures outside the temporal lobe (Cavanagh and Meyer, 1956; Falconer et al., 1964; Margerison and Corsellis, 1966). Although these studies reported variability in both the severity of the lesion and different brain regions affected, attention rapidly became focused on the hippocampus because of evidence that appeared to point to this region as an important contributor to the development of some cases of TLE. Similar neuronal death occurs in most animal models of acquired epilepsy in the *adult* brain, and these models have been used to study how the hippocampus may be involved in epileptogenesis (Buckmaster and Dudek, 1997; Cavalheiro et al., 1991; Fujikawa, 1996; Hirsch et al., 1992; Mello et al., 1993; Obenaus et al., 1993; Pollard et al., 1994; Turski et al., 1983). However, in animal models of acquired epilepsy in *juvenile* animals, where, in general, the immature brain is more resistant to neuronal loss and less likely to develop epilepsy (for reviews see Lado et al., 2002; Stafstrom and Sutula, 2005), the evidence that neuronal death in the hippocampus contributes to epileptogenesis is less clear. Evidence both for and against a role for hippocampal neuronal loss has been provided in several studies using many different techniques, and the controversy has been summarized in recent reviews addressing this topic (Baram et al., 2011; Dudek et al., 2010). Interestingly, although not as well studied as the hippocampus, most of the animal models of acquired epileptogenesis in both the immature and adult brain also have damage in other brain regions similar to what was observed in the initial neuropathology studies in humans (Cavalheiro et al., 1987; Dudek et al., 2006; Fernandes et al., 1999; Kubova et al., 2001; Nairismagi et al., 2006; Sankar et al., 1998; 1997; Tuunanen et al., 1999). The absence of unequivocal evidence of hippocampal neuronal death raises the possibility that some of these other cerebral structures, which often are not rigorously examined, may also be important in the development of epilepsy in the immature brain. This study uses the chemoconvulsant lithium pilocarpine model of status epilepticus in the immature rat (post-natal day 20=P20) to assess which structures outside the hippocampal formation are injured acutely during prolonged status epilepticus. Compared to previous reports (Cavalheiro et al., 1987; Fernandes et al., 1999; Kubova et al., 2001; Nairismagi et al., 2006; Sankar et al., 1998; 1997), we have attempted to provide a more detailed description of the distribution within specific nuclei. Injury was assessed using the histochemical marker Fluoro-jade B, a marker for degenerating neurons. We demonstrate that acute neuronal degeneration does occur in several extrahippocampal areas after status epilepticus, which may have important implications for epileptogenesis.

EXPERIMENTAL PROCEDURES

Surgical procedure for implantation of electrodes

Sprague-Dawley (Harlan Laboratories, Indianapolis IN) rat pups were housed as a litter with their dam, and kept in a standard light/dark cycle (12/12 h). At P18, pups were anesthetized with 2.5–3.0% isoflurane and placed into a stereotaxic frame. Using sterile surgical techniques, an incision was made midline on the scalp and the skull exposed. With a high-speed dremel drill, a craniotomy was performed over the left parietal cortex (centered around a point 2 mm rostral and 2 mm lateral to bregma) and five burr holes were drilled for placement of the electrode wires and anchoring screws. Two electrode wires and a grounding wire were placed in three burr holes such that the wires were adjacent to the dura over the left and right parietal cortex. Two anchoring screws were placed in the remaining distal burr holes. The screw-electrode complex was fixed securely to the skull with cold-curing dental cement, and the incision site was closed with 4.0 coated vicryl absorbable suture (polyglactin 910-Ethicon). Post-surgery, the animals received 0.1 ml Marcaine injected directly in the suture site and 1.0 ml sub-cutaneously injected lactated Ringer's solution. The rats were then allowed to recover in a temperature-controlled heating element (Gaymar TP500 water circulating heating pad), prior to returning to their dam and litter.

mates. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Utah and conducted in accordance with its guidelines.

Induction of status epilepticus

On P19 animals were pretreated with intraperitoneal lithium (LiCl 127 mg/kg). Status epilepticus was induced on P20 with intraperitoneal pilocarpine (60 mg/kg); scopolamine (1 mg/kg) was given 30 min prior to injection. Age-matched control animals also received LiCl and scopolamine, but were administered saline instead of pilocarpine. The EEG signals were stored on a computer (Acknowledge software, BIOPAC systems, Inc), which allowed simultaneous recordings from 8 rats with individual EEG electrodes. EEG ictal discharges were defined as repetitive spiking (amplitude 3 times baseline) lasting longer than 15 sec. Video data were analyzed to identify behavioral correlates of ictal EEG activity. Video monitoring was performed using EZWatch pro, Version 3.1HD, a video surveillance system with infrared cameras. Ictal activity was correlated with behavioral seizure activity, which was rated using a modified Racine scale (Racine 1972; stage 1, mouth and facial movements; stage 2, head nodding; stage 3, forelimb clonus; stage 4, rearing; stage 5, rearing and falling).

Fluoro-jade B staining

Seventy-two hours after initiation of status epilepticus, animals were deeply anesthetized with the oral inhalation anesthetic isoflurane (Fluriso-Vet One MWI Veterinary Supply, Boise ID) by placing animals in an enclosed glass desiccator jar with inhalation agent separated from animal by porous floor grid. Animals then underwent cardiac perfusion-fixation with 10% neutral-buffered formalin (Sigma-Aldrich) followed by incubation in 30% sucrose for freezing. Brain sections 40- μ m thick were cut on a cryostat in both coronal and horizontal sections in order to visualize optimal orientations of specific brain regions (as defined in Paxinos and Watson, 1986). Every third section was examined with Fluoro-jade B (F-JB, Histo-chem Inc., Jefferson, AR, U.S.A). Sections were incubated with 0.06% KMnO₄ followed by 0.001% Fluoro-Jade B according to the protocol described by Schmued et al. (1997). Initial images were obtained using a Hamamatsu Nanoscope 2.0 HT (Olympus). More detailed widefield imaging was performed using a Zeiss Axio Imager, and confocal images were obtained using an Olympus FV1000 microscope.

Scoring of Fluoro-jade B staining

Scoring of Fluoro-jade B was initially based on a system of 0–3 points. For this system, “0” indicated no stained cells in the nuclei or region of interest, “1” indicated low density of staining (~ 33% of cells), “2” indicated moderate staining density (~33–67% of cells), and “3” indicated a high density of staining (~>67% of cells). For each animal, the number of sections analyzed for each region varied between 3 and 12 sections depending on the size of the area of interest. The score from each section of a region of interest as detailed in Table 1 was averaged and given a 1–6 asterisk (star) rating based on the following numbers: 1 star was for a score 0.50, 2 stars was for 0.51–1.00, three stars was for 1.01–1.50, four stars was for 1.51–2.00, five stars was for 2.01–2.50, and six stars was for 2.51–3.00. Sections that showed no staining were ranked as 0. In the following text descriptions, 1–2 stars will be defined as low/little staining, 3–4 stars as medium/moderate staining, and 5–6 stars as high/heavy staining.

RESULTS

Lithium-pilocarpine-induced status epilepticus in P20 rats

Status epilepticus was induced in 11 of 12 animals treated with lithium pilocarpine. The seizure activity had the characteristic gradual increase in amplitude with spike and spike-wave activity typical of lithium pilocarpine-induced status epilepticus recorded in adult rats (Fig. 1). Continuous spiking activity persisted for 3–5 hours, followed by intermittent activity over the next 12 hours. Clinical correlates of seizure activity, observed in all 11 animals, consisted of initial behavioral arrest, single-limb clonus, followed by generalized tonic-clonic activity in all limbs with rearing and loss of stability (Racine class 5; Racine, 1972). The single animal given pilocarpine that did not show EEG evidence of seizure activity was observed to have increased scratching, lip-smacking, and increased grooming typical of systemic effects of pilocarpine without behavioral changes seen in any Racine stages. All 12 animals treated with lithium pilocarpine as well as 5 controls were processed for histological analysis with Fluoro-jade B.

Acute neuronal degeneration after status epilepticus

Seventy-two hours after lithium pilocarpine treatment, brain sections were examined with Fluoro-jade B for evidence of neuronal degeneration. All 11 animals that had electrographic and behavioral seizure activity showed variable, but widespread Fluoro-jade B staining. Staining was not present in control animals or in the single animal given lithium pilocarpine that did not have seizures and progress to status epilepticus (data not shown). Specific nuclei and other described brain regions were identified by examining Nissl-stained sections adjacent to Fluoro-jade B sections. All abbreviations are from Paxinos rat brain atlas (Paxinos and Watson, 1986), and other definitions of anatomical nomenclature are described below.

Amygdala—As previously delineated (Pitkanen et al., 1997a), the amygdaloid nuclei were divided into three groups: deep, superficial, and other. In general, there was marked staining in every animal in several nuclei, with the more caudal and medial nuclei showing the most degeneration. Within the deep nuclei, the lateral ventral nucleus (LaV) and anterior and posterior aspects of the basomedial nuclei (BMA and BMP) were consistently the most highly stained (Fig. 2C, Table 1). Other nuclei also showed variably low to moderate staining such that individual animals might have moderate Fluoro-jade B labeling present, but this was less consistent. Within the superficial nuclei, the highest staining was observed in the posterior median nuclei (MeP), bed nucleus of the accessory olfactory tract (BAOT), and also variably in the posteromedial cortical nuclei (PMCo) (Fig. 2B–D, Table 1). Other amygdaloid nuclei not traditionally classified as deep or superficial that also had consistently marked staining included the amygdalostratial transition area (AStr), intercalated masses (I), and the central medial nucleus (CeM) (Fig. 2A–C, Table 1). Labeled neuronal soma had, for the most part, non-specific multipolar morphology of indeterminant classification. However, the staining of both medial and intercapsular intercalated masses (Fig. 2E) strongly resembled the staining observed in these cell groups in the GAD67-GFP mice described by Tamamaki et al., (2003). Taken together, these results demonstrate a robust and reliable pattern of neuronal degeneration observed in every animal, particularly in the caudal and medial nuclei, with some support for selective loss of interneurons in at least the intercapsular intercalated masses and possibly other regions.

Other limbic regions—The hippocampal formation showed the greatest variability in Fluoro-jade B staining, consistent with our previous report on this structure (Ekstrand et al., 2011). Again, a clear increase in neuronal damage was apparent along the septo-temporal axis, with moderate to marked Fluoro-jade B staining in the ventral hippocampus (Fig. 3A),

but wide variations in staining (no staining to moderate) in the dorsal hippocampus (Table 1). By contrast, other limbic areas showed less variable marked staining in several regions including parasubiculum (PaS) (Fig. 3B), lateral septal area (LSD, LSI, and LSV), bed stria terminalis nuclei (BSTM), acumbens shell (AcbsH) (Fig. 3D), and dorsal endopiriform nucleus (DEn) (Fig. 2A–C). Finally, several limbic-associated cortices also had more consistent labeling compared to the dorsal hippocampal formation. The entorhinal cortex (Ent) was moderately-heavily labeled (Fig. 3B). The perirhinal cortex (PRh) was well stained in layers II/III, V, and VI, with the heaviest staining in layers II/III (Fig. 2D, 3F, Table 1). The piriform cortex (Pir) also showed robust Fluoro-jade labeling, mostly in deeper parts of layer II and layer III (Fig. 2A–C). As with some other regions described, a clear rostral-caudal gradient was observed with the most labeling of neurons in the caudal portion of piriform cortex. Cell soma were mostly multipolar with some pyramidal profiles, especially in superficial layer III (Fig. 2F). Overall, these results demonstrate a consistent pattern of neuronal degeneration particularly in the caudal region of many limbic regions that are more reliably observed than degeneration in the dorsal hippocampus.

Other Neocortices—Mild to moderate damage was observed in layers II/III and sometimes V in several regions of neocortex (Fig. 4) in every animal, although the pattern was inconsistent across individual animals. Somatic profiles included mostly pyramidal cells, with occasional multipolar shapes.

Thalamus—In general, there was prominent Fluoro-jade labeling in many regions of the thalamus, resulting in stained nuclei that, when labeled, were well delineated. Divisions of the thalamus were defined according to Druga et al. (2005). The most consistent heavy staining, occurring in every animal, was in the medial and midline nuclei including the mediodorsal (MD), paratenial (PT), and reuniens (Re) nuclei (Fig. 5A–C, Table 1). Somatic profiles had stellate or fusiform morphologies (Fig. 5E). The lateral nuclei (LDDM, LDVL, LPMR, and LPLR) were more variable in the degree of Fluoro-jade B staining, but still showed moderate to marked labeling in every animal. Other nuclei were distinctive and easy to identify because of the clear absence of Fluoro-jade labeling including parafascicular (PF), ventral lateral geniculate (VLG), and anterior nuclei (AD and AV). The remainder of thalamic nuclei had tremendous variability between animals ranging from no staining to heavy labeling often with little discernible pattern, although averaging across animals did result in general trends suggesting low to moderate degeneration in these areas. Overall, consistent damage in the medial nuclei, with more variable degeneration in lateral structures best characterized neuronal degeneration in the thalamus.

Basal Ganglia—The staining in the caudate putamen was moderate, and appeared throughout the entire rostral-caudal axis. In contrast, other regions of the basal ganglia, including the nucleus accumbens (NAc), the globus pallidus (GP), the subthalamic nucleus (STh), the medial globus pallidus (MGP), and the substantia nigra pars reticulata (SNR), had no or little staining (Table 1).

Hypothalamus—The hypothalamus was divided into the preoptic, anterior, tuberal, and mammillary regions as previously described (Simerly, 1994). In general, little to no staining was observed in any region of the hypothalamus except in the central portion of the anterior hypothalamic area (AHC) (Table 1), medial tuberal nucleus, and pre mammillary nucleus (PMV) (Fig. 6A). In the latter region, somatic profiles were consistent with neurosecretory cells (Fig. 6B).

DISCUSSION

The goal of this study was to identify in more detail which structures other than the hippocampus have acute neuronal cell loss after lithium pilocarpine-induced status epilepticus in P20 rat pups. Status epilepticus was confirmed by both behavioral correlates and EEG monitoring. All animals that underwent status epilepticus had evidence of neuronal degeneration in areas outside the hippocampus. The severity of injury was variable across animals, but still revealed a consistent pattern of damage that identified several brain regions and nuclei in the thalamus, amygdala, endopiriform nuclei, hypothalamus, and other paralimbic cortices. Somatic profiles were observed that, although not cyto-chemically confirmed as glutamatergic or GABAergic, were still consistent with both principal excitatory cells and inhibitory interneurons. Many of the areas with neuronal degeneration corresponded with similar regions first described in early neuropathology studies of patients with TLE, suggesting a possible role in epileptogenesis for these structures in the immature brain.

Technical considerations

A variety of techniques have been utilized to document neuronal cell loss in animal models of epileptogenesis. We used the immunohistochemical marker Fluoro-jade B, an acidic dye with a high affinity to strongly basic molecules expressed during neuronal degeneration (Schmued et al., 1997; Schmued and Hopkins, 2000). Fluoro-jade B is more sensitive than traditional cell counting methods at detecting subtle potential neuronal cell loss that is below the variability of cell counts between tissue sections. While all biochemical markers of degenerating cells are open to the criticism that the labeled neuron may be injured, but not destined to die, the few studies that have attempted to assess the final status of Fluoro-jade labeled neurons suggest that they are on an irretrievable path to death. Analysis of electron microscopy ultrastructure in Fluoro-jade B labeled cells in both adult (Pitkanen et al., 2002) and immature brain (Kubova et al., 2001) have shown a pattern consistent with dying neurons. Multiple studies have also shown either a direct co-localization with other markers of apoptosis and/or necrosis, or correspondence with Nissl-stain loss or markers of necrosis and/or apoptosis (Anderson et al., 2005; Frank et al., 2003; Larsson et al., 2001; Sato et al., 2001; Scallet et al., 2004). Taken together, these studies support the conclusion that Fluoro-jade B labels dying neurons, not reversibly injured ones.

The goal of this study was to identify areas outside the hippocampus that show acute neuronal degeneration after LiPC induced SE. While the scoring system and descriptions provide information about the affected regions, it can be difficult to directly compare quantitative differences when specific structures vary in size, neuronal density, and consistency of neuronal degeneration. Although each region had at least 3 sections per animal examined, small regions and nuclei were represented with fewer sections, thus affecting the robustness of the averaged measurements, especially when there was variability among sections. Likewise, subtle neuronal degeneration is more easily detected and measured in densely populated regions. The results, therefore, must be evaluated in the context of regional differences in size and neuronal density, as well as variability in neuronal degeneration.

Differences in methodology may account for some disparities between previous work examining neuronal loss after LiPC induced SE in immature animals. Age (Dube et al., 2001; Priel et al., 1996; Sankar et al., 1997), strain (Xu et al., 2004), pilocarpine dose (Cavalheiro et al., 1987; Priel et al., 1996), presence of lithium (Clifford et al., 1987), electrode implantation techniques (Boast et al., 1976; Loscher et al., 1995), attenuation of SE with benzodiazepine (Hasson et al., 2008) or paraldehyde (Kubova et al., 2005), as well as both timing and type of injury assessment have all been postulated to affect the degree of

detectable neuronal loss. Table 2 summarizes some of the parameters used previously to assess for neuronal damage outside the hippocampus in immature animals after LiPC induced SE (Table 2).

Lithium pilocarpine-induced status epilepticus as a model of human TLE

Many researchers have used pilocarpine, with or without lithium, to induce status epilepticus in rodents with the goal of examining the relationship between pathological changes in structure and the development of TLE (Cavalheiro et al., 1991; Fernandes et al., 1999; Fujikawa, 1996; Hirsch et al., 1992; Mello et al., 1993; Nairismagi et al., 2006; Raol et al., 2003; Roch et al., 2002; Sankar et al., 1998; Turski et al., 1983). This model is attractive because it reliably recapitulates both the phenomena of TLE (i.e., generation of recurrent spontaneous seizures) and damage in the hippocampus, similar to that observed in many patients with this type of epilepsy (Wieser, 2004). The apparent association of damage in the hippocampus with TLE has often resulted in a de-emphasis of other injured brain structures, even though others using evidence from both animal models (including studies with lithium pilocarpine) and human TLE patients have advocated a role for other structures also being involved in the development of epilepsy (Bertram, 2009; Gloor, 1992; Margerison and Corsellis, 1966; Spencer, 2002). Several neuropathological studies in patients with TLE have reported, in addition to the hippocampus, lesions in other areas including thalamus, amygdala, and cerebellum (Bruton, 1988; Cavanagh and Meyer, 1956; Falconer et al., 1964; Hudson et al., 1993; Margerison and Corsellis, 1966; Miller et al., 1994). Studies using the lithium pilocarpine-induced status epilepticus model, although often focusing primarily on the hippocampal lesion, have also demonstrated injury to other structures (Bertram and Scott, 2000; Cavalheiro et al., 1987; Fernandes et al., 1999; Fujikawa, 1996; Kubova et al., 2002; 2001; Nairismagi et al., 2006; Pitkanen et al., 1997b; Sankar et al., 1998; 1997; Turski et al., 1983), including many of the regions identified in human pathology reports. Therefore, this model, with its widespread injury outside the hippocampus observed but often not described in detail, is still consistent with human TLE.

The hippocampus vs. other regions as the primary area involved in TLE

The historical focus on the hippocampal formation as the primary lesion in TLE has been based mostly on clinical studies that demonstrated that (1) the hippocampus is the most common damaged area in patients with temporal lobe epilepsy, (2) onset of seizure activity is in or near the temporal lobe, and (3) surgical resection of the hippocampus often results in a “cure” of epilepsy. Early pathology studies found evidence for damage to the hippocampus (i.e. hippocampal sclerosis) in 50–70% of patients with TLE (Falconer et al., 1964; Margerison and Corsellis, 1966). Depth electrode studies also have shown evidence for ictal onset within the hippocampal region (Quesney et al., 1988; So et al., 1989). Surgical resection of the anterior portion of the temporal lobe, including the hippocampal formation, did eliminate or reduce seizures in 60–70% of patients in some case studies (Goldring et al., 1992; Spencer et al., 1984; Wyler et al., 1995). However, several researchers (for review see Bertram, 2009; Gloor, 1992) have challenged the unique role of the hippocampus, asserting that because of technical issues favoring observations of neuronal damage and ictal onset in the hippocampus (i.e., laminar cellular organization, and the relative ease in obtaining *en bloc* surgical resections), the epileptogenic role of this structure has been overemphasized. TLE can occur in the absence of discernible damage to the hippocampus, sometimes with isolated pathology in extrahippocampal structures such as amygdala and other regions (Cavanagh and Meyer, 1956; Falconer et al., 1964; Margerison and Corsellis, 1966). Indeed, when the importance of the amygdala is reevaluated by criteria similar to the hippocampus, evidence for a structural lesion or for ictal onset (either isolated or in conjunction with the hippocampus), suggest that this structure may also contribute to the development of epilepsy (Gloor, 1992; Kullmann, 2011; Pitkanen et al., 1998). Current surgical resection practices

for TLE usually remove both amygdala and hippocampus, making it difficult to distinguish which structure's removal has the greatest impact on seizure reduction. However, one study (Feindel and Rasmussen, 1991) found no difference in seizure reduction after removal of the amygdala with substantial sparing of hippocampus, as compared to larger resections that included the hippocampus. Additionally, while not as well studied as the hippocampus or even the amygdala, several anatomical and physiological studies have demonstrated perturbations in mid-thalamic nuclei and extrahippocampal regions that influence excitability and/or the expression of TLE (Andre et al., 2003; Bertram et al., 2001; Bertram and Scott, 2000; Bertram et al., 1998; Du et al., 1993; Francois et al., 2011; Spencer and Spencer, 1994). The inability of lesional or physiological alterations in the hippocampal formation to account for all cases of TLE, along with documented cases of isolated damage to other regions that result in the clinical features of TLE indistinguishable from those with clear damage in the hippocampus, suggest that while the hippocampus may be important in a subset of TLE patients, other regions and mechanisms may also be involved.

Pilocarpine model of TLE: neuronal damage and epilepsy during development

An intriguing finding in many previous studies of animal models of epilepsy comparing the adult and immature brain is the observation that the immature brain is more resistant to neuronal loss and less likely to develop epilepsy (for review see Baram et al., 2011; Dudek et al., 2010; Lado et al., 2002; Stafstrom and Sutula, 2005). Most studies have not shown any evidence of hippocampal neuronal loss in animals less than 2 weeks of age, and when neuronal injury or neuronal loss has been documented (either in this age group or in slightly older but still immature animals), it has always been less than observed in adults (Cavalheiro et al., 1987; Sperber et al., 1991; Stafstrom et al., 1992). Also, the proportion of young animals that later develop epilepsy is dramatically less compared to the proportion of adult animals (Priel et al., 1996; Sankar et al., 1998; Kubova et al., 2004). Despite this apparent relationship between the severity of neuronal damage and epileptogenesis across developmental ages, researchers have had difficulty demonstrating a direct correlation of hippocampal neuronal loss in immature animals that develop epilepsy. Using the lithium pilocarpine model in the P21 age group, Raol and colleagues (2003) reported that only 2 of 9 epileptic animals had discernible evidence of neuronal loss in the dorsal hippocampus from cell counts of Nissl stained sections. While the authors qualified their conclusion to account for the possibility that more subtle neuronal loss may have been present in the hippocampus and that other cortical areas not examined may have had neuronal loss, this study has been used with other work from animal models of febrile seizures and hypoxia to claim that acquired epileptogenesis in the immature brain may not require neuronal death (Baram et al., 2011). While it is uncertain how comparable the hypoxia and febrile seizure models are to the lithium pilocarpine model of prolonged SE, the results from the present study show that even when neuronal loss is not observed in the dorsal hippocampus, neuronal degeneration is present in several other cortical regions, thus allowing for the possibility that epileptogenesis from lithium pilocarpine-induced SE may still require a mechanism that involves neuronal death. Indeed, some of the earliest studies of pilocarpine-induced seizures in the immature brain also reported neuronal death in extra-hippocampal regions (Table 2). Examining cresyl violet-stained sections, Cavalheiro and colleagues (1987) stated that, while very little damage could be observed in animals younger than P14, neuronal death was present in PN15-21 rats in amygdala, olfactory cortex, neocortex, and certain thalamic nuclei, in addition to neuronal loss in the hippocampus (Cavalheiro et al., 1987; Priel et al., 1996). Sankar and colleagues (1997) also confirmed seizure-induced neuronal damage after lithium pilocarpine to extrahippocampal structures including neocortex, caudate, septum, amygdala, and thalamus in P21 animals assessed with serum neuron-specific enolase, however limited detail was provided about specific nuclei affected. More recently, Kubova and colleagues have separately analyzed different extrahippocampal regions both outside

and within the temporal lobe using neuronal counting techniques and histochemical markers for neuronal death, including Fluoro-jade B. They demonstrated neuronal degeneration in amygdala and perirhinal cortex (Nairismagi et al., 2006), cerebral cortex (Mares et al., 2005), and thalamus (Kubova et al., 2001). Unlike previous studies, they were able to demonstrate damage to the medial dorsal thalamic nuclei as early as PND 12 (Kubova et al., 2001). Our results extend these early studies and taken together support the conclusions that 1) neuronal death does occur in the immature brain after pilocarpine-induced status epilepticus, and 2) studies that do not show an association of hippocampal neuronal loss with the development of epilepsy do not exclude the possibility that neuronal death is involved in epileptogenesis from other brain regions.

Mechanisms of epileptogenesis resulting from degeneration of neurons

Numerous mechanisms have been hypothesized to contribute to epileptogenesis (for review see Jasper's Basic Mechanisms of Epilepsy, 2012), some which rely on a component of neuronal injury and/or death. One of the most direct mechanisms involves the loss of inhibition from specific populations of GABAergic interneurons that disrupt the balance of excitation and inhibition such that seizures are generated. Previous studies have identified loss of interneurons in some models of TLE in adult animals (Drexel et al., 2011; Knopp et al., 2005; Kobayashi et al., 2003; Tuunanen et al., 1996; van Vliet et al., 2004), with many focusing mostly on the hippocampal formation (Andre et al., 2001; Cossart et al., 2001; Dinocourt et al., 2003; Fritsch et al., 2009; Houser and Esclapez, 1996; Morin et al., 1998; Obenaus et al., 1993). Although not confirmed by GABAergic markers, some of the neurons described in this study have somatic profiles that could be consistent with inhibitory neurons. While it is possible that morphology may be affected when a neuron undergoes degeneration, and further studies that directly confirm inhibitory interneuron loss are necessary, this study identifies potential regions that may be important in assessment for loss of GABAergic interneurons. Other indirect effects of degenerating neurons include altered receptor-ion channel function, upregulation of genes, axonal sprouting, loss of excitatory inputs to interneurons, and neurogenesis, all of which could affect regional excitability.

Network mechanisms of epileptogenesis resulting from degeneration of neurons

The observation that multiple brain regions have neuronal degeneration after status epilepticus suggests that the mechanisms of epileptogenesis may be distributed across these areas. As described in several reviews (Bertram, 2009; Laufs, 2012; Spencer, 2002), TLE may be a network phenomenon requiring the activity of many structures to manifest the features of a clinical seizure. One of the earliest descriptions of network activity in TLE identified the hippocampus, amygdala, entorhinal cortex, lateral temporal neocortex, medial thalamus, inferior frontal cortex as essential structures involved in this process (Spencer, 2002). All of these regions show neurodegeneration in the present study. Most of them have direct and often reciprocal connections to each other, where perturbations in one region as a result of neuronal loss will likely affect the excitability of other connected areas. Thus, there is an anatomic and functional substrate for altered excitability between these regions that could allow for localized seizure activity in the temporal lobe even when hippocampal neuronal degeneration is modest or inconsistently observed.

Conclusions

Our results demonstrate that neuronal degeneration occurs in several areas outside the hippocampus, any of which may contribute to TLE. The regions and pattern of degeneration show striking similarity to analogous regions damaged in human pathological specimens of TLE patients. These experiments serve as a foundation to explore further the relationship between neuronal death in these areas and the development of TLE.

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Abbreviations

AA	anterior amygdaloid area
ACH	anterior hypothalamic area, central
ACo	anterior cortical nucleus
Acb	acumbens
AcbSh	acumbens shell
AD	anterodorsal nucleus
AHC	anterior hypothalamic area
AI	agranular insular cortex
AM	anteromedial
AO	anterior olfactory nucleus
APir	amygdalopiriform transition area
AStr	amygdalostratial transition area
AV	anteroventral nucleus
BAOT	bed nucleus accessory olfactory tract
BLA	basolateral nucleus, anterior
BLP	basolateral nucleus, posterior
BLV	basolateral nucleus, ventral
BMA	basomedial nucleus, anterior
BMP	basomedial nucleus, posterior
BSTIA	bed nucleus stria terminalis, intraamygdaloid division
BSTM	bed stria terminalis nuclei
CA	cornu ammonis
CeL	central nucleus, lateral
CeM	central nucleus, medial
Cg1-3	anterior cingulate
CL	centrolateral nucleus
CM	centromedial nucleus
CPu	caudate putamen
DEn	dorsal endopiriform nucleus
DG	dentate gyrus
DI	dysgranular insular cortex
DLG	dorsolateral geniculate nucleus

DP	dorsal peduncular
EEG	electroencephalogram
Ent	entorhinal cortex
Fr1-3	frontal cortex
GABA	γ -aminobutyric acid
GI	granular insular cortex
GP	globus pallidus
HC	hippocampus
Hil	hilus
I	intercalated masses
IL	infralimbic
LaD	lateral nucleus, dorsal
LaV	lateral nucleus, ventral
LDDM	laterodorsal nucleus, dorsomedial
LDVL	laterodorsal nucleus, ventrolateral
LHb	lateral habenula
LM	lateral mammillary
LO	lateral orbital cortex
LOT	nucleus lateral olfactory tract
LPLR	lateroposterior nucleus, lateral rostral
LPMR	lateroposterior nucleus, medial rostral
LSD	lateral septal, dorsal
LSI	lateral septal, intermediate
LSV	lateral septal, ventral
MD	mediodorsal nucleus
MeA	medial nucleus, anterior
MePD	medial nucleus, posterodorsal
MePV	medial nucleus, posteroventral
MGD	medial geniculate nucleus, dorsal
MGM	medial geniculate nucleus, medial
MGP	medial globus pallidus
MGV	medial geniculate nucleus, ventral
MHb	medial habenula
MO	medial orbital cortex
MS	medial septal
MTu	medial tuberal

NAc	nucleus accumbens
Oc2L	occipital cortex
P	post-natal day
Par1	parietal cortex
PaS	parasubiculum
PC	paracentral
PF	parafascicular
Pir	piriform cortex
PLCo	posterolateral cortical nucleus
PMCo	posteromedial cortical nucleus
PMD	premmammillary nucleus, dorsal
PMV	premmammillary nucleus, ventral
Po	posterior nucleus
PRh	perirhinal cortex
PrS	presubiculum
PT	paratenial
PVA	paraventricular nucleus, anterior
PVP	paraventricular nucleus, posterior
Re	reuniens nucleus
Rh	rhomboid nucleus
RSA	retrosplenial agranular cortex
RSG	retrosplenial granular cortex
Rt	reticular nucleus
SG	supragenulate nucleus
SI	substantia innominate
SNR	substantia nigra pars reticulate
STh	subthalamic nucleus
S	subiculum
Te1,3	temporal cortex
TLE	temporal lobe epilepsy
VL	ventrolateral nucleus
VLG	ventrolateral geniculate nucleus
VLO	ventrolateral orbital cortex
VM	ventromedial nucleus
VP	ventral pallidum
VPL	ventral posterolateral nucleus

VPM	ventral posteromedial nucleus
vRe	ventral reuniens nucleus
VTR	ventral tegmental area
ZI	zona incerta

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Highlights

1. Experimental status epilepticus in 20-day-old rats causes extrahippocampal damage
2. Prominent neuronal degeneration occurs in thalamus, amygdala, and paralimbic cortices
3. The neuronal damage is highly variable across brain areas and individual animals

Figure 1.

EEG recording from P20 rat during status epilepticus. Arrows indicate beginning of expanded time scale for tracings prior to seizure activity (A) and at initiation of status epilepticus (B). C represents expanded time scale for beginning of region B.

Figure 2.

Fluoro-jade B staining in amygdala, piriform cortex, and adjacent regions. Coronal sections at approximate bregma level -1.40 (A), -2.12 (B), -3.14 (C), and -4.80 (D). Note the intense staining in posterior medial nucleus (MeP), intercalated cells (I), dorsal endopiriform (DEn), posteromedial cortical nucleus (PMCo), and the bed nucleus of the accessory olfactory tract (BAOT) as opposed to the much reduced staining of the basolateral nucleus (BLA), lateral olfactory nucleus (LOT2), and anterior cortical nucleus (ACo). (E) Detail of (putative interneuron) cells in the medial intercalated mass. (F) Multipolar pyramidal cells from layer III of the piriform cortex. AA, anterior amygdaloid area; ACo, anterior cortical nucleus; APir, amygdalopiriform transition area; AStr, amygdalostratial transition area;

BAOT, bed nucleus accessory olfactory tract; BLA, basolateral nucleus, anterior; BLP, basolateral nucleus, posterior; BLV, basolateral nucleus, ventral; BMA, basomedial nucleus, anterior; BSTIA, bed nucleus stria terminalis, intraamygdaloid division; CeL, central nucleus, lateral; CeM, central nucleus, medial; CPu, caudate putamen; DEn, dorsal endopiriform nucleus; GP, globus pallidus; I, intercalated masses; La, D/V, lateral nucleus, dorsal/ventral; LOT, nucleus lateral olfactory tract; MeA, medial nucleus, anterior; MeP, D/V, medial nucleus, posterodorsal/posteroventral; Pir, piriform cortex; PLCo, posterolateral cortical nucleus; PMCo, posteromedial cortical nucleus.

Figure 3.

Fluoro-jade B staining in other limbic regions. (A) Horizontal section through ventral hippocampus (HC), approximately bregma -6.6 . (B) Horizontal section through entorhinal cortex (Ent) and parasubiculum (PaS), approximately bregma -6.6 . (C) Perirhinal cortex (PRh) at approximately bregma -6.04 . Staining is evident in layers II/III, V, and VI. (D) Absence of labeling in Acb (considered part of basal ganglia) but robust labeling in the AcbSh (limbic system). Coronal section, approximately 0.70 Bregma.

Figure 4. Mild-moderate cell injury in the neocortex. (A) Retrosplenial granular cortex (RSG) and retrosplenial agranular cortex (RSA), approximately bregma -6.3. (B) Parietal cortex 1 (Par1), showing labeling in layers II/III and V; approximately bregma -2.3. Both sections coronal.

Figure 5.

Fluoro-jade B staining in thalamus. Coronal sections at approximate bregma level -2.12 (A), -2.56 (B), -3.60 (C), and -5.80 (D). Note the intense staining in medial dorsal nuclei (MD) and midline reuniens (Re). Other nuclei also show evidence of degeneration, but this is more variable. Little to no damage is observed in interlaminar (CL, CM) nuclei. (E) Detail of MD cells showing fusiform morphology. AD, anterodorsal nucleus; AV, anteroventral nucleus; CL, centrolateral nucleus; CM, centromedial nucleus; DLG, dorsolateral geniculate nucleus; LDDM, laterodorsal nucleus, dorsomedial; LDVL, laterodorsal nucleus, ventrolateral; LHb, lateral habenula; LPMR, lateroposterior nucleus, mediorostral; MD, mediodorsal nucleus; MGD, medial geniculate nucleus, dorsal; MGM, medial geniculate nucleus, medial; MGv,

medial geniculate nucleus, ventral; MHb, medial habenula; Po, posterior nucleus; PVA, paraventricular nucleus, anterior; PVP, paraventricular nucleus, posterior; Re, reuniens nucleus; Rh, rhomboid nucleus; Rt, reticular nucleus; SG, supragenulate nucleus; VL, ventrolateral nucleus; VLG, ventrolateral geniculate nucleus; VM, ventromedial nucleus; VPL, ventral posterolateral nucleus; VPM, ventral posteromedial nucleus; vRe, ventral reuniens nucleus; ZI, zona incerta.

Figure 6.

Fluoro-jade B staining in hypothalamus. (A) Little to no staining is observed except in ventral preammillary nucleus (PMV) and dorsal preammillary nucleus (PMD). (B) Neurosecretory cell in PMV. Sections are coronal, approximately bregma -3.8 .

Severity of damage at 72 h following Li-pilocarpine status epilepticus. V denotes regions with inter-animal variability that differed by at least 2 score point values.

Table 1

Region	Subregion	Abbr.	% Injured	score
<i>Amygdala</i>				
<i>Deep nuclei</i>	lateral, dorsal	LaD	100	***V
	lateral, ventral	LaV	100	*****
	basolateral, anterior	BLA	100	***
	basolateral, posterior	BLP	100	***
	basolateral, ventral	BLV	100	**
	basomedial, anterior	BMA	82	***V
<i>Superficial nuclei</i>	basomedial, posterior	BMP	100	****
	nucleus lateral olfactory tract	LOT	100	**
	bed nucleus of the accessory olfactory tract	BAOT	100	*****
	anterior cortical	ACo	100	***
	medial, anterior and posterior	MeA, MeP	100	*****
	posterolateral cortical	PLCo	100	****
<i>Other amygdaloid nuclei</i>	posteromedial cortical	PMCo	91	***V
	amygdalopiriform transition area	APir	100	***
	anterior amygdala area	AA	100	****
	central, lateral	CeL	73	*
	central, medial	CeM	100	****
	amygdalostratial transition area	AStr	100	*****
Other limbic regions	intercalated masses	I	100	*****
	CA1	CA1	82	***V
	CA2	CA2	82	***V
	CA3	CA3	64	***V
	dentate gyrus	DG	73	***V
	hilus	Hil	64	**V
<i>ventral HC</i>	CA1	CA1	100	*****

Region	Subregion	Abbr.	% Injured	score
<i>Extra-HC limbic</i>	CA2	CA2	100	*****
	CA3	CA3	100	*****V
	dentate gyrus	DG	64	***V
	hilus	Hil	55	**V
	subiculum	S	82	***V
	parasubiculum	PaS	100	*****
	presubiculum	PrS	54	*
	lateral septal, dorsal and intermediate	LSD, LSI	100	*****
	lateral septal, ventral	LSV	100	*****
	medial septal	MS	36	*
	dorsal endopiriform	DEn	100	*****
	perirhinal cortex	PRh	100	*****
	entorhinal cortex	Ent	100	*****
	piriform cortex	Pir	100	***
	infralimbic	IL	100	*****
	bed nucleus stria terminalis, interamygdala	BSTIA	100	*****
Hypothalamus	bed nucleus stria terminalis, major nucleus	BSTM	100	*****
	accumbens shell	AcbSh	100	*****
	substantia innominata (ventral pallidum)	SI	0	0
	habenula, lateral and medial	LHb, MHb	0	0
	lateral mammillary	LM	18	*
	premamillary, ventral	PMV	100	*****
Thalamus	premamillary, dorsal	PMD	100	***
	anterior hypothalamic area, central	AHC	100	*****
	medial tuberal	MTu	100	*****
	mediodorsal	MD	100	*****
	paratenial	PT	100	*****
	reuniens	Re	100	*****
<i>medial and midline</i>			73	**

Region	Subregion	Abbr.	% Injured	score
<i>Intralaminar</i>	centrolateral	CL	73	**V
	central medial	CM	73	**
	paracentral	PC	64	**
	parafascicular	PF	100	**
	paraventricular	PV	82	**
<i>Caudal</i>	posterior	Po	100	***
	dorsal lateral geniculate	DLG	91	*****V
	ventral lateral geniculate	VLG	0	0
	medial geniculate, dorsal	MGD	100	*****
	medial geniculate, ventral	MGV	82	***V
<i>Anterior</i>	subgeniculate	SG	100	*****
	anterodorsal, anteroventral	AD, AV	0	0
	anteromedial	AM	45	**V
	laterodorsal, dorsal medial	LDDM	100	*****
	laterodorsal, ventral lateral	LDVL	100	*****
<i>Ventral</i>	lateral posterior, medial and lateral rostral	LPMR, LPLR	100	*****
	ventrolateral	VL	100	*****V
	ventromedial	VM	100	*****V
	ventral posterolateral	VPL	100	***
	ventral posteromedial	VPM	100	**
Basal Ganglia	reticular	Rt	73	**
	caudate putamen	CPu	100	****
	globus pallidus	GP	0	0
	substantia nigra pars reticulata	SNR	0	0
	ventral tegmental area	VTA	0	0
Other Neocortex	ventral pallidum	VP	100	**
	accumbens	Acb	9	*
	anterior cingulate	Cg1-3	91	*****V
	agangular insular	AI	82	***V

Region	Subregion	Abbr.	% Injured	score
	dysgranular insular	DI	73	***V
	granular insular	GI	73	***V
	anterior olfactory nucleus	AO_	82	***V
	dorsal peduncular	DP	100	*****
	lateral orbital	LO	100	*****
	ventrolateral orbital	VLO	100	*****
	medial orbital	MO	100	*****
	parietal	Par1-2	91	***
	frontal	Fr1-3	91	**V
	occipital	Oc2L	82	***V
	temporal	Tel, 3	91	***V
	retrosplenial, granular and agranular	RSA, RSG	100	***V

Table 2

Summary of previous studies that assessed for neuronal damage outside of hippocampal formation in immature rat using lithium pilocarpine model of SE

Reference	Age Sex & Strain	Pilo Dose & Other Parameters	Injury Assessment	Affected Regions Identified
Cavalheiro et al., 1987	P3-P90 Male & Female Wistar	100–380 mg/kg No LiCl Electrode implantation pre SE	Examination of Cresyl violet sections assessed 1–3 d post SE	P3-P10 No discernible damage P11-P21 Thalamus, Olfactory Cortex, Septum, Hypothalamus, Amygdala, & Neocortex. P24-90 adult pattern
Hirsch et al., 1992	P7-P30 Male & Female Sprague-Dawley	3–60 mg/kg Electrode implantation pre SE	Examination of hematoxylin-eosin sections assessed 3 d post SE	No discernible damage
Priel et al., 1996	P7-P120 Male Wistar	170–380 mg/kg No LiCl	Cell counting of Nissl stained sections assessed 120 d post SE	P7-P11 No discernible damage P12-P17 Medial Thalamus, Olfactory Cortex, Septum, Hypothalamus, Amygdala, & neocortex. P18-24 Previous structures as well as Lateral Thalamus & Substantia Nigra P25-120 adult pattern
Sankar et al., 1997	P7– P28 Male & Female Wistar	60 mg/kg	Serum specific enolase Hematoxylin-eosin staining and eosin fluorescence assessed 24 h post SE	P7 No discernible damage P14 Thalamus, Amygdala, & Septum P21-P28 Thalamus, Amygdala, Septum, Cortex, & Caudate
Sankar et al., 1998	P14-P28 Male & Female Wistar	60 mg/kg Paraldehyde 0.3 ml/kg @ 2h post SE	Hematoxylin-eosin staining & eosin fluorescence assessed 24 h post SE	Neocortex, Caudate, Septum, Amygdala, & Thalamus
Kubova et al., 2001	P12 Male Wistar	40 mg/kg Paraldehyde 0.3 ml/kg @ 2h post SE	Silver impregnation & Fluoro-Jade B assessed 12 h to 1 wk post SE	Thalamus
Dube et al., 2001	P10 & P21 Male Sprague-Dawley	P10 60 mg/kg P21 30 mg/kg Electrode implantation 50 days post SE	Cell counting Cresyl violet sections assessed 6 mo post SE	P10 No discernible damage P21 Amygdala, Thalamus, Entorhinal Cortex, Primary Olfactory Cortex, & Piriform Cortex
Roch et al., 2002	P21 Male & Female Sprague-Dawley	30 mg/kg Diazepam 2 mg/kg @ 2 h post SE	MRI T2 signal changes assessed serially 6 h to 4 mo post SE	Piriform & Entorhinal Cortex
Mares et al., 2005	P12 & P25 Male Wistar	40 mg/kg Paraldehyde 0.3 ml/kg @ 2h post SE	Fluoro-Jade B assessed 24 h post SE	P12 Sensorimotor Cortex P25 Motor, Primary and Secondary Somatosensory Cortex
Nairismagi et al., 2006	P12 Male Wistar	40 mg/kg Paraldehyde 0.3 ml/kg @ 2h post SE	Silver impregnation & Fluoro-Jade B assessed 8 h post SE Volume reduction assessed 16 wk post SE	Amygdala & Perirhinal Cortex