

IS NEURONAL DEATH NECESSARY FOR ACQUIRED EPILEPTOGENESIS IN THE IMMATURE BRAIN?

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A central question concerning acquired epileptogenesis in the immature brain is whether neuronal death is required for the development of epilepsy after a brain insult. Results from three different animal models of brain injury during early development have been used to develop the hypothesis that status epilepticus, prolonged febrile seizures, or hypoxia-induced seizures can lead to chronic epilepsy without the occurrence of neuronal death. This brief review will summarize the evidence supporting the hypothesis in each model and then critique the data and published interpretations. A case will be made that the evidence to date neither rules out the occurrence of neuronal death nor demonstrates that epileptogenesis (i.e., spontaneous recurrent seizures) has actually occurred in these animal models of acquired pediatric epilepsy. We also review evidence for the opposing hypothesis: acquired epileptogenesis in the immature brain requires, or at least often involves, neuronal death.

One of the most fundamental questions in epilepsy research is whether neuronal death is a prerequisite for the development of acquired epilepsy after a brain injury. The present review focuses on this issue in the immature brain, although the same question needs to be answered for the adult brain. An extensive body of animal model-based experimental data has been used to support the hypothesis that *neuronal death in the immature brain is not necessary for epileptogenesis* (1–5). A corollary to

this hypothesis is that a neuronal insult (e.g., repetitive seizures) and subsequent recovery of the neurons from the insult is sufficient for the development of acquired epileptogenesis—even if none of the neurons subjected to the insult undergo cell death (1,2,4,5). An opposing hypothesis is that *neuronal death is a necessary step in acquired epileptogenesis*, in both the mature and the developing brain. Data supporting this latter hypothesis would suggest that subtle neuronal death was not detected and/or chronic epilepsy did not occur in those studies reporting acquired epileptogenesis without neuronal death after an insult to the immature brain. Published data concerning these two different views (or opposing hypotheses) will be assessed in different animal models of acquired pediatric epilepsy.

Status Epilepticus

One line of research suggesting that epileptogenesis does not require neuronal death involves the induction of status epilepticus with lithium pilocarpine at postnatal day 20 (P20) (4). At this age, of the treated animals, roughly one-half develop epilepsy and the other half do not. In this study by Raol et al., several lithium pilocarpine-treated animals showed spontaneous recurrent seizures without apparent hippocampal neuron loss, based on neuronal counts in histological sections. Their results led to the conclusion that neuronal death in the hippocampus after status epilepticus at P20 was not necessary for subsequent epileptogenesis. However, it can be difficult to *detect modest neuronal loss* with Nissl or other histopathological staining techniques and traditional neuronal-counting methods; these approaches can be variable under the best of conditions. The study by Raol et al. also did not include positive controls to show that neuronal loss could be demonstrated quantitatively in adult animals with the same methods. One potential cause of a false-negative result (in this instance, an inability to detect neuronal loss) arises from problems associated with relatively small-scale, nonstereological neuronal-counting techniques. When neuronal loss is patchy, as it often is, variance in cell counts can lead to a failure to detect neuronal loss. In addition, neuronal loss could have occurred in any of many different brain structures other than the hippocampus (6–8) and could be variable from animal to animal, thus neuronal loss may not have been detected because the wrong structure or part of a structure was analyzed. The study by Raol et al. examined *only* the dorsal hippocampus, an area that is less prone to neuronal loss after status epilepticus, even in adults (9,10). Therefore, neuronal death may have occurred in other areas more likely to show detectable neuronal loss after status epilepticus, such as

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the ventral hippocampus (9,11), thalamus (12,13), amygdala (14), and perirhinal cortex (14).

Furthermore, other studies, including work using histochemical markers of degenerating neurons in the same model used by Raol et al., have found strong evidence for the presence of neurons that appear to be in the process of degeneration (14–17). Histochemical markers are much more sensitive to detecting subtle neuronal loss than are neuronal counts in histological sections, because they are able to label a relatively small proportion of neurons that are destined to die. The small fraction of dying neurons might be below the inherent variability observed in comparisons between groups of experimental and control animals. Finally, the study by Raol et al. did not examine the possibility of loss of vulnerable GABAergic interneurons, which is known to occur in the pilocarpine (18) and kainate models (9) and would require different and more specialized techniques than the general counting of unidentified neurons. Therefore, although the evidence seems solid in the Raol et al. study that some of the animals developed epilepsy (i.e., chronic spontaneous recurrent seizures), the evidence that no neuronal death occurred is equivocal for several reasons discussed here. Future studies on this issue should address the possibility that a failure to find neuronal loss using cell-counting methods is potentially a false-negative result, and thus the approach offers only weak evidence that neuronal loss did not occur in animals that later developed epilepsy after an insult to the immature brain.

Complex Febrile Seizures

Retrospective epidemiological studies suggest that children who have experienced prolonged febrile seizures are at risk for the development of temporal lobe epilepsy later in life (19). Thus, several studies have used induction of hyperthermia via a heated airstream as an animal model of prolonged (i.e., ≥ 30 minutes) or complex febrile seizures (2,20). With the goal of making this an age appropriate animal model of complex febrile seizures during childhood, animals in these studies were subjected to hyperthermia at P10–12. Initial studies with this model reported hyperexcitability in brain slices and an increase in seizure susceptibility (measured as more robust responses to kainate injections), but found no electrographic evidence for seizures, even with prolonged EEG recording (21). Threshold for seizure generation to kainate injection—a potential surrogate marker for epileptogenesis in these studies—is an indirect measure of chronic epilepsy and does not meet the ultimate requirement of spontaneous recurrent seizures known to occur with temporal lobe epilepsy (22). A subsequent study reported evidence for hippocampal seizures (23), but these electrographic events were quite brief (i.e., only a few seconds, with a maximum duration of 10–15 seconds) and had properties more like theta rhythm

(24) than hippocampal seizures (25). The preponderance of published EEG data using this model so far raises concerns that indirect measurements of hyperexcitability and equivocal examples of spontaneous seizure activity provide only weak evidence that these animals have developed epilepsy after prolonged periods of hyperthermia at P10–12. Thus, these data could represent a false-positive result, in which epileptogenesis is inferred without demonstrating bone fide spontaneous recurrent seizures.

Furthermore, in this model, no evidence of hippocampal neuronal loss (using similar Nissl-stain counting methods) was found, which is similar to studies using pilocarpine-induced status epilepticus at P20 (4). Many of the issues concerning the histopathology and cell counting previously discussed with the induction of status epilepticus (4) apply to these studies (26,27) as well and raise concerns of false-negative results in regard to acquired epileptogenesis without neuronal loss after prolonged hyperthermia at P10–12. Furthermore, because apoptosis is a prominent feature of normal brain development (28,29), the issue of ensuring that neuronal death has not occurred after an insult to the immature brain is more complicated than with an adult brain (i.e., repetitive seizures may affect which neurons undergo apoptosis). In summary, the available data from this hyperthermic seizures model fail to prove that neuronal loss in the immature brain is unnecessary for the development of chronic epilepsy, because: 1) the seizure data suggesting that the animals develop epilepsy could be a false-positive result and 2) the histopathological evidence that neuronal loss did not occur could be a false-negative result. The false-positive conclusions could have arisen from: 1) problems associated with using seizure threshold to kainate injection as a surrogate marker for epilepsy and 2) misidentification of brief rhythmic electrographic events, which appear to be theta rhythm, as being seizures. Finally, exogenously induced hyperthermia, as used in these studies, may be a better model of heatstroke than fever (30).

Hypoxia–Ischemia

Hypoxic–ischemic brain insults are a prominent cause of neonatal morbidity and mortality, often leading to developmental disabilities, cerebral palsy, and intractable epilepsy. Animal models involving hypoxia at P7–10 have been used to study both acute neonatal seizures and the subsequent development of epilepsy. Electrophysiological and behavioral studies with models of neonatal hypoxia have reported hyperexcitability in brain slices and increased sensitivity to fluorothyl-induced seizures, occurring days to weeks after the hypoxia-induced seizures (31). A recent study reported the occurrence of spontaneous recurrent seizures as long-term sequelae (i.e., epilepsy) to hypoxia-induced seizures at P10; however, the reported electrographic

events were relatively brief (1–5 seconds) and thus may not be actual seizures (32). Again, neuronal loss was not observed, using Nissl cell-counting techniques (31). Therefore, the same problems concerning the histopathology, outlined in the previous sections for pilocarpine-induced status epilepticus at P20 and prolonged hyperthermic seizures at P10–12, also apply to this model. The failure to observe neuronal death after hypoxia-induced seizures at P10 could be a false-negative result, and the evidence that epileptogenesis has actually occurred may represent a false positive.

Recent studies using a hypoxia–ischemia insult, in which unilateral carotid occlusion was combined with hypoxia at P7, explore further the question of whether neuronal death (or lack of it) in the immature brain is associated with the subsequent development of epilepsy. In one particular animal model of perinatal stroke, roughly one-half of the experimental animals subjected to the hypoxia–ischemia treatment show a macroscopic infarct involving the neocortex and underlying structures and about half do not exhibit any obvious signs of an infarct or neuronal loss (33). When these animals were studied with nearly continuous radio-telemetric recordings of the EEG, over approximately 5 months (i.e., EEG recordings were obtained for 97% of the experimental period), all of the animals (i.e., 10 of 10 animals) with an infarct showed progressive epilepsy that persisted for at least 6–12 months (34). Furthermore, *no spontaneous recurrent seizures were recorded* in any of the animals subjected to the hypoxia–ischemia insult *without a detectable infarct* (i.e., eight of eight animals). These data, using a traditional hypoxia–ischemia model of perinatal stroke and associated encephalopathy, support the hypothesis that neuronal death is essential for the development of acquired epilepsy after perinatal brain insults (34). It is important to note that this conclusion also rests on the failure to observe a particular, critical result—in this case, seizures in animals with no infarction. However, the prolonged, continuous monitoring in this study provided sufficient statistical power to conclude that seizures were, indeed, not present in those animals lacking an infarct; thus, a false-negative result arising from undetected seizures appears highly unlikely. These data, therefore, represent evidence against the hypothesis that acquired epilepsy occurs without neuronal death after an insult to the immature brain, and they support (but do not prove) the opposing hypothesis that neuronal death after an insult to the immature brain is required for development of acquired epilepsy.

The Rationale for the Alternative Hypothesis

It has long been known from studies with kainate- and pilocarpine-induced status epilepticus in immature rats that when status epilepticus is induced in progressively younger animals, less neuronal death is observed in the hippocampus and

other limbic structures. Furthermore, these younger animals are also less likely to develop epilepsy, manifested as the subsequent development of spontaneous recurrent seizures (15,35–38). Such work on models of status epilepticus in immature animals provides the basis for an alternative explanation (i.e., for the data currently reported in support of the hypothesis that epileptogenesis, after an injury to the immature brain, does not require neuronal death). Potential lines of reasoning for an alternative hypothesis include the facts that: 1) younger animals (or humans) have a lower threshold for seizure generation, 2) repetitive seizures, however, are less likely to cause neuronal death in younger animals than adults, and therefore, 3) chronic epilepsy is less likely to develop after repetitive seizures in younger animals, because younger animals (and humans) are more resistant to seizure-induced neuronal death (3,39–41). Thus, in this hypothesis, when repetitive seizures are particularly severe and/or are coupled with fever, ischemia, or other circumstances that cause neuronal death, then subsequent epileptogenesis may occur; however, if an insult to the immature brain does not cause neuronal death, subsequent epileptogenesis, with spontaneous recurrent seizures, does not occur. This interpretation of the data leads to the general hypothesis that insults to the immature brain do not lead to epilepsy unless neuronal death (either glutamatergic principal neurons or GABAergic interneurons) has occurred.

Conclusion

The aim of this review is not to demonstrate that acquired epilepsy after an insult to the immature brain requires neuronal death (Table 1). Rather, it seeks to provide constructive criticism and generate an alternative interpretation to published experimental data concerning the basis for acquired pediatric epilepsy. Indeed, the goal is to raise scientific doubt about published interpretations of those data and advance re-analysis. As already outlined, a false-negative result concerning a lack of neuronal death can occur in numerous ways, even in well-designed studies, which therefore emphasizes the need to exercise caution regarding any interpretation of the failure to detect neuronal death. Similarly, a false-positive result for the presence of epileptogenesis can readily occur in studies of animal models of acquired epilepsy, particularly when the reported seizures are equivocal, surrogate markers of changes in seizure threshold are designated, and/or hyperexcitability is used. Future research on the issue of acquired epilepsy in the immature brain in animal models should provide clear electrographic and behavioral evidence for spontaneous recurrent seizures as the final common denominator of epilepsy. Thus, this review encourages additional research concerning the hypothesis that loss of neurons (i.e., frank neuronal death) is unnecessary for epileptogenesis after an insult to the immature brain.

TABLE 1. Summary of Pro and Con Evidence for Epilepsy without Neuronal Death in the Immature Brain

	STATUS EPILEPTICUS AT P20	HYPERTHERMIC CONVULSIONS FOR 30 MIN AT P10	HYPOXIA AT P10	HYPOXIA– ISCHEMIA AT P7
Neuronal death/loss?	Yes (other labs)	Minimal to none, but false-negative findings possible	Minimal to none, but false-negative findings possible	Half with infarct; half without
Spontaneous recurrent seizures?	Yes, in some animals	No, reported seizures were probably theta rhythm	No documented seizures	Animals with infarct had seizures
Reference	Raol et al. (4)	Dube et al. (20, 23); Toth et al. (26); Bender et al. (27)	Rakhade et al. (32)	Kadam et al. (34)

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