



Mortality with brainstem seizures from focal 4-aminopyridine–induced recurrent hippocampal seizures

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SUMMARY

Objective: Sudden unexplained death in epilepsy is the leading cause of death in young adult epilepsy patients, typically occurring during the early postictal period, presumably resulting from brainstem and cardiorespiratory dysfunction. We hypothesized that ictal discharges in the brainstem disrupt the cardiorespiratory network, causing mortality. To study this hypothesis, we chose an animal model comprising focal unilateral hippocampal injection of 4-aminopyridine (4-AP), which produced focal recurrent hippocampal seizures with secondary generalization in awake, behaving rats.

Methods: We studied ictal and interictal intracranial electrographic activity (iEEG) in 23 rats implanted with a custom electrode array into the hippocampus, the contralateral cortex, and brainstem. The hippocampal electrodes contained a cannula to administer the potassium channel blocker and convulsant (4-AP). iEEG was recorded continuously before, during, and after seizures induced by 4-AP infusion into the hippocampus.

Results: The control group ($n = 5$) was monitored for 2–3 months, and the weekly baseline iEEG recordings showed long-term stability. The low-dose group (1 μL 4-AP, 40 mM, $n = 5$) exhibited local electrographic seizures without spread to the contralateral cerebral cortex or brainstem. The high-dose group (5 μL 4-AP, 40 mM, $n = 3$) had several hippocampal electrographic seizures, which spread contralaterally and triggered brainstem discharges within 40 min, and were associated with violent motor seizures followed by dyspnea and respiratory arrest, with cortical and hippocampal iEEG flattening. The group that received high-dose 4-AP without brainstem implantation ($n = 5$) had similar seizure-related respiratory difficulties. Finally, five rats that received high-dose 4-AP without EEG recording also developed violent motor seizures with postictal respiratory arrest. Following visualized respiratory arrest in groups III, IV, and V, manual respiratory resuscitation was successful in five of 13 animals.

Significance: These studies show that hippocampal seizure activity can spread or trigger brainstem epileptiform discharges that may cause mortality, possibly mediated by respiratory network dysfunction.

KEY WORDS: Intrahippocampal 4-AP, Freely behaving rats, Recurrent generalized seizures, Brainstem seizures, Mortality.



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KEY POINTS

- In vivo focal seizures were acutely generated by intrahippocampal 4-AP in behaving rats
- With prolonged intrahippocampal seizures (~150 s) and contralateral neocortical generalization of these seizures, brainstem seizures were also recorded
- Prolonged brainstem seizures (>30 s) were associated with visualized dyspnea and respiratory arrest followed by mortality

Sudden unexpected death in epilepsy (SUDEP) is the leading cause of death in young adults with treatment-resistant epilepsy.¹ Risk factors include recent generalized tonic-clonic seizures (GTCSs), nonadherence with anti-seizure medication, subtherapeutic antiseizure drug levels, intellectual disability, nocturnal seizures, and lack of supervision.^{2–5} Unfortunately, the mechanisms of SUDEP are not well understood. When recorded in an epilepsy monitoring unit, SUDEP typically follows an early postictal, centrally mediated, severe alteration of respiratory and cardiac function following a GTCS. However, the sequence of events, variability of mechanisms and factors across patients, and precise neural mechanisms remain poorly defined.^{2,5,6}

Video-electroencephalographic (vEEG) and electrocardiographic (EKG) recordings of patients in epilepsy monitoring units who died from SUDEP provide important insights into pathophysiology. SUDEP typically occurs after a focal onset or generalized onset GTCS, with combinations of respiratory dysfunction, arousal failure, nontachyarrhythmic cardiac dysfunction, and postictal generalized EEG suppression. Notably, all 11 SUDEPs recorded on vEEG followed a GTCS.^{2,7–10}

Due to the difficulty of recording brainstem neuronal activity in freely behaving animals or in humans, there is little evidence that generalized seizures spread to the brainstem. Ictal loss of consciousness may involve seizure spread into the bilateral thalamus and upper brainstem areas,^{11,12} but whether this spread could also cause the mortality events associated with SUDEP is unknown. Brainstem nuclei are likely to be affected by seizures and related hypoxia because both seizures and hypoxia induce Fos immunoreactivity in neurons with similar anatomical distributions to the nucleus tractus solitarius, the dorsal motor nucleus of the vagus, and the ventrolateral medulla.¹³ Brainstem serotonergic raphe neurons maintain breathing (medulla) and arousal (mid-brain), and in animal models, brainstem serotonergic activity is impaired after seizures.¹⁴ The selective serotonin reuptake inhibitor, fluoxetine, reduced the incidence of ictal respiratory arrest during audiogenic seizures in mice.¹⁵ Because the brainstem is hypothesized to generate barrel rotations¹⁶ and some types of generalized seizures,^{11,12} it is likely that the brainstem is affected by seizures, although

what physiological changes occur in the brainstem during and after a GTCS remain poorly defined.⁶

In this study, our primary objective was to create a relevant animal model of supratentorially (cerebrally) originating seizures to study the underlying possible brainstem involvement during and after seizure occurrence, hypothesizing that a potentially fatal outcome is associated with brainstem seizure activity.

METHODS

Terminology

Nonconvulsive and convulsive motor seizures were recorded behaviorally with video monitoring. The witnessed mortality was nontraumatic, with evidence of a prior brainstem seizure on intracranial EEG (iEEG) recordings. Some animals survived by successful respiratory resuscitation. Discharge describes any abnormal epileptiform electrographic activity (duration ≥ 10 s) associated with relatively high frequency and amplitude of spikes. The “nonconvulsive seizures” were defined according to the Racine scale (class 0: behavioral arrest [motionlessness], hair raising, excitement, and rapid breathing; class I: mouth movement of lips and tongue, vibrissae movements, and salivation or seizures), whereas the “convulsive seizures” were class II (head clonus and eye clonus), class III (forelimb clonus, “wet dog shakes”), class IV (clonic rearing), and class V (clonic rearing with loss of postural control and uncontrollable jumping).¹⁷

Animals

Fifty-five male Wistar rats (275–400 g) were used in these experiments. Of these, 32 rats were not used for the experiments for the following reasons: 25 rats died during brainstem implantation, two rats lost their electrode headcaps, and five rats did not show spontaneous seizures after 4-aminopyridine (4-AP) injection. With greater experience in brainstem implantation techniques and with smaller electrodes, the mortality greatly decreased. Twenty-three rats were randomly divided into five groups (Fig. 1A): (I) control (n = 5), (II) low-dose 4-AP (n = 5), (III) high-dose 4-AP with brainstem implantation (n = 5), (IV) high-dose 4-AP without brainstem implantation (n = 5), and (V) high-dose 4-AP without EEG recording. The control group was monitored for 2–3 months, and the weekly baseline EEGs were recorded to investigate the impact of implantation damage (especially in the brainstem) on the EEG and the long-term stability of the recording system. The other groups received an intrahippocampal dose of 4-AP (1–5 μ L at 40 mM) and were monitored for 3–4 h. Artificial ventilation was performed on rats demonstrating evident respiratory distress. Rat experiments were performed according to the protocols approved by the Animal Care Committee of the University Health Network (Toronto, Canada) according to the Canadian Guidelines for Animal Care.

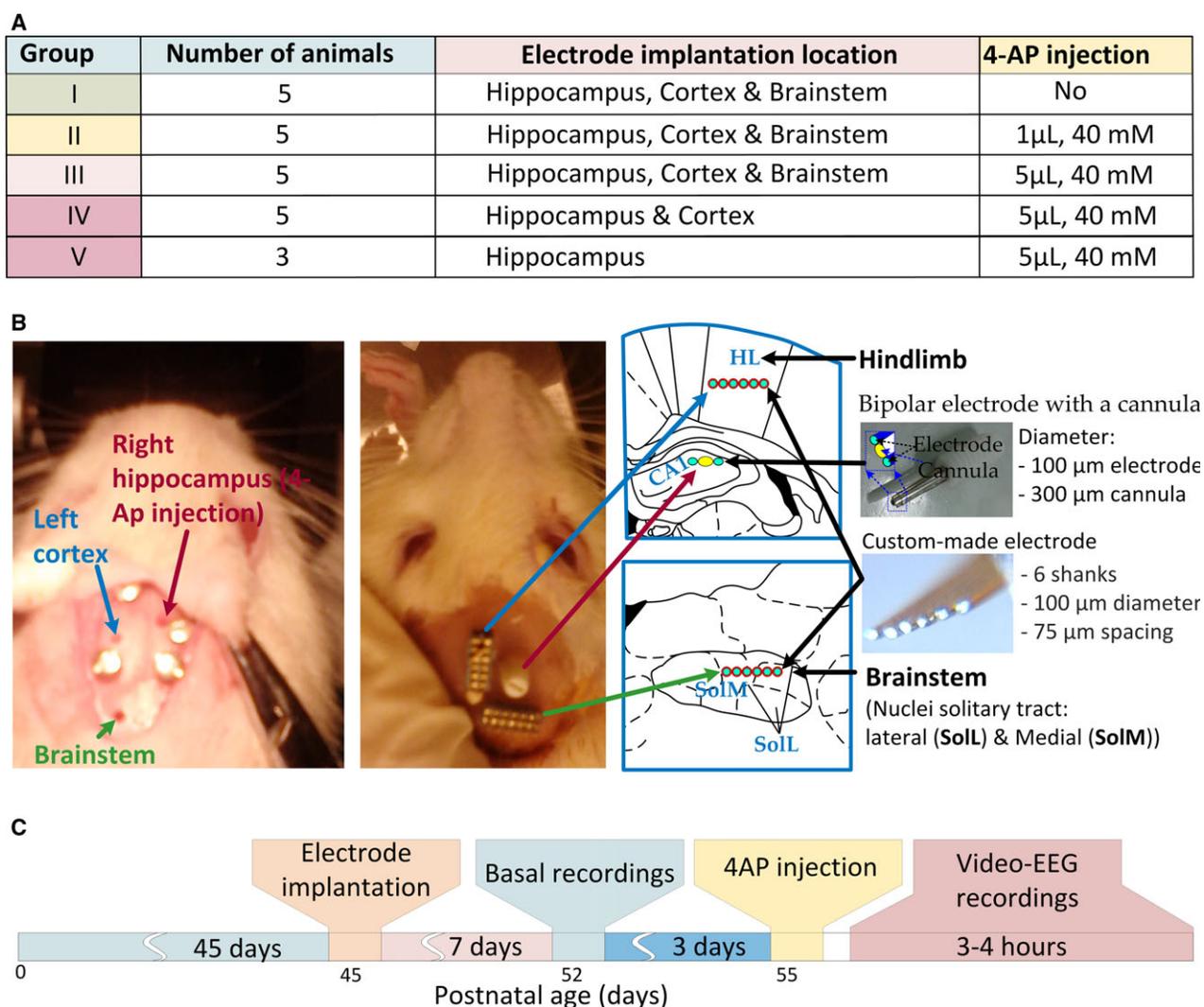


Figure 1. Methodology for this study. (A) Study groups, electrode implantation locations, and 4-aminopyridine (4-AP) injection doses. (B) Craniotomies, implanted electrode locations, and diameters. (C) Experimental timelines. EEG, electroencephalographic. *Epilepsia* © ILAE

Electrode implantation

Rats were anesthetized with isoflurane and oxygen, and placed into a stereotaxic frame for the electrode implantation. Electrodes were placed into three regions of interest using standard stereotaxic coordinates¹⁸: hippocampus, cortex, and nucleus of the solitary tract (lateral [SolL] or medial; contains respiratory nuclei).¹⁹ Figure 1A shows the location of electrodes for each group. A bipolar electrode with a microcannula (Plastics One, Roanoke, VA, U.S.A.) was implanted into the right hippocampus (Fig. 1B), using a stereotaxic micromanipulator with a steady forceps arms (Stoelting, Germany). On the other side, an assembly of six custom-made microwires (Plastics One) was implanted into the left cortex (hindlimb area). Another similar custom-made set of microwires was implanted into the right brainstem (nuclei solitary tract lateral and medial) using

stereotactic techniques. The electrodes were fixed to the skull using dental acrylic. Finally, the surface of the surgical site (i.e., skull, anchor screws, microwires) was covered and sealed with dental cement.

There were technical challenges to these experiments. The brainstem cardiorespiratory network is surrounded by major blood vessels, making electrode implantation hazardous. Our brainstem electrodes were custom-made depending on the animal's size. Our initial attempts at brainstem implantation using commercial electrodes failed due to their size and rigidity, which caused blood vessel damage and massive bleeding, leading to rat death within a few minutes. In this study, the number of electrodes, dimensions, and interelectrode spacing were optimized based on our brainstem implantation experience and by implementing a customized brainstem electrode.²⁰ In our experiment,

at least six contacts were required for the brainstem implantation due to human and stereotactic projection error. Among the six implanted contacts in the brainstem, on average 2–3 contacts reached the desired nuclei solitary tract location, confirmed by histological examination.

To avoid brainstem damage, the microwire (electrode) diameter was a major criterion in the design. The lower diameter electrode had several limitations: (1) inconsistent recording after a few weeks, (2) higher impedance, (3) degraded local field potential (LFP) recording, and (4) recording single units without the LFP. The optimal diameter was 100 μm for brainstem implantations, providing adequate LFP quality recording for 3 months.

The interelectrode spacing was vital for bipolar recordings from the SolL region. We found 75- μm spacing enabled EEG rhythm recording from the SolL region while reducing blood vessel damage and movement artifact. The conventional monopolar brainstem recording was often contaminated with movement artifact. Thus, all our recordings were bipolar, which subtracted all common signals in the two electrodes and reduced the movement artifact, especially in the brainstem recordings.

Seizure induction

A well-characterized rodent seizure model was used to reproduce some features of human temporal lobe epilepsy.²¹ 4-AP powder was dissolved in 0.9% saline to make a 40-mM 4-AP solution. This 4-AP solution was diluted into 2 mL of sterile 0.9% saline and was sonicated for 2 min to produce a uniform suspension. Each rat was anesthetized with isoflurane, and 1–5 μL of the 40-mM 4-AP solution were injected through a microcannula into the right hippocampus in 23 rats. Following injection, within minutes, the rats recovered consciousness and initially normal behavior. Within 10–20 min following injection, 18 rats demonstrated spontaneous recurrent seizures.

In vivo intracerebral and behavioral recording

The iEEG recordings were performed in an electrically screened plexiglass cage. Rats were connected to an AI 402 \times 50 Ultra Low Noise Differential Amplifier (Axon Instruments, Foster City, CA, U.S.A.), a Cyber-Amp 380 signal conditioner (Axon Instruments), and an analog digital converter (MP100; Biopac; Harvard Instruments, St. Laurent, Quebec, Canada) and were monitored by two video cameras for clinically associated behaviors. The seizure behaviors were noted according to the modified Racine scale.¹⁷ Following the implantation and recovery period (1 week), rats underwent basal recordings for 4 h (Fig. 1C). The control group (five rats) was monitored biweekly for 2–3 months. The other groups were injected with 4-AP and vEEG monitored for 3–4 h. Respirations were viewed visually, and artificial resuscitation was attempted with observed respiratory distress.

Statistical analysis

Statistical tests were performed in MATLAB using the Statistics Toolbox. Results are expressed as mean \pm standard deviation. The statistical significance of differences in discharge duration, signal amplitude, and spectral power during normal condition and respiratory arrest were evaluated using a one-way repeated-measure analysis of variance. A Fisher's least significant difference test was used to determine the significance between the various group means. The level of significance was set to $p < 0.05$.

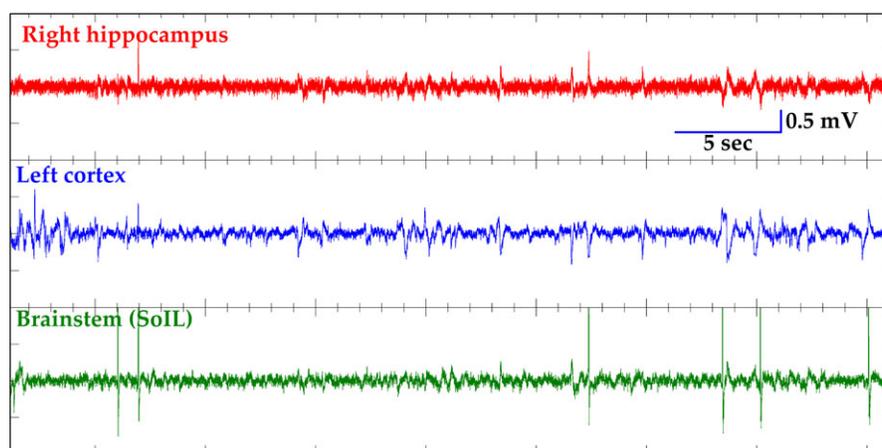
RESULTS

The control group (five rats) demonstrated no abnormal behavior or side effects due to the long-term brainstem implantation. The basal iEEG recordings from this group showed long-term stability (Fig. 2) from hippocampus, cortex, and brainstem. The brainstem electrodes contained six contacts for adjacent bipolar recordings. Electrode pairs in the SolL region showing clear EEG rhythms were selected for further analysis. Rats in group II ($n = 5$) were injected with a low dose (1 μL) of 4-AP (40 mM) into the right hippocampus and exhibited local electrographic seizures with limited contralateral spread and no evident brainstem seizure-like activity (Fig. 3A), which suggested that a higher dose was required. There were hippocampal discharges (mean 50 s) with seizure behavior class I and II.

Group III (five rats) and group IV (five rats) each received 5 μL of 4-AP (40 mM) and had recurrent hippocampal electrographic seizures within 10–20 min following injection (Fig. 3B). The number of electrographic seizures per animal in each of the high-dose groups (III and IV), measured from the hippocampus and lasting >10 s, was on average eight, with 70% of them spreading to the cerebral cortex, and in group III into the brainstem. The initially localized hippocampal seizures quickly spread to the contralateral cortex and became secondarily generalized convulsive seizures (e.g., Figs. 3B,C, 4, and 5A). Only when longer hippocampal seizure-like discharges (~ 150 s) associated with tonic-clonic seizures occurred (30–40 min postinjection; e.g., Figs. 3B and 4) did brainstem discharges (10–15 s) measured in group III become apparent. This brainstem activity was associated with violent motor seizures (Fig. 5A) and dyspnea.

Occasionally brainstem seizure-like discharges were identified occurring before the supratentorial cerebral epileptiform discharges. Following a series of hippocampal epileptiform discharges, the rat had several GTCSs and the brainstem discharges became longer (30–80 s), within 45 min of 4-AP injection. The longer brainstem discharges were associated with a respiratory arrest with or without prior hippocampal and cortical discharges. This activity was also associated with hippocampal and cortical iEEG flattening (Figs. 3B,C and 4). The respiratory arrest was commonly seen during the longer brainstem discharges (>30 s),

Figure 2. Intracerebral electroencephalogram (iEEG) from control group (group I). iEEG recordings from hippocampus, cortex, and brainstem are shown. SoLL, solitary tract, lateral. *Epilepsia* © ILAE



and dyspnea without respiratory arrest was observed during the briefer brainstem discharges (~15 s). Group IV (five rats) also received 5 μ L of 4-AP (40 mM), and all rats had similar electrographic seizures to those of group III with ensuing respiratory arrest (Fig. 3C). Group V (three rats), which did not have implanted EEG recordings but did receive a high dose of intrahippocampal 4-AP, showed the typical respiratory arrests after severe motor seizures. No respiratory arrests were noted in groups I and II.

High-dose 4-AP in all rats in groups III, IV, and V led to respiratory arrest and mortality except for those that were successfully resuscitated by manual artificial respiration (group III: 1, group IV: 2, and group V: 2 animals). In all rats, respiratory arrest preceded death (presumably from cardiopulmonary arrest). The respiratory arrests were commonly seen following or at the end of the longer hippocampal discharges (~150 s) together with brainstem discharges (Figs. 3B and 5).

DISCUSSION

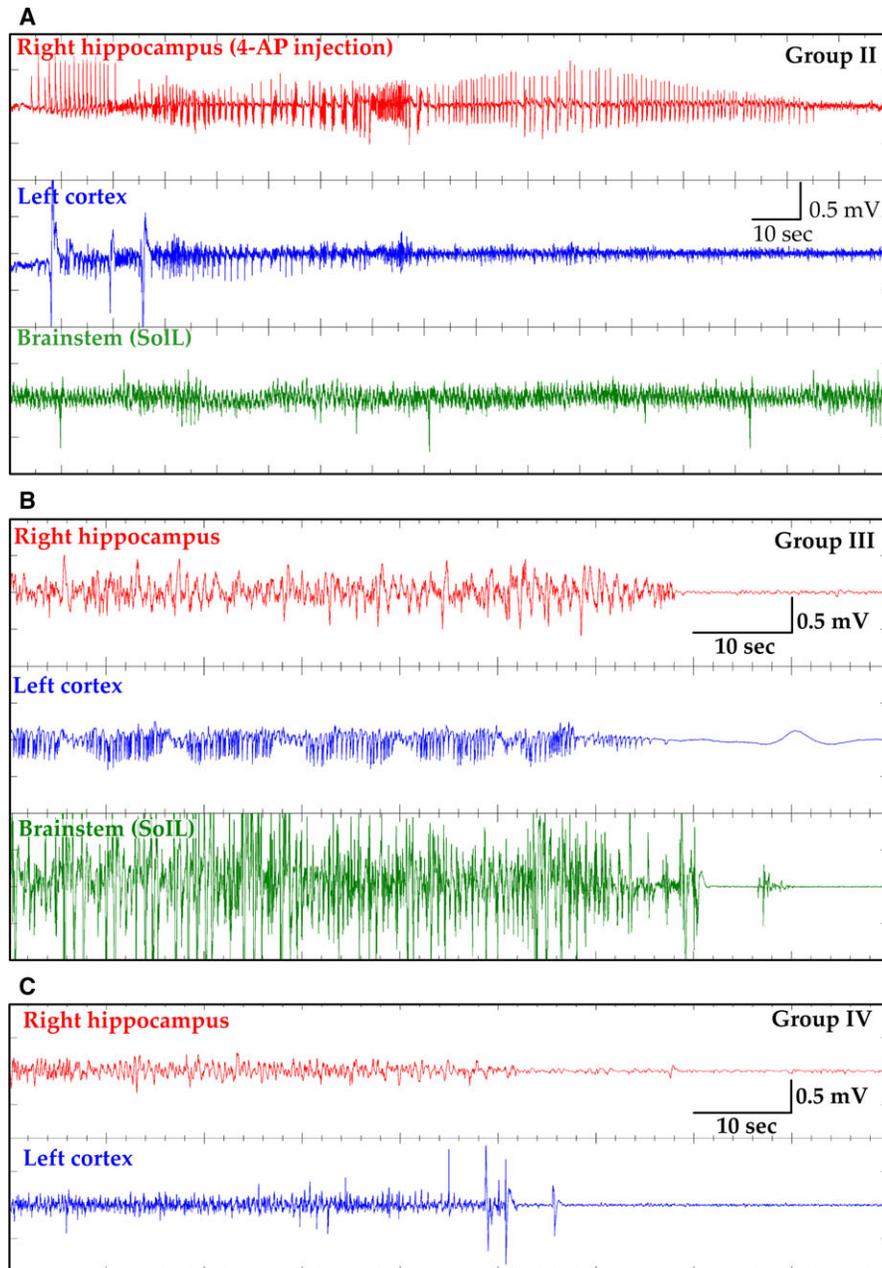
We show, for the first time in behaving animals, with directly recorded brainstem EEG, that hippocampal seizure activity can spread or trigger brainstem epileptiform discharges. These brainstem discharges are associated with violent motor seizures followed by dyspnea and respiratory arrest. This brainstem activity is also associated with cortical and hippocampal iEEG flattening, which could be a marker for subsequent respiratory arrest in our animal model. The acute recurrent hippocampally driven generalized seizures with brainstem spread are not a direct model of human SUDEP, which most often occurs in patients with spontaneous seizures, usually over many years. However, our data show that generalized seizures are associated with brainstem seizurelike activity and respiratory failure, which is a favored putative mechanism for SUDEP. Our findings reveal a potentially important mechanism—brainstem seizure activity—causing respiratory failure. Future studies, with accurate concomitant measures of respiration and

cardiac function, should explore the role of brainstem seizure activity, as well as spreading brainstem depression²² and other mechanisms, in producing ictal and postictal cardiorespiratory dysfunction, and how to modulate or prevent these effects. We did not record spreading depression in the brainstem prior to respiratory dysfunction and attempted resuscitation.

Although we think that the brainstem seizures were a result of spread of seizure activity originating in the supratentorial cerebrum, one has to consider the alternative possibilities that the high dose of 4-AP might reach the brainstem via recirculation or diffusion to cause the observed effects directly rather than from network effects or propagation from the forebrain. In our experiments, it took >20 min to see brainstem seizure activity, which goes against rapid recirculation or diffusion of 4-AP from the hippocampal injection site. It is also to be noted that the brainstem is resistant to kindling of seizures.¹²

The mechanisms underlying human SUDEP are unknown. Partly, this may reflect the absence of animal models of epilepsy using brainstem recordings. SUDEP often occurs in younger people with incompletely controlled epilepsy and likely results from seizure-induced cardiac and respiratory dysfunction. There is evidence that cardiac dysfunction contributes to SUDEP,²³ with myocyte hypertrophy and focal myocardial fibrosis commonly seen pathologically, and compared to control cases, there are significantly more cardiac channelopathy mutations²⁴ and abnormal ventricular conduction patterns²⁵ in SUDEP cases. Respiratory mechanisms are more directly implicated in SUDEP.^{10,26} Periictal apnea and hypoxia occur commonly with GTCSs and, to a lesser degree, with complex partial seizures.²⁷

Our data suggest that ictal hypoxemia and hypercapnia may be the primary mechanism of respiratory arrest in these rodents. In human patients, there is a close temporal relationship between ictal spread to the contralateral hemisphere and the onset of seizure-associated apnea,²⁸ suggesting that patients with temporal lobe epilepsy, or even

**Figure 3.**

Intracerebral electroencephalographic recordings from groups II, III, and IV. **(A)** Lower-dose (1 μ L 4-aminopyridine [4-AP], 40 mM) intracerebral injection induced local hippocampal discharges after 5–10 min, associated with diminished and briefer activity in the left cortex and no clear-cut epileptiform activity in the brainstem. **(B)** Higher-dose (5 μ L 4-AP, 40 mM) intrahippocampal injection induced secondary generalized tonic-clonic seizures, which were also associated with epileptiform brainstem discharges, followed by respiratory arrest. **(C)** The same higher-dose 4-AP injection without a brainstem electrode induced similar generalized seizures and respiratory arrest. SolL, solitary tract, lateral. *Epilepsia* © ILAE

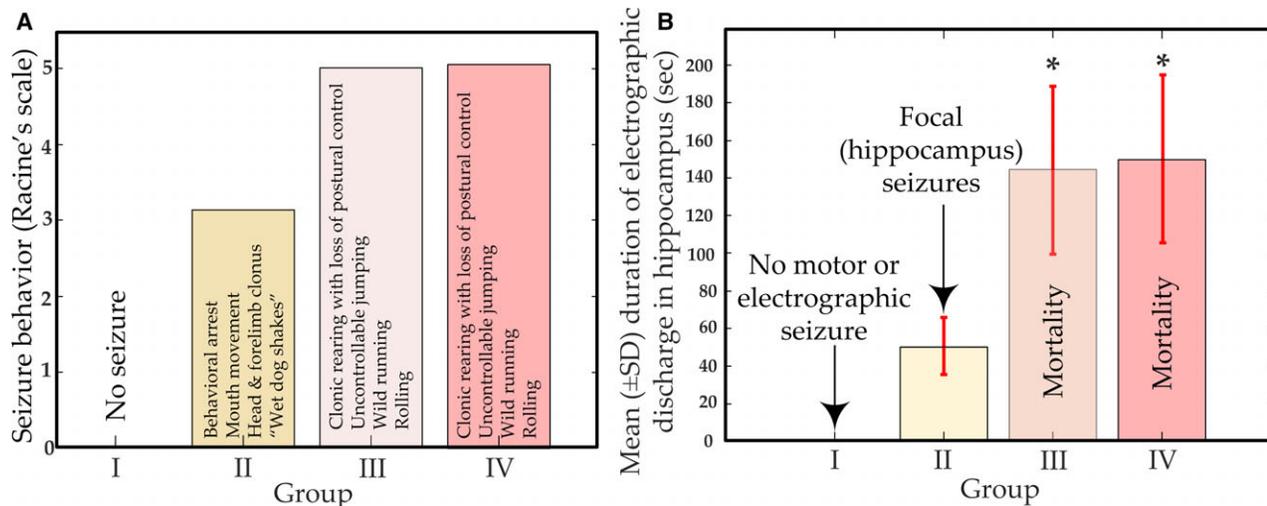
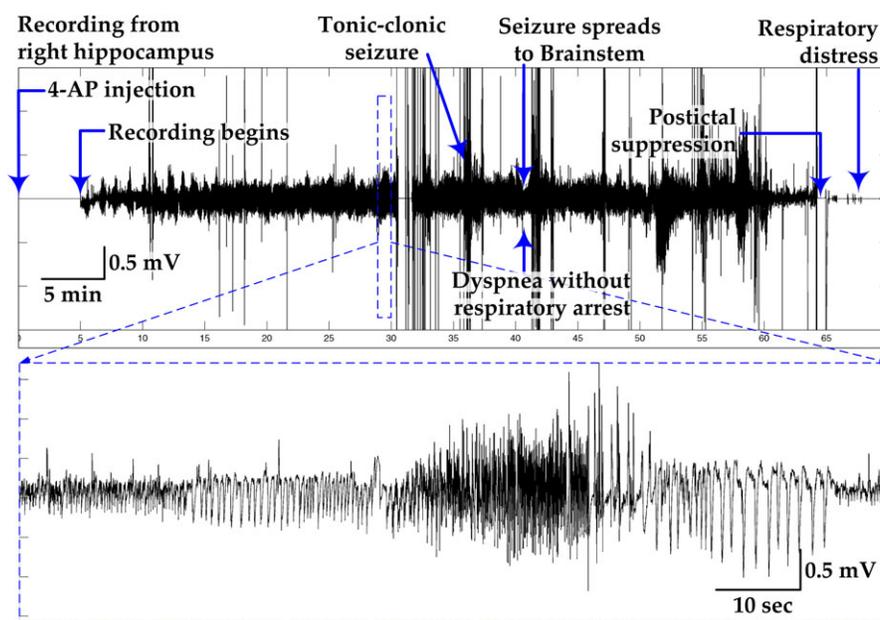
with epilepsy originating from other focal cerebral sites, with contralateral seizure spread, may be at higher risk for ictal respiratory dysfunction than those whose seizures remain unilateral. In the model used here, focal hippocampal discharges spread contralaterally and became a secondary GTCS, followed by dyspnea and respiratory arrest associated with brainstem epileptiform EEG activity.

In summary, reliable brainstem recordings are vital to understand mechanisms underlying mortality associated with seizures. In our rodent seizure model, the animals experienced many seizures. Only those seizures that spread from the hippocampus contralaterally and became secondary generalized seizures triggered the brainstem

discharges that sometimes were associated with respiratory arrest and death if respiratory resuscitation was unsuccessful. The longer hippocampal discharges (~150 s) were associated with epileptiform activity in the brainstem. The durations of brainstem discharges varied. Dyspnea was observed during brief brainstem discharges (~15 s) and the respiratory arrests were seen only during the longer brainstem discharges (>30 s). The brainstem discharges appear to disturb the respiratory network. The prolonged periods of brainstem epileptiform discharges associated with the respiratory arrests/mortality were also associated with a concomitant shutdown of hippocampal and cortical activity.

Figure 4.

Time course of electrographic seizures and concomitant behaviors following a focal high-dose injection of 4-aminopyridine (4-AP) into the right hippocampus. Bottom trace is an enlargement of hippocampal electrographic seizure activity. *Epilepsia* © ILAE

**Figure 5.**

(A) Seizure behaviors in the different groups assessed using Racine's scale and some other abnormal behaviors (e.g., wild running and rolling) commonly seen in groups III and IV. (B) Mean hippocampal seizure discharges (lasting >10 s) of all recorded seizures in each animal group were determined to establish a relation between the hippocampal discharge duration and the respiratory arrest. The hippocampal discharge duration was significantly higher prior to the respiratory arrest ($*p < 0.05$ for groups III and IV vs. I and II). SD, standard deviation.

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This study demonstrated that respiratory arrests occurred during the first part of the SUDEP episode, followed by death presumed to be due to combined pulmonary–cardiac arrest, a temporal sequence shown by Aiba and Noebels.²² The respiratory and cardiac systems are tightly related and cannot be easily dissociated, which makes it difficult to determine which system fails first. A few studies have looked at cardiac activity and SUDEP. Heart rate variability is not associated with SUDEP and is not a biomarker for SUDEP.²⁹ Ictal bradycardia and sinus arrest occur in

epilepsy patients,^{30–32} but have not been identified as precursors to SUDEP in epilepsy monitoring unit–recorded cases. In humans, ictal apnea typically occurs before and less often during the cardiac arrhythmia.⁷ Further studies employing detailed measures of respiration and EKG are required. Szurhaj et al.³³ demonstrated that a prolonged impairment of parasympathetic tone was correlated with greater postictal hypoxemia in patients monitored in an epilepsy unit. Respiratory dysfunction is common postictally, and subsequent oxygenation can prevent sudden death in

mice and reduce postictal hypoxia (with suctioning and stimulation) in humans.³⁴ Our study showed similar death-preventing results by artificial ventilation of the rats during apnea. Our hypothesis is that the respiratory impairment is due to the brainstem discharges causing central apnea. Artificial ventilation continuing oxygenation during brainstem respiratory failure sometimes permits recovery of brainstem function and prevents progression to cardiac dysfunction. Further delineation of the role of brainstem seizure activity in other animal models of SUDEP and in human SUDEP, particularly coupled with measures of respiration and cardiac function, will provide critical insights to understand why some seizures are fatal and how to develop effective preventive or therapeutic strategies.

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DISCLOSURE

The authors declare no conflicts of interest. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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