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Title: Postictal death is associated with tonic phase apnea in a mouse model of SUDEP

Running Head: Tonic phase apnea and SUDEP

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Abstract (249 out of 250)

Objective: Sudden unexpected death in epilepsy (SUDEP) is an unpredictable and devastating comorbidity of epilepsy that is believed to be due to cardiorespiratory failure immediately after generalized convulsive seizures. **Methods:** We performed cardiorespiratory monitoring of seizure-induced death in mice carrying either an p.Arg1872Trp or p.Asn1768Asp mutation in a single *Scn8a* allele – mutations identified from patients that died from SUDEP – and by seizure-induced death of pentylenetetrazole-treated wild type mice. **Results:** The primary cause of seizure-induced death for all mice was apnea, as 1) apnea began during a seizure and continued for tens of minutes until terminal asystole, and 2) death was prevented by mechanical ventilation. Fatal seizures always included a tonic phase that was coincident with apnea. This tonic phase apnea was not sufficient to produce death, as it also occurred during many nonfatal seizures; however, all seizures that were fatal had tonic phase apnea. We also made the novel observation that continuous tonic diaphragm contraction occurred during tonic phase apnea, which likely contributes to apnea by preventing exhalation, and this was only fatal when breathing did not resume after the tonic phase ended. Finally, recorded seizures from a patient with developmental epileptic encephalopathy with a previously undocumented *SCN8A* likely pathogenic variant (p,Leu257Val) revealed similarities to those of the mice; an extended tonic phase that was accompanied by apnea. **Interpretation:** We conclude that apnea coincident with the tonic phase of a seizure, and subsequent failure to resume breathing, are the determining events that cause seizure-induced death in *Scn8a* mutant mice.

Keywords: apnea; SCN8A; seizure-induced death; SUDEP; tonic phase.

Abbreviations: SUDEP = sudden unexpected death in epilepsy; AUC = area under the curve; PGES = postictal generalized EEG suppression; PTZ = pentylenetetrazole; EMU = epilepsy monitoring unit; ipm = inspirations per minute; bpm = beats per minute.

Introduction (450 out of 500)

Sudden unexpected death in epilepsy (SUDEP) is defined as the sudden, unexpected, nontraumatic, nondrowning, death of a person with epilepsy, which is not due to status epilepticus nor does a postmortem examination reveal another cause of death[1]. SUDEP is the most common cause of death associated with epilepsy, accounting for between 8 and 17% of all epilepsy-related deaths[2]. This number increases to 50% for patients whose seizures are refractory to treatment[3,4].

Although numerous mechanisms have been proposed for SUDEP[3,5,6], a growing body of evidence supports postictal respiratory dysfunction as the primary cause of death for many cases. Apnea and oxygen desaturation have been reported in a large percentage of patients during and after convulsive and nonconvulsive seizures[7–12], and in 9 cases of SUDEP with adequate postictal cardiorespiratory monitoring, terminal apnea occurred prior to terminal asystole[13]. It is believed that most SUDEP cases occur after generalized convulsive seizures[13–17]; thus, mouse models of SUDEP include those in which death occurs immediately after convulsive seizures. In such models, including *Cacna1a*^{S218L}, *Lmx1b*$^{f/f/p}$, and *Scn1a*^{R1407X} transgenic mice, apnea has also preceded terminal asystole[18–21]. Of these, only the *Scn1a*^{R1407X} mice represent a patient population known to be susceptible to SUDEP (i.e. Dravet Syndrome)[18,22].

The current study utilizes three transgenic mouse lines carrying gain-of-function *Scn8a* mutations, which were identified in several patients with developmental epileptic encephalopathy (DEE) that died of SUDEP[23,24]; either the germline p.Asn1768Asp (referred to as "D/+ mice") or conditional p.Arg1872Trp (referred to as "W/+ mice"). In the presence of Emx1-Cre the conditional p.Arg1872Trp mutation is expressed in forebrain excitatory neurons (referred to as "W/+$^{Emx1\text{-}Cre}$ mice") and in the presence of EIIA-Cre it is expressed globally (referred to as "W/+$^{EIIA\text{-}Cre}$ mice")[25]. Phenotypically, expression of either mutation recapitulates clinical features seen in patients, including spontaneous tonic-clonic seizures and seizure-induced death[25–27]. Prior to the current study, peri-ictal cardiorespiratory monitoring of these pertinent mouse models had not been performed. We utilized the natural epileptogenesis of the W/+$^{Emx1\text{-}Cre}$ mice to examine the semiology of spontaneous nonfatal and fatal seizures, the inducible fatal seizures of the W/+$^{EIIA\text{-}Cre}$ mice to examine a causal relationship between apnea and seizure-induced death, and the inducible nonfatal seizures of the D/+ mice to observe tonic diaphragmatic contraction during the tonic phase.

We demonstrate that 1) sudden death in the *Scn8a* mutant mice and pentylenetetrazole (PTZ)-treated mice occurs after convulsive seizures in which apnea occurred coincident with the tonic phase; 2) death can be prevented by mechanical ventilation; 3) tonic phase apnea is necessary, but not sufficient, to produce seizure-induced death; 4) tonic diaphragm contraction may contribute to the fatal apnea; and 5) seizures recorded from a DEE patient with a novel *SCN8A* mutation also present with apnea during the tonic phase.

Materials and Methods

Mutant Mice

All mice were housed and cared for in accordance with NIH guidelines in a 12-hour light/dark cycle with food and water *ad libitum,* and all procedures were carried out at the University of Virginia and approved by the Animal Care and Use Committee. Male mice homozygous for the conditional R1872W *Scn8a* mutant allele[25] (Fig. 1A) were crossed with female mice homozygous for either Emx1-Cre (Jax 005628)[28] or EIIA-Cre (Jax 003724)[29] to produce mice heterozygous for both alleles, resulting in expression of the R1872W mutation in forebrain excitatory neurons (W/+$^{Emx1\text{-}Cre}$) or globally (W/+$^{EIIA\text{-}Cre}$) (Fig. 1B). Mice with germline N1768D *Scn8a* mutant allele were also used[27]. Genotyping was performed to confirm the presence of the R1872W and N1768D mutant alleles as described previously[25,27], and for Emx1-Cre and EIIA-Cre alleles as directed by the Jackson Laboratories website. Male and female W/+$^{Emx1\text{-}Cre}$ and wild type mice were used with no difference in seizure phenotype. Due to the pre-weaning age of the W/+$^{EIIA\text{-}Cre}$ mice, sex was not recorded.

Continuous recording of EEG, ECG/EMG and breathing

As previously reported, the W/+$^{Emx1\text{-}Cre}$ mice develop spontaneous tonic-clonic seizures at 4 weeks of age and 75% succumb to seizure-induced death by 2 months[25]. D/+ mice have a milder phenotype with seizure onset at 8-16 weeks and only 50% lethality by 6 months[27]. Custom headsets (PlasticsOne, Inc.,) were implanted in 4-5 week-old W/+$^{Emx1\text{-}Cre}$ and 8-10 week old D/+ mice using approved surgical techniques as previously described by our laboratories[18,25]. Briefly, EEG electrodes were placed over the parietal lobe unilaterally and ECG electrodes were placed in an approximate lead II configuration. In a subset of D/+ mice, ECG leads were replaced with leads

that extended to contact the diaphragm, so as to record diaphragm EMG as previously described[30]. Reference electrode was placed over the cerebellum. Normal saline (8 mL/kg, i.p.) and ketoprofen (5 mg/kg, i.p.) were given post-operatively. Mice were monitored and additional ketoprofen was administered every 24 hours as needed. After recovery from surgery (minimum of 4 days), mice underwent continuous monitoring of EEG, ECG/EMG, and breathing in custom-designed plethysmography chambers that comply with guidelines for continuous housing described in the Guide for the Care and Use of Laboratory Animals[31], and as described previously[18]. The same techniques were used for recording seizures from W/+$^{EIIA\text{-}Cre}$ mice at postnatal age P15. However, because W/+$^{EIIA\text{-}Cre}$ mice were used prior to weaning, due to their early lethality, they could only be allowed 24 hours to recover from surgery, since degradation of ECG signals was common due to parental grooming.

Audiogenic seizures were induced in W/+$^{EIIA\text{-}Cre}$ and D/+ mice with a 15 kHz pure tone (~80 to 100 dB), generated using Tone Generator software (NCH Software, Inc.), amplified with a Kinter K3118 stereo amplifier (Kinter USA), and converted to sound using a small 3-watt speaker lowered into the plethysmography chamber. For mechanical ventilation experiments, a sonicator (Branson 200 ultrasonic cleaner) was placed next to the mouse cage and turned on to induce audiogenic seizures (see Supplemental Movie 5).

Pentylenetetrazole (PTZ)-induced seizures were generated by i.p. injection of 90 mg/kg PTZ, which resulted in 100% lethality, as previously reported[32,33]. PTZ (Sigma Aldrich) was dissolved in normal sterile saline on the day of administration.

SCN8A patient Video-EEG monitoring and breathing analysis

Human studies were approved by the University of Virginia Institutional Review Board and performed under supervision of a faculty neurologist (Dr. Howard Goodkin). A patient with a pathogenic variant of *SCN8A* was admitted to the University of Virginia Medical Center (Charlottesville, VA) for continuous video-EEG scalp recordings (Natus, Pleasanton, CA) with electrodes placed according to the 10-20 system. Breathing movement was visualized using phase-based Eulerian video magnification motion processing with open-source software (http://people.csail.mit.edu/mrub/vidmag/) written for MATLAB (Mathworks, Inc.) as done previously[18].

Statistical analysis and blinding

Since only mice expressing *Scn8a* mutations were used in this study, blinding to genotype was not necessary. For the quantification in Figure 5, measurements of tonic phase and apnea were done independent of one another. Quantification reported in Figure 7 was done with the researcher blinded to seizure outcome (i.e. fatal or non-fatal). All statistics were performed using GraphPad Prism 8 (GraphPad Software, Inc.), presented as mean ± SEM and defined as statistically detectable when $p < 0.05$. Comparison of survival proportions in Figures 2 and 4 was done using a one-sided Fisher's exact test. Comparisons between two groups were assessed by unpaired, two-tailed Student's t-test when residuals passed the D'Agnostino-Pearson Omnibus normality test and by the nonparametric Mann-Whitney test when residuals did not pass the D'Agnostino-Pearson Omnibus normality test. Comparisons between more than two groups were assessed by one-way ANOVA followed by Dunnett's multiple comparison test when residuals passed D'Agnostino-Pearson Omnibus normality test and by the nonparametric Kruskal-Wallis test for those that did not pass the D'Agnostino-Pearson Omnibus normality test. Details for statistical comparisons that are graphically depicted in figures are described in the figure legends. For comparisons only mentioned in the results section, statistics are reported in line.

Results

Monitoring seizures and sudden death in *Scn8a* mutant mice

Eight W/+$^{Emx1\text{-}Cre}$ mice were monitored for ECG, cortical EEG, and whole-body plethysmograph which captured 44 nonfatal and 8 fatal seizures. Only seizures qualifying as R5 or above (i.e. R5-R8[32,33]) on a modified Racine scale were analyzed. These showed rearing and falling, tail-raising, clonic and/or tonic convulsions, and increased ictal EEG amplitude followed by postictal generalized EEG suppression (PGES). Seizures were always separated by at least an hour, and never qualified as *status epilepticus*. Three subcategories of R5 or greater seizures were identified in the W/+$^{Emx1\text{-}Cre}$ mice: fatal tonic seizures (i.e. seizure-induced death; Supplemental Movie 1), nonfatal tonic seizures (Supplemental Movie 2), and nonfatal clonic seizures (Supplemental Movie 3). Fatal tonic seizures were recorded from 8 W/+$^{Emx1\text{-}Cre}$ mice. A total of

44 spontaneous nonfatal seizures were recorded from 6 of the 8 W/+$^{Emx1\text{-}Cre}$ mice, of which 15 were tonic and 29 were clonic.

Four W/+$^{EIIA\text{-}Cre}$ mice were monitored for ECG, EEG, and plethysmography during audiogenic seizure-induced death. We found that high frequency sound routinely caused seizures in W/+$^{EIIA\text{-}Cre}$ mice. In all cases these seizures resulted in sudden death with the same sequence of events as those experienced by W/+$^{Emx1\text{-}Cre}$ mice during spontaneous seizures. All W/+$^{EIIA\text{-}Cre}$ mice had convulsive seizures that included wild running followed by a tonic phase and death. The audio stimulus never induced observable seizure behavior in W/+$^{Emx1\text{-}Cre}$ or wild type mice (data not shown).

In order to compare results obtained from the *Scn8a* mutant mice to a more widely used model of seizure-induced death, we monitored seven wild-type (C57/B6J) mice for ECG, EEG, and plethysmography immediately before and after i.p. injection of 90 mg/kg PTZ. All mice died from convulsive seizures with similar semiology to the fatal seizures of the *Scn8a* mutant mice, which included wild running followed by a tonic phase and death (Supplemental Movie 4).

Finally, three D/+ mice were monitored for ECG, EEG, and plethysmography during 14 spontaneously occurring nonfatal tonic seizures. Three additional D/+ mice had EEG, plethysmography, and diaphragm EMG measured (as previously described[30]) during audiogenic nonfatal tonic seizures. As per our previous report, both spontaneous and audiogenic seizures in D/+ mice were characterized by wild running followed by tonic phase and recovery (manuscript under review).

Apnea causes sudden death in *Scn8a* mutant mice

In all cases of sudden death, breathing was absent during the tonic phase of a convulsive seizure and never resumed (Fig. 1C, D, and E). During and after the fatal seizure, cardiac activity continued, albeit at a lower rate (Fig. 1C_2, D_2, and E_2). For W/+$^{Emx1\text{-}Cre}$, W/+$^{EIIA\text{-}Cre}$, and PTZ-treated WT mice, terminal asystole followed breathing cessation by 15.4 ± 1.7 mins, 34.4 ± 12.4, and 13.8 ± 3.0 mins, respectively (Fig. 2A). Thus, the sequence of events did not differ when the *Scn8a* mutation was expressed globally (W/+$^{EIIA\text{-}Cre}$ mice) or selectively in forebrain neurons (W/+$^{Emx1\text{-}Cre}$ mice), or exogenously induced in otherwise genetically wild type mice (i.e. PTZ-treated WT mice).

Based on these observations, we hypothesized that apnea was the primary cause of death. To directly test this hypothesis, we induced audiogenic seizures in W/+$^{EIIA\text{-}Cre}$ mice that routinely resulted in death (0/8 survived with no intervention; Supplemental Movie 5, left side). When we induced audiogenic seizures in W/+$^{EIIA\text{-}Cre}$ mice and performed mechanical ventilation using a transfer pipet adapted to fit snugly around the nose and mouth (Fig. 2B; Supplemental Movie 5, right side), survival markedly improved (7/8 survived when ventilated; Fig. 2C). Ventilation was initiated within 6 s after the start of the tonic phase (Fig 2D) and continued until the mouse began breathing on its own. The one mouse that did not survive was ventilated for 43 seconds before stopping due to lack of recovery. Since another mouse that survived was ventilated for 80 s, the single fatality may have resulted from premature termination of mechanical ventilation.

Tonic phase apnea is not sufficient for seizure-induced death

Nonfatal tonic seizures in W/+$^{Emx1\text{-}Cre}$ mice appeared nearly identical to fatal seizures, except that breathing resumed immediately postictal (compare Fig. 3A to Fig. 1C-D; Supplemental Movies 1 and 2). Nonfatal seizures had a pronounced tonic phase, concurrent with apnea and bradycardia (Fig. 3A$_2$). Following the tonic phase of nonfatal seizures, mice experienced tachycardia and tachypnea (Fig. 3A$_3$), which was different from the bradycardia and apnea observed after fatal tonic seizures (Fig. 3B and C). Our analysis likely underestimates heart rate during the tonic phase due to EMG activity obscuring the QRS complexes. However, at the end of the tonic phase, when QRS complexes were distinguishable from the EMG signal, heart rate was clearly reduced (Fig. 3A$_2$). Interestingly, there were no mice that had intermediate responses between complete and permanent respiratory failure, and rapid recovery. The mechanisms for conversion from reversible apnea to terminal apnea are unknown.

Tonic phase apnea is necessary for seizure-induced death

Clonic seizures were similar to tonic seizures with respect to EEG activity and most behavioral semiology (compare Fig. 4A to Fig. 3A; Supplementary Movies 2 and 3). However, instead of a tonic phase denoted by hindlimb extension, clonic seizures had repetitive clonic movements in the upper extremities, visible in the ECG signal as small repetitive bursts of EMG activity (Fig. 4A$_2$). Instead of apnea, tachypnea occurred during clonic seizures (Fig. 4B), with the

minimum respiratory frequency (Rf) during clonic seizures being detectably higher than for tonic seizures (329 ± 12 and 75 ± 27 ipm, respectively; $p < 0.0001$, Mann-Whitney test). Both tonic and clonic seizures presented with bradycardia (Fig. 4C), that did not differ statistically (70 ± 21 versus 279 ± 60 beats per minute (bpm), respectively; $p = 0.1244$, Mann-Whitney test). Although clonic seizures represented a significant proportion of nonfatal seizures (29 out of 44), no clonic seizure was fatal, and the probability of mortality was higher for tonic compared to clonic seizures (Fig. 4D).

Tonic diaphragm contraction occurs during tonic phase apnea

In W/+$^{Emx1\text{-}Cre}$ mice, ictal apnea is coincident with the tonic phase. Apnea did not occur during clonic seizures. Analysis of the minimum Rf for all nonfatal seizures from W/+$^{Emx1\text{-}Cre}$ mice (n = 43 seizures) identified two distinct groups; those with breathing rates of < 100 or > 100 ipm, corresponding to tonic and clonic seizures, respectively (Fig. 5A). We hypothesized the tonic phase and apnea share an underlying mechanism, and thus a longer tonic phase should be correlated with longer apnea. We compared duration of the tonic phase and apnea, with data obtained from spontaneous tonic seizures recorded from D/+ mice included, as they present with the same behavioral and electrographic signatures, but with shorter apnea than W/+$^{Emx1\text{-}Cre}$ mice (9.0 ± 0.5 and 16.4 ± 1.8 for D/+ and W/+$^{Emx1\text{-}Cre}$ mice, respectively; $p = 0.0045$; unpaired t-test, $t = 3.182$, $df = 21$). This allowed us to assess the relationship of tonic phase and apnea duration over a wider range. To estimate tonic phase duration, we rectified and smoothed the ECG/EMG signal and identified the lowest points preceding and following the tonic extension phase (Fig. 5B, black trace on top). Across all apneic seizures of the two mouse models, apnea duration was directly proportional to tonic phase duration (Fig. 5C; $R^2 = 0.6866$). It is important to note that wild running occurs immediately prior to the tonic phase. The running produces noise in the plethysmography signal, making it impossible to know whether there is breathing during this short period. For this, and other reasons, our determination of the time of onset of breathing cessation is imprecise, and it is possible that apnea starts prior to the tonic phase in some cases.

We hypothesized that tonic muscle contraction prolongs apnea. To test this, we recorded diaphragm muscle activity (diaEMG; as recently described[30]) during audiogenic seizures in D/+ mice. Our diaEMG was reliably synchronized with inspiratory activity recorded by

plethysmography (Fig. 6A). When we stimulated an audiogenic seizure, we observed a large amount of tonic diaEMG activity during the tonic phase (Fig. 6B). This observation was replicated in a total of 8 audiogenic seizures recorded from 3 D/+ mice (Figs. 6C).

Comparison of fatal and nonfatal seizure dynamics

The ictal duration and amplitude of PGES were compared between clonic, tonic, and fatal seizures from W/+$^{Emx1\text{-}Cre}$ mice (Fig. 7A). There was no difference in ictal duration among the three seizure types (Fig. 7B). PGES, defined as the lowest EEG amplitude within 30 seconds of seizure termination as a percentage of pre-ictal level, was detectably greater for fatal compared to clonic seizures, but was not detectably different between nonfatal tonic and fatal tonic seizures (Fig. 7C).

Duration, peak, and area under the curve (AUC) of EMG tonic activity were analyzed for tonic nonfatal and fatal seizures in W/+$^{Emx1\text{-}Cre}$ mice (Fig. 7D). Duration of tonic phase was longer for fatal seizures compared to non-fatal tonic seizures (Fig. 7E); however, no detectable differences in peak or AUC EMG activity were identified between the seizure types (Fig. 7F and G).

Tonic phase apnea in a patient with *SCN8A* epilepsy

An 18-month-old male patient was monitored for seizure characterization in the epilepsy-monitoring unit (EMU) at the University of Virginia Health System. Exome sequencing detected the heterozygous likely pathogenic variant p.Leu257Val (L257V) located in the pore-lining transmembrane segment S5 in domain 1 of $Na_v1.6$ (Fig. 8A). The corresponding leucine residue is conserved in the 3 paralogous human neuronal sodium channels as well as vertebrate and invertebrate homologs (Fig. 8B). Val257 is not present in the GNOMAD database of 120,000 control exome sequences lacking early onset neurological disorders. Prediction software rates the L257V substitution as possibly detrimental (Polyphen2 score 0.9) or damaging (SIFT 0.037), with a high CADD score of 25.4 indicative of deleterious effects.

During EMU monitoring, a 12-minute cluster of three electroclinical seizures was recorded, and reviewed by a neurologist (HPG). Each seizure in the cluster had a pronounced tonic phase, during which breathing motions were either absent or markedly diminished. The position

of the patient during the first seizure provided clear visibility of the left side of the body, permitting video analysis of breathing movements (Fig. 8C, Supplemental Movie 6). This seizure lasted approximately 80 seconds, of which 60 seconds involved pronounced tonic muscle contraction (Fig. 8D), as indicated by the presence of a large amount of EMG activity in the ECG signal. The patient had tachycardia upon seizure onset followed by bradycardia at the tonic phase onset (Fig. 8D, bottom trace of HR).

To assess respiratory frequency and pattern, visualization of respiratory movements were aided by Eulerian video magnification[34] as described previously[18]. Dark pixels between the patient's left arm and body (red line in Fig. 8H) were plotted over time to approximate breathing motions (Figs. 8I and J). We were able to reliably analyze breathing with this method during two time periods during the seizure when view of the patient was not obstructed by caregivers. During the first period, occurring just after the start of the tonic phase (box "E" in Fig. 8D), there were minimal breathing movements, clear tonic muscle contraction and bradycardia. The second period occurred at the end of the seizure (box "F" in Fig. 8D), with minimal breathing motions for more than 10 seconds prior to clear hyperpnea as breathing resumed (Fig. 8F; Supplemental Movie 6, apnea/hypopnea begins at 0:29 and hyperpnea begins at 1:26). These two periods of breathing analysis were different than the eupneic breathing observed several minutes after the seizure when PGES had subsided (Figs. 8G). Fourteen breaths were identified during this 26 second period, resulting in 32 breaths per minute, which is within the normal breathing frequency range for a child of 18 months (29 to 48 bpm for 10^{th} and 90^{th} percentiles, respectively)[35].

Discussion (1070 out of 1500)

In the present study, we characterize tonic phase apnea and demonstrate its role in spontaneous and evoked seizure-induced deaths using three mouse models of *SCN8A* epilepsy. Recordings of audiogenic seizure-induced deaths from $W/+^{EIIA\text{-}Cre}$ mice allowed us to identify breathing cessation as the primary cause of death. Chronic recording of $W/+^{Emx1\text{-}Cre}$ captured spontaneous nonfatal and fatal seizures, which demonstrate that tonic phase apnea is necessary but not sufficient for seizure-induced death. Death only occurred when the animal did not recover from postictal apnea. Recording of diaphragm EMG during audiogenic seizures of D/+ mice allowed us to determine that tonic phase apnea is coincident with tonic respiratory muscle contraction. Finally,

data acquired from a patient with a novel *SCN8A* mutation demonstrates that tonic phase apnea can also occur in patients with *SCN8A* epilepsy.

Tonic Phase Apnea

In *Scn8a* mutant and PTZ-treated wild-type mice, a tonic phase was present in each case in which apnea and death occurred. We further show that tonic phase apnea occurs in a patient with a novel *SCN8A* mutated allele. This is consistent with the limited clinical literature assessing respiratory function during tonic muscle contractions that occur during seizures[36]. Tonic phase apnea is likely common to many mouse models of epilepsy. Specifically, mouse models of Dravet Syndrome[18,37] and *Kcna1*$^{-/-}$[38] can demonstrate some apneas coinciding with elevated tonic EMG activity.

Our findings suggest that tonic phase apnea occurs, at least in part, due to tonic contraction of muscles involved in inspiration (i.e. the diaphragm), which prevents the normal rhythmic contraction needed for alveolar ventilation. Although this concept is described in Neurology textbooks[39], relevant breathing muscles have not been recorded during tonic seizures in humans or mice. While tonic diaphragm contraction alone could produce an inspiratory breath hold, effectively an apnea, it should be noted that other respiratory muscles could be affected, including those that could cause airway obstruction, as has been reported for seizures induced with kainic acid in anesthetized rats[40].

It is important to note that breathing did not resume immediately upon termination of diaphragm contraction for nonfatal seizures. In addition, wild running creates noise in the plethysmography signal immediately before the tonic phase begins, obscuring our ability to determine precise timing of apnea initiation. It is possible, even probable, that apnea is initiated before the tonic phase in some seizures. These periods of apnea outside the tonic phase indicate that mechanisms of central apnea independent of diaphragm contraction must also exist. One such mechanism could be inhibition of brainstem respiratory neural circuitry, as is observed by stimulation of the amygdala[41,42]. Whatever the mechanism(s) of apnea, our evidence demonstrates a close temporal relationship between the tonic phase and apnea in both the *Scn8a* mutant and PTZ-treated mice; however, the directionality of this relationship is not clear. It is possible that apnea causes tonic contraction or that an unknown third process produces both effects. Since it is

currently impossible to selectively suppress the tonic phase, it is not possible to determine directionality of this relationship.

Apnea is the primary cause of death for *Scn8a* mutant mice

The MORTEMUS study is the most extensive compilation of cardiorespiratory recording during clinical SUDEP events to date. In nine patients with adequate cardiorespiratory monitoring, terminal apnea occurred before terminal asystole[13]. This sequence of events was replicated by both the spontaneous seizure-induced deaths of W/+$^{Emx1\text{-}Cre}$ mice and the audiogenic seizure-induced deaths of W/+$^{EIIA\text{-}Cre}$ mice; terminal apnea preceded terminal asystole by tens of minutes. Our interpretation is that apnea resulted in anoxia during and after the seizure, which eventually caused cardiac failure. This is supported by our report that wild-type mice exposed to anoxia succumb to cardiac failure on a similar time scale[18]. In the present study, we were further able to demonstrate a causal relationship between apnea and sudden death by utilizing the audiogenic seizure-induced death of W/+$^{EIIA\text{-}Cre}$ mice. These audiogenic seizures were routinely fatal; however, mechanical ventilation greatly increased survival, underscoring the importance of apnea for seizure-induced death in this model.

Tonic phase apnea always occurs with seizure-induced death in Scn8a mutant mice and other models of SUDEP

All observed instances of seizure-induced death of *Scn8a* mutant mice and PTZ-treated wild type mice were preceded by seizures with a tonic phase, denoted by hindlimb extension, and coincident apnea. A number of SUDEP mouse models, including *Scn1a*^{R1407X}, DBA1/2, *Kcna1*$^{-/-}$, 129/SvTer and *Cacna1a*^{S218L} mice, experience fatal seizures that are classified as tonic, generally based on the observation of hindlimb extension[18–20,38,43–45]. However, in many studies of seizure-induced death, seizure-type is not specified, and we have observed a subset of fatal heat-induced seizures of *Scn1a*^{R1407X} mice that were clonic[18]. Thus, the relative predominance of tonic phase apnea during seizure-induced death across other epilepsy models remains to be determined. It is well known that ictal apnea does occur in many patients with nonconvulsive seizures[8–11], so the same may be true of mouse models.

Tonic phase apnea is not sufficient to produce seizure induced death in Scn8a mutant mice

Although all cases of seizure-induced death in the *Scn8a* mutant mice were preceded by a tonic seizure, there were many occurrences of tonic phase apnea in the W/+$^{Emx1\text{-}Cre}$ mice that were not fatal. Thus, tonic phase apnea appears necessary but not sufficient for death to occur in the *Scn8a* and PTZ models used in this study. For the W/+$^{Emx1\text{-}Cre}$ mice, recovery from tonic phase apnea was an all or none phenomenon: either breathing rapidly and fully recovered within seconds of tonic phase termination, or it failed completely to recover and death followed. There were no examples of partial or slow recovery. This suggests that a secondary central process responsible for breathing regulation must also fail in order to produce seizure-induced death. It is not unexpected that mechanisms of seizure-induced death and SUDEP will be multifactorial, and different SUDEP mechanisms may exist for different epilepsy etiologies. Other mechanisms that may inhibit recovery from tonic phase apnea include spreading depolarization to the brainstem, impaired homeostatic breathing (i.e. central apnea), and cardiovascular dysfunction[2,46–48]. In fact, cardiac dysfunction appears to vary considerably in different mouse SUDEP models. For example, in W/+$^{Emx1\text{-}Cre}$ mice tonic seizures induce transient asystole that occurs as rapidly as apnea (Figure 3B & C), whereas in *Scn1a*^{R1407X} mice fatal seizures are associated with rapid, irreversible apnea, but with much slower development of bradycardia[18]. Thus, future investigation should focus on mechanisms of tonic phase apnea, and uncovering the coexisting factors that impede breathing recovery after the tonic phase.

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Author Contributions

IW, MP, FT, GR, and MM contributed to the conception and design of the study; EW, PW, JW, PP, PS, HG, and BB contributed to the acquisition and analysis of data; IW, MP, FT, GR, and MM contributed to drafting the text and preparing the figures.

Potential Conflicts of Interests

The authors report no competing interests.

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Figures

Fig. 1. Seizure-induced death occurs when breathing does not resume after the tonic phase. (**A**) Diagram of *SCN8A* protein structure with location of R1872W mutation depicted. (**B**) Breeding scheme to generate W/+$^{Emx1\text{-}Cre}$ (top) and W/+$^{EIIA\text{-}Cre}$ (bottom) mice. Mice homozygous for the floxed R1872W mutant allele were bred with mice homozygous for Emx1-Cre or EIIA-Cre. The resulting offspring were heterozygous for both alleles. (**C**) Representative traces showing time from seizure onset to terminal asystole. (**C, D, and E**) Recording of EEG, ECG, and plethysmography (Pleth) during fatal spontaneous seizures of W/+$^{Emx1\text{-}Cre}$ (C), fatal audiogenic seizures of W/+$^{EIIA\text{-}Cre}$ (D) mice, and fatal PTZ-induced seizures of wild-type mice (E). **C_1 and C_2)** expanded traces from corresponding boxes in C. **D_1 and D_2)** expanded traces from corresponding boxes in D. **E_1 and E_2)** expanded traces from corresponding boxes in E.

Fig. 2. Apnea is the determining cause of seizure-induced death. A) In the events of seizure-induced death, terminal apnea preceded terminal asystole in all mouse models. **B and C)** Mechanical ventilation significantly increased survival of audiogenic seizures for W/+$^{EIIA\text{-}Cre}$ mice ($p = 0.0205$; two-sided Chi-square = 5.367). **D)** For each mouse, ventilation was initiated within 6 s of the beginning of the tonic phase and was continued for 21 to 80 s.

Fig. 3. Transient apnea and bradycardia also occur during nonfatal tonic seizures. (A) Recording of EEG, ECG, and plethysmography (pleth) during a convulsive seizure in a W/+$^{Emx1\text{-}Cre}$ mouse. **(A_1-A_3)** Expanded views of traces from panel A, depicting EEG, ECG, and breathing, at different timepoints. In A_2, note the bradycardia, EMG infiltration of the ECG, and apnea. **(B and C)** Respiratory frequency (Rf) and heart rate (HR) binned every second from 50 seconds before, to 150 seconds after seizure initiation for all tonic (red/blue) and fatal (black) seizures. For B and C, solid lines = mean, shaded region = SEM. Note, in nonfatal seizures both breathing and heart rate increase beyond resting levels immediately after the seizure.

Fig. 4. Clonic seizures do not produce death or apnea. A) Recording of EEG, ECG, and Pleth during a clonic seizure in a W/+$^{Emx1\text{-}Cre}$ mouse. **(A_1-A_3)** Expanded views of boxes in panel A, depicting EEG, ECG, and breathing prior to, during, after the seizure. **(B)** Respiratory frequency (Rf) and **(C)** heart rate (HR) binned every second from 50 seconds before to 150 seconds after seizure initiation for tonic and clonic seizures. (**D**) Pie charts show tonic seizures are more likely to cause fatality than clonic seizures ($p = 0.007$; two-sided Fisher's exact test). For B and C, solid lines = mean, shaded region = SEM.

Fig. 5. The tonic phase is coincident with apnea. (A) Histogram of the nadir of breathing rate for all nonfatal seizures. Breathing rate nadir fell into two distinct groups; tonic and clonic, with low (near zero) and high Rf, respectively. **(B)** Depiction of measurement of tonic phase and apnea delay and duration made from all nonfatal tonic seizures. Delay was measured from the seizure onset (i.e. the first spike-wave) to apnea or tonic phase start (spike-wave/EEG data not shown in image). Black line superimposed on the ECG/EMG trace is the same signal after rectification and smoothing. Lower points of black line were used to determine the start and end of the tonic phase. **(C)** The duration of apnea is directly proportional to the duration of the tonic phase itself ($R^2 = 0.6886$; n = 14 and 9 for W/+$^{Emx1\text{-}Cre}$ and D/+ mice, respectively.)

Fig. 6. Tonic diaphragm activity is coincident with tonic phase apnea. (A) Plethysmography (pleth), raw diaphragm EMG (diaEMG), and RMS amplitude of diaEMG (RMS) recorded from a D/+ mouse. **(B)** EEG, Pleth, diaEMG, and RMS recorded during an audiogenic seizure from the same D/+ mouse as in A. Some of the diaEMG signal is clipped (indicated by black horizontal lines) to better depict lower amplitude, pre-ictal signal. **(C)** Left, expanded 1 s traces from pre-ictal and tonic phase time points indicated in B. Note inspiratory motor oscillation occurs during the pre-ictal period and tonic contraction during the tonic phase. Middle and right, the same observation was made during seizures in two additional D/+ mice.

Fig. 7. EEG and EMG dynamics are similar for fatal and nonfatal tonic seizures in W/+$^{Emx1\text{-}Cre}$ mice. (**A**) Depiction of seizure duration, pre-ictal amplitude, and PGES amplitude measurements from a peri-ictal EEG trace. **(B)** Seizure duration was no different across the three

observed seizure types (Kruskal-Wallis test, K = 1.166, p = 0.5583). (**C**) PGES amplitude (as a fraction of pre-ictal amplitude) was smaller for fatal compared to clonic seizures; however, there was no difference between tonic and fatal seizures (p = 0.0373 by Dunnett's multiple comparison test after significant one-way ANOVA, $F_{2,45}$ = 4.065, p = 0.0238). **(D)** Depiction of tonic phase duration, peak amplitude, and area under the curve (AUC) measurement from a peri-ictal ECG/EMG trace from a tonic seizure. **(E-G)** Tonic phase duration was longer for fatal seizures (p = 0.0013, T = 3.729, df = 20, unpaired t-test).; however, there was no detectable difference in peak (p = 0.2407, T = 1.209, df = 20; unpaired t-test) or AUC EMG activity (p = 0.7583, T = 0.3120, df = 20; unpaired t-test). * and ** indicate p < 0.05 and p < 0.01, respectively. Values in parentheses indicate number of seizures analyzed in that dataset.

Fig. 8. **Apnea and bradycardia occur during tonic seizures in a patient with *SCN8A* epilepsy.** **(A)** Diagram of *SCN8A* gene product ($Na_v1.6$) structure with location of L257V mutation depicted. **(B)** Evolutionary conservation of residue L257 in multiple species. h, human; m, mouse; c, chicken; a, anole; z, zebrafish; f, fugu; dpara, drosophila 'paralytic'. Amino acids are indicated by the single letter code; dots represent identical to the human amino acid. **(C)** Image of 18-month-old patient during tonic phase of seizure. Note the tonic muscle contraction apparent in the quadricep and latissimus muscles (white arrows). (**D**) Recording of C3 and C4 EEG, EMG, and ECG leads during a convulsive seizure. See Supplemental Movie 6 for full video of seizure. This seizure has a pronounced tonic phase, as indicated by elevated EMG activity (green signal). (**E**) At the beginning of tonic phase of the seizure, there was apparent apnea/hypopnea and bradycardia (box E in panel D). Signals shown are EEG, ECG, and breathing assessed with Eulerian video magnification analysis of breathing (blue trace, see below for description of analysis). **(F)** Same analysis at the end of the seizure showing a period of apnea/hypopnea followed immediately by hyperpnea when breathing recovers (box F in panel D). **(G)** Breathing analysis several minutes after the seizure, depicting normal, eupneic breathing in addition to normal ictal activity and ECG. **(H)** Expanded image of region in black box in panel C depicts column of pixels (red line segment) that were analyzed for breathing movements. **(I and J)** Greyscale time series of pixels of location depicted in H at the end of apnea (I) and several minutes postictal (J). Blue line corresponds to the midpoint of the band of lowest pixel intensity (binned over 5 pixels). Corresponding signals in F and G were bandpass filtered between 1 and 5 Hz.

Supplementary Movies:

Supplemental Movie 1. Video of a fatal tonic seizure in a W/+$^{Emx1\text{-}Cre}$ mouse with EEG, ECG, and breathing (pleth) signals synced to video. The mouse having the seizure is indicated by red box.

Supplemental Movie 2. Video of nonfatal tonic seizure in a W/+$^{Emx1\text{-}Cre}$ mouse with EEG, ECG, and breathing (pleth) signals synced to video. The mouse having the seizure is indicated by red box.

Supplemental Movie 3. Video of clonic seizure in a W/+$^{Emx1\text{-}Cre}$ mouse with EEG, ECG, and breathing (pleth) signals synced to video. Mouse having seizure is indicated by red box.

Supplemental Movie 4. Video of a fatal tonic seizure induced by PTZ (90 mg/kg) in a wild type mouse with EEG, ECG, and breathing (pleth) signals synced to video. The mouse having the seizure is indicated by red box.

Supplemental Movie 5 Side-by-side videos of audiogenic seizures induced in two littermate P15 W/+$^{EIIA\text{-}Cre}$ mice. On the left, the mouse was handled, but not ventilated, resulting in sudden death. On the right, the mouse was mechanically ventilated and survived.

Supplemental Movie 6. Video of a DEE patient with an L257V *SCN8A* mutation before, during, and after the seizure analyzed in Figure 7.

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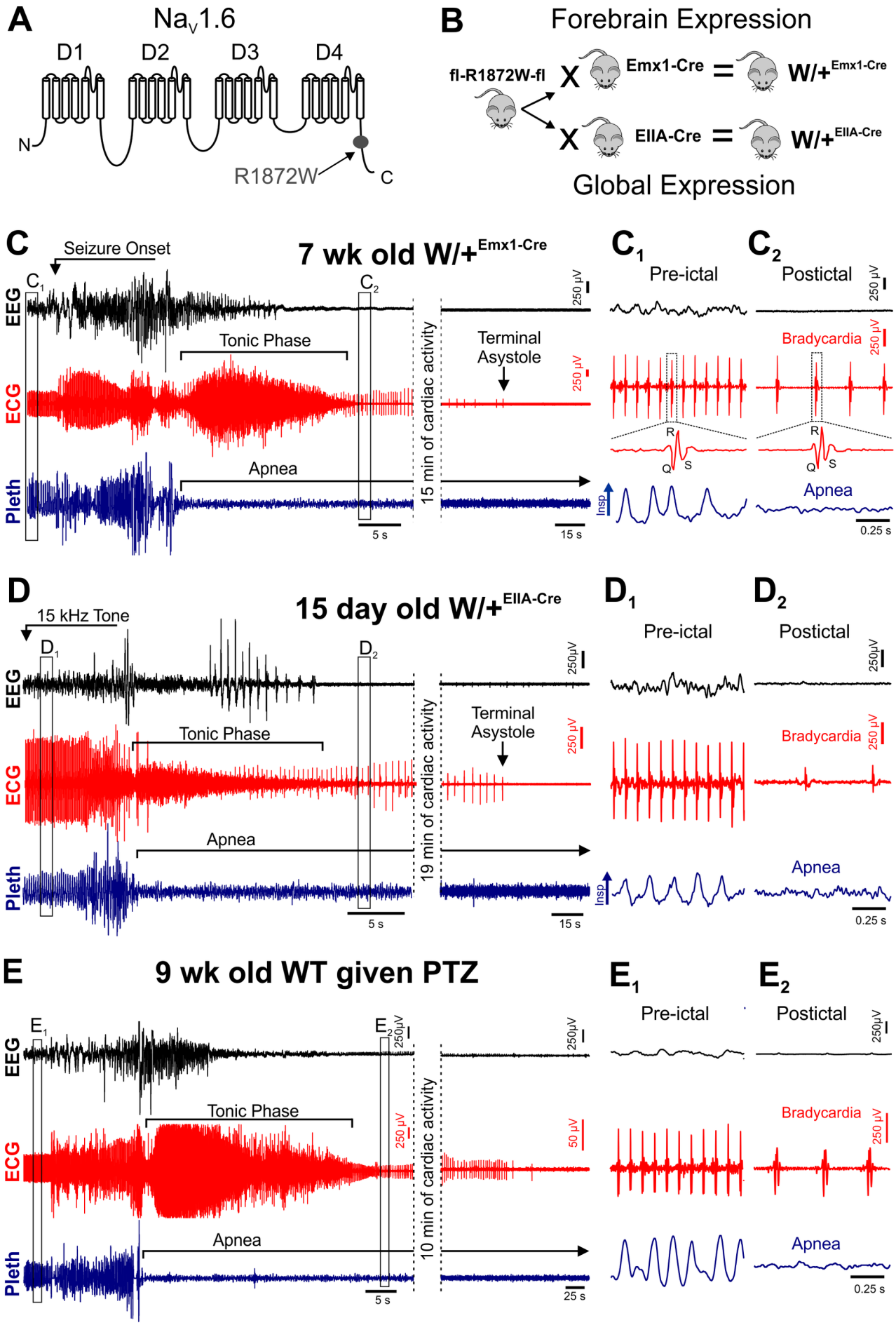

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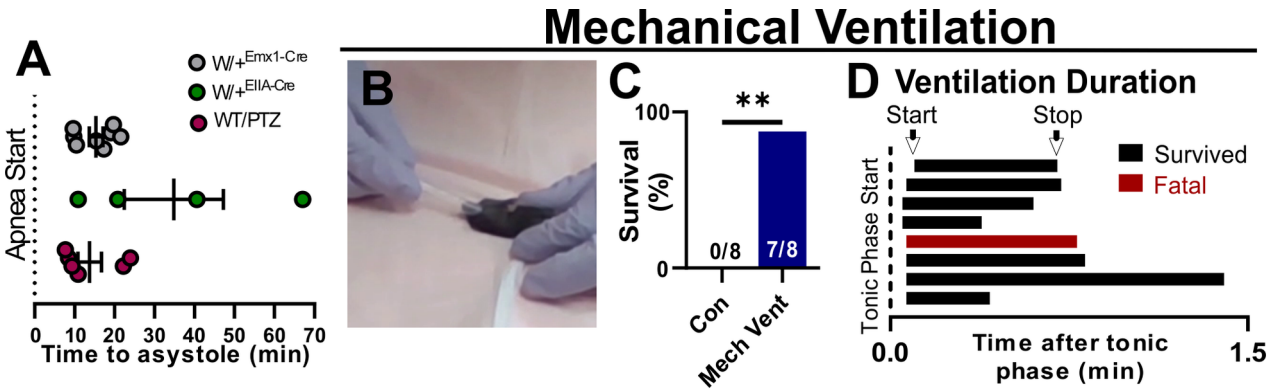

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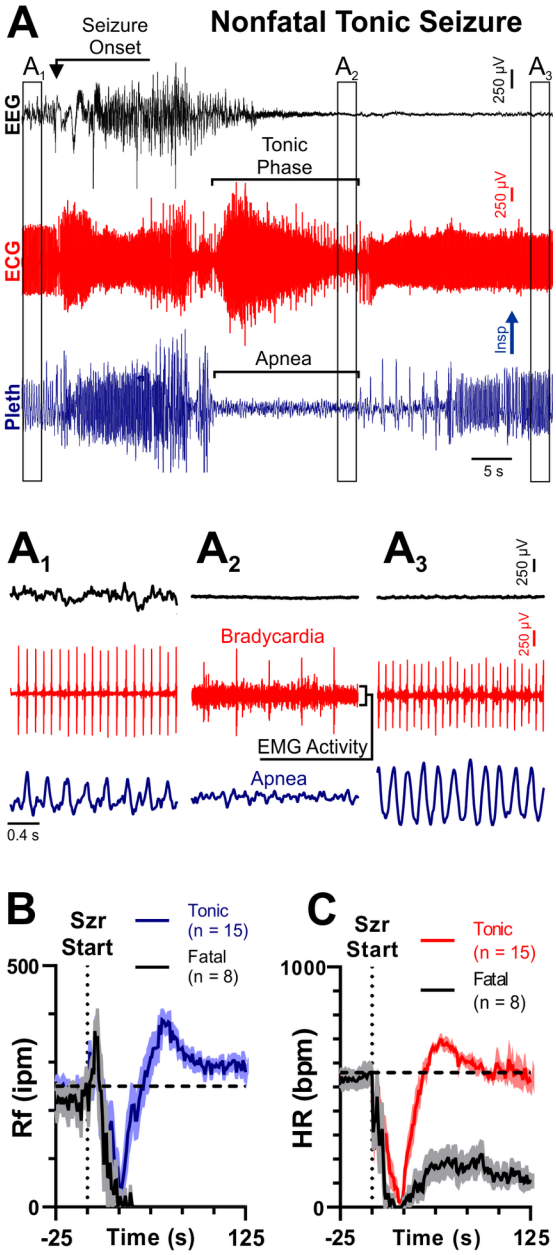

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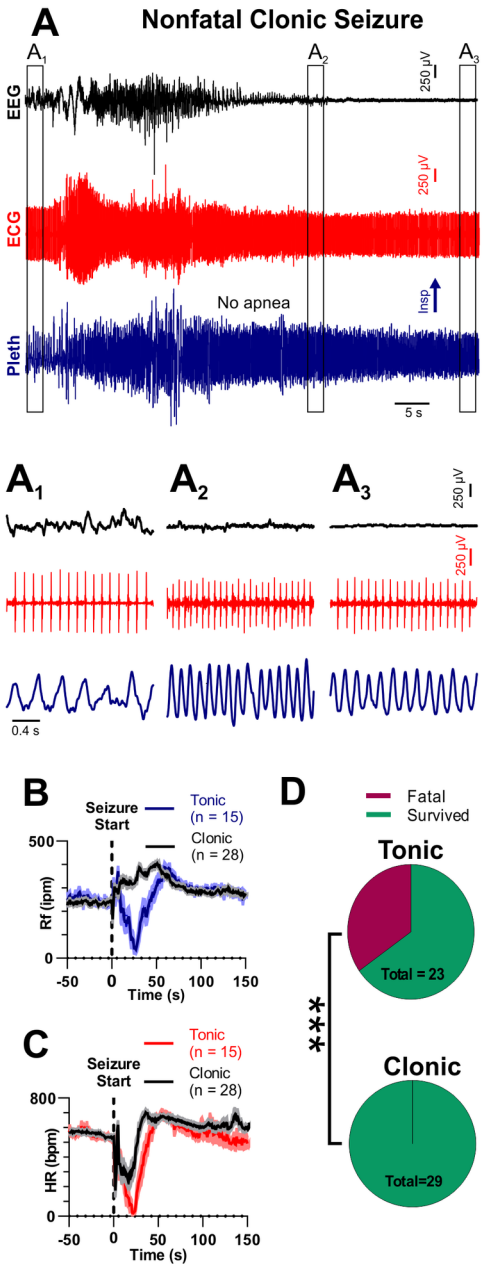

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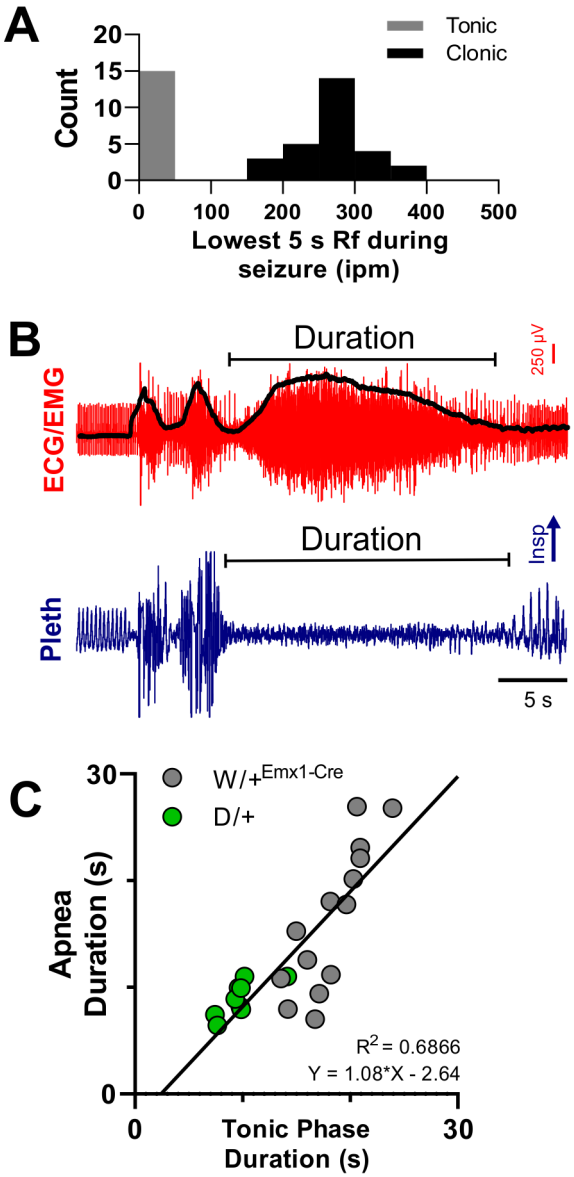
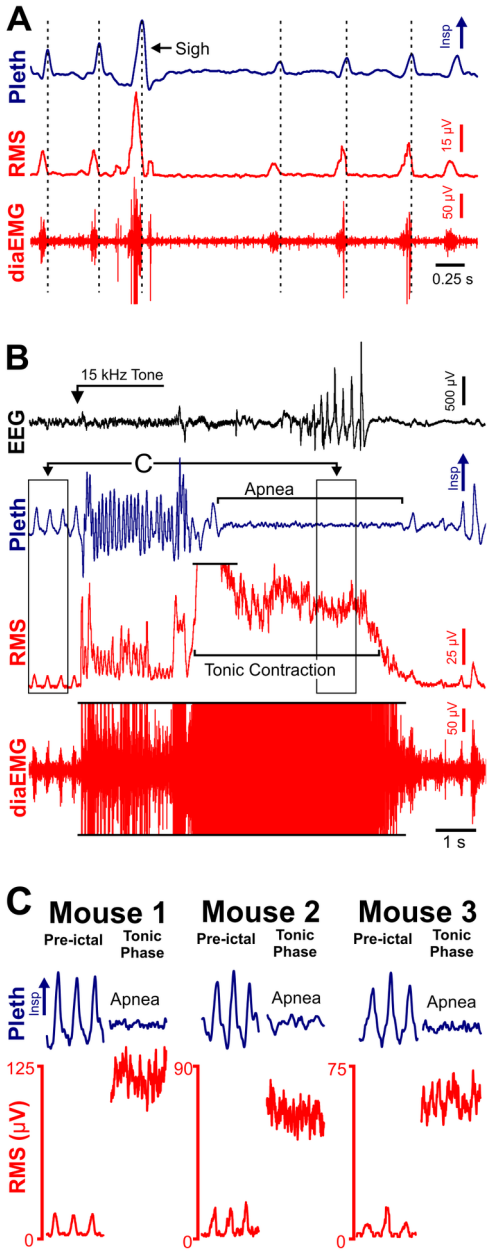
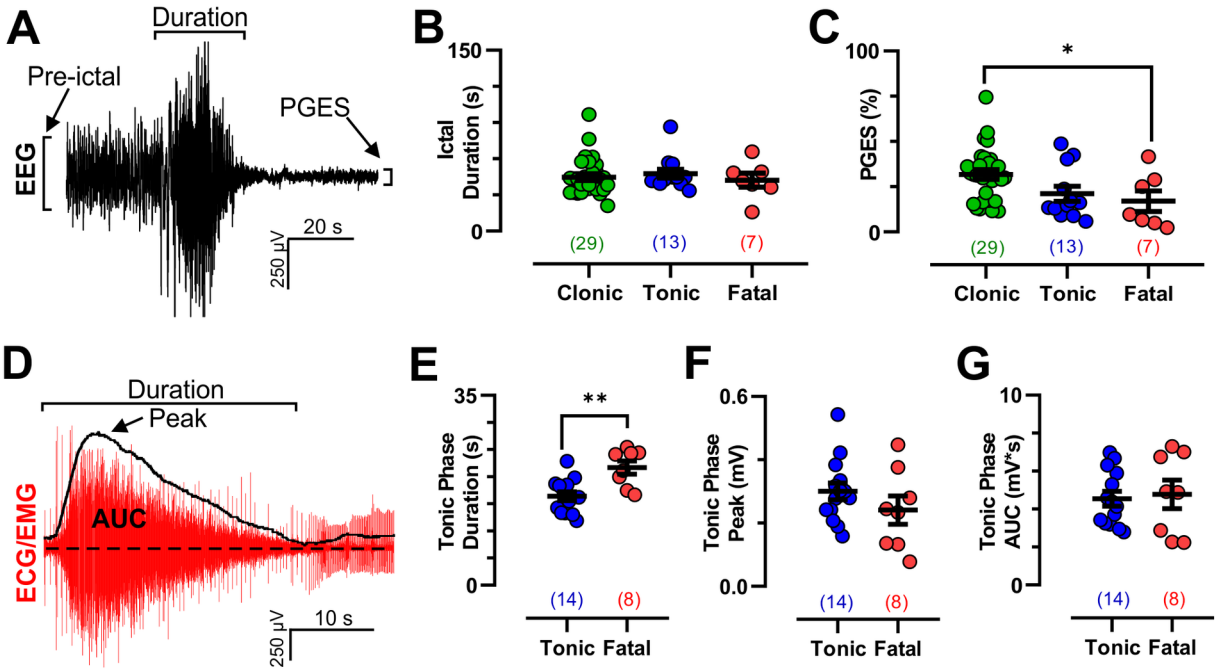


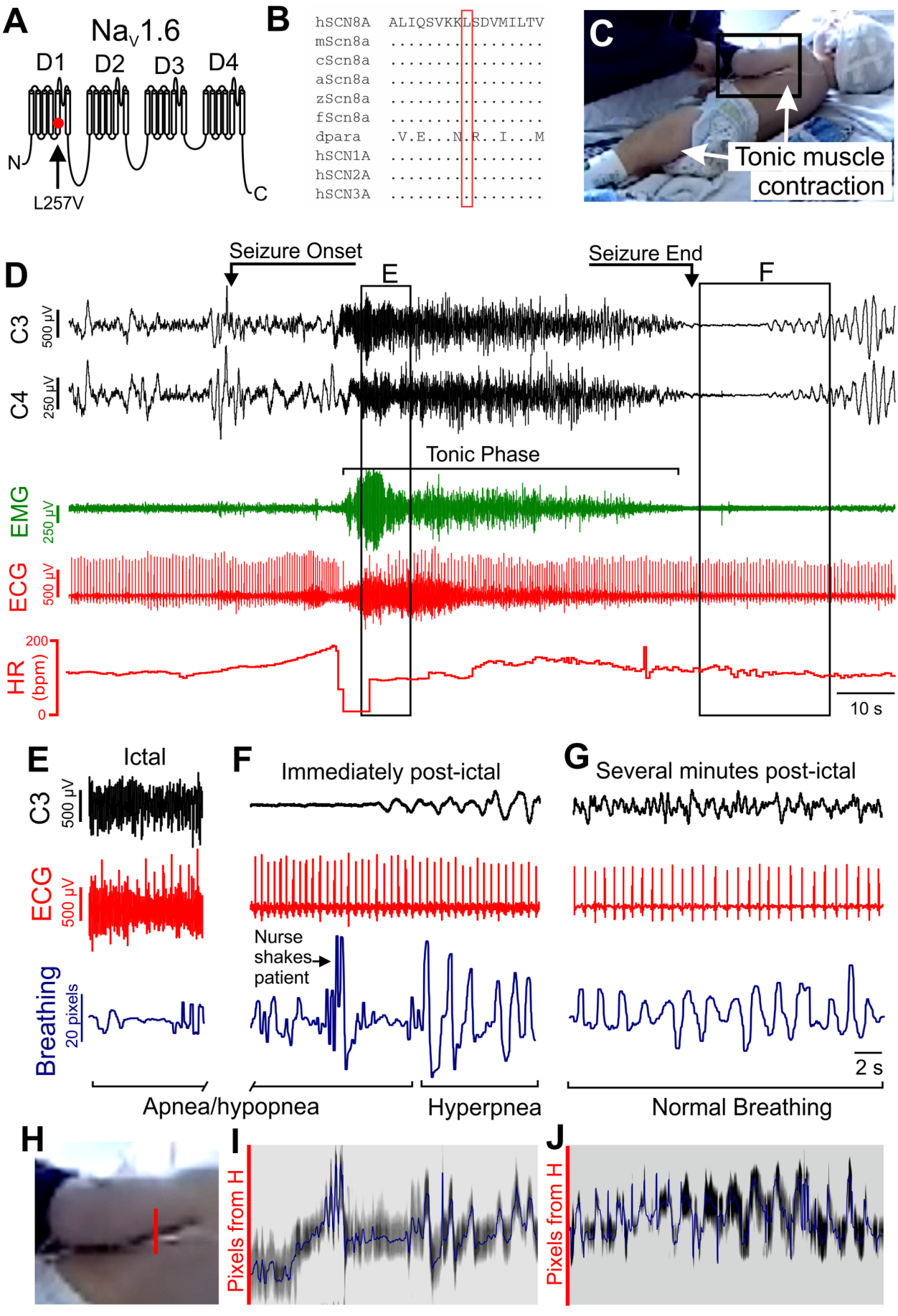
ANA_26053_Fig5.tif



ANA_26053_Fig6.tif



ANA_26053_Fig7.tif



ANA_26053_Fig8.tif