Brainstem activity, apnoea and death during seizures induced by intrahippocampal kainic acid in anaesthetised rats.

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Abstract

Objective: To investigate how prolonged seizure activity impacts on cardiorespiratory function and activity of pre-Bötzinger complex, leading to sudden death.

Methods: Urethane-anaesthetized female Long-Evans rats were implanted with: nasal thermocouple, venous and arterial cannulae, and electrodes for ECG, hippocampal, cortical and brainstem recording. Kainic acid injection into the ventral hippocampus induced status epilepticus.

Results: Seizures caused hypertension, tachycardia and tachypnoea punctuated by recurrent transient apnoeas. Salivation increased considerably: in 11/12 rats, liquid with alkaline pH consistent with saliva was expelled from the mouth.

Most transient apnoeas were obstructive: nasal airflow ceased, while, in 83%, efforts to breathe persisted as continued rhythmic activity of respiratory pre-Bötzinger neurons, inspiratory EMG, and excursions of chest wall and abdomen. Blood pressure oscillated in time with respiratory efforts. This pattern also occurred in a minority of cases (16%) of incomplete apnoea, but not in rare cases (1%) of transient central apnoeas. During transient obstructive apnoeas the frequency of all inspiratory efforts decreased abruptly by ~30% suggesting a re-setting of the central respiratory rhythm generator.

22/31 rats died, due either to obstructive apnoea (12) or central apnoea following progressive slowing of respiration (10). Most rats dying of central apnoea had experienced several transient obstructive apnoeas.

Negative DC field potential shifts of the brainstem followed the final breath, consistent with previous reports on spreading depolarization in mouse models. Timing suggests the DC shift is a consequence rather than cause of respiratory collapse. Cardiac activity continued for tens of seconds.

Significance: Seizure activity in forebrain induces pronounced autonomic activation and disrupts activity in medullary respiratory centres, resulting in death from either obstructive or central apnoea. These results directly inform mechanisms of death in status epilepticus, and indirectly provide clues to mechanisms of SUDEP.
Introduction

Systemic epileptogenic agents directly access all parts of the brain, including those involved in cardiorespiratory control. Seizures are associated with significant autonomic and respiratory changes, which provide a potential cause of sudden unexpected death in epilepsy (SUDEP).\(^1\) The MORTEMUS study\(^2\) revealed 11 cases of SUDEP with cardiorespiratory monitoring. Respiration ceased within 11 minutes of a final generalised tonic-clonic seizure, following both cardiac and respiratory postictal abnormalities. This implicates respiratory failure as a major factor in sudden death.

Animal models are used to investigate how seizure activity may predispose to fatal cardiorespiratory events. Systemic kainic acid (KA) is commonly used to induce seizure activity in rodents, usually status epilepticus followed by repeated shorter-lasting seizures over several hours. Systemic KA produces sympathoactivation with pressor response, tachycardia and lengthening of QT interval.\(^3\)\(^-\)\(^5\) Respiration changes to shallow irregular breathing with gasping breaths interspersed with apnoeas, followed by sudden death due to laryngospasm in some animals.\(^6\)\(^,\)\(^7\)

Two further hypotheses proposed to explain seizure-related apnoeas and fatal cardiorespiratory collapse are: (1) spreading depolarization (SD; similar to spreading depression) propagating from the forebrain into the brainstem, demonstrated in several mouse models;\(^8\)\(^-\)\(^10\) (2) laryngospasm triggered by gastric acid reflux; gastric acid reflux has been shown following systemic KA.\(^11\)

Although autonomic consequences of systemic KA have been attributed to the effects of seizure activity,\(^5\) and sympathetic and parasympathetic brainstem are activated during seizure activity,\(^4\) a contribution from KA acting directly on the brainstem cannot be excluded. Sympathoexcitatory glutamatergic actions at medullary sites are well established,\(^12\) and direct application of KA to the ventral caudal medulla initially stimulates respiration followed by apnoea.\(^13\) The relative contribution to autonomic and respiratory changes of seizure activity versus direct effects of systemically-administered agents on brainstem control centres remains an open question, as does the fundamental question of whether seizure-related death is primarily caused by cardiac or respiratory malfunction.

To test the hypothesis that reported cardiorespiratory consequences, including deaths, of systemic KA\(^6\)\(^,\)\(^7\);\(^11\);\(^14\) were due to seizures, rather than to its direct actions on brainstem sites, we induced seizures by local microinjection of KA directly into the hippocampus of
anaesthetised rats.\textsuperscript{15} Identical injections of dyes and other toxins show they are limited to the hippocampus.\textsuperscript{16} We recorded changes in cardiorespiratory variables that accompanied seizures, together with activity of respiratory-related neurons in the pre-Bötzinger complex, a key brainstem area for generating respiratory rhythm.\textsuperscript{17,18}

Status epilepticus evoked in acute preparations cannot, by definition, model SUDEP directly, but such models do present the opportunity for investigating fatal cardiorespiratory consequences associated with seizure activity. They are directly relevant to potential mechanisms of death in clinical status epilepticus, and may lead to hypotheses on SUDEP that should be tested in chronic models of self-limiting seizures.

\textbf{MATERIALS AND METHODS}

Experiments complied with Institutional Animal Care and Use Committee (IACUC) regulations, and used procedures approved by the Purdue Animal Care and Use Committee (PACUC). Rats lived under a 12:12 dark/light cycle, lights on at 0700, with free access to food and water until induction of anaesthesia.

Thirty-one female Long Evans rats (265-330 g; Envigo, Indianapolis, IN) were anaesthetised with urethane (1.4 g kg\textsuperscript{-1} i.p.), a femoral artery was cannulated to measure blood pressure (BP), as was a femoral vein to administer saline (0.9\%, 0.5 ml bolus i.v.) as required to maintain systolic pressure >100 mm Hg. We excluded 1/31 rats, because its baseline BP (102/54 mm Hg, mean 64 mm Hg) was much lower than the group mean (83.9±10.4 mm Hg) and failed to respond to saline injection. A thermostatically-controlled heating blanket maintained rectal temperature at 37\degree C (507±2\degree F, Harvard Apparatus, Cambridge, UK).

Urethane anaesthesia can lead to hyperglycaemia in fasted rats,\textsuperscript{19} but this effect is not significant in fed rats.\textsuperscript{20} Therefore the proconvulsant effect of hyperglycaemia\textsuperscript{21} is not a confound in the present studies, which do not involve fasting. The oestrous cycle of the female rats could affect seizure susceptibility and increase variability in this study, however, oestrous cycle is reported not to affect duration or severity of status epilepticus induced by intrahippocampal KA.\textsuperscript{22}

The animal was mounted in a stereotaxic frame with the bregma-lambda axis horizontal. Measurements of nasal air temperature oscillations monitored respiration, using a thermocouple (5TC-TT-K-36-36, Omega, Egham, UK) in the nasal passage: in 12 rats
inserted through a small bur hole 7 mm rostral to the suture with the frontal bone, in 19
inserted into the nares. Rats are obligate nose-breathers, so that respiratory airflow can be
detected by changes in temperature in the nasal passage or outside the nares. This inferred
airflow parallels movements of the chest wall. ECG was recorded by percutaneous needle
electrodes inserted at right shoulder and left caudal thorax. In 11 rats the ECG contained a
component of inspiratory EMG activity, extracted by thresholding low-amplitude troughs
(−0.01 mV, c.f. −1 mV ECG; Fig. 1). EMG-related events dominated sufficiently to make it
unnecessary to remove ECG-related events (compare red and black events during first breath
in Fig. 1). A respiration-related waveform was obtained using a Gaussian kernel.

Several bur holes were drilled in the skull for probes. A Hamilton microsyringe containing
KA (10 mg.ml−1) was positioned with its tip in right ventral hippocampus (5.4 mm caudal, 4.5
mm lateral to bregma, 6.8 mm below cortical surface); the target was CA3 stratum radiatum.
ECoG was recorded from pairs of skull-screw electrodes inserted 2.5 mm caudal and 1.0 mm
rostral to bregma, both 2.0 mm lateral of midline. In some rats we also recorded local field
potential activity from ipsilateral dorsal hippocampus using bipolar 125-µm diameter
platinum-iridium twisted-pair wire electrodes 3.6 mm caudal and 3.0 mm right of bregma,
and 3.0 mm below cortical surface. Filters were 0.1-1000 Hz. Respiratory unit activity was
recorded with 25-µm diameter parylene-insulated Pt/Ir wire, inserted using a system which
rotates the electrode to allow it to penetrate the brain without bending. The initial target in
right brainstem pre-Bötzinger complex was 12.8 mm caudal and 0.6 mm left of bregma, for a
10° angle in the coronal plane to reach the target 1.9 mm right of bregma. If no respiratory-
related neurons were found in the first track, the angle of subsequent tracks was varied up to
14°. Track length to recorded units was 6.7–7.8 mm below cerebellar surface. Recording
bandwidth was 300-3500 Hz. In 5 experiments we also recorded DC to 3.5 kHz from the
contralateral pre-Bötzinger region using a Teflon-coated 125-µm silver electrode conditioned
by passing current for 6x1-minute periods (0.3 mA, normally requiring 2 V, sufficient for
electrolysis), alternating direction to finish with the silver electrode negative. Reference
electrode was placed in neighbouring scalp.

After preparation was complete, the brainstem recording electrode was advanced into the pre-
Bötzinger complex until a neuron was isolated, with activity phase-locked to the nasal
thermocouple signal. After ≥15 min baseline recording, 1-2 µl of KA was injected into the
hippocampus over 5 min to induce seizure activity. Concern over the potency of our samples
of KA led to use of higher doses, however dose had no significant effect on any of the
cardiorespiratory changes (Pearson correlation significance p>0.1 for each). Time between induction of anaesthesia and injection of KA. Time between induction of anaesthesia and injection of KA averaged 295±107 min, the main source of variation being time required to find units.

Data were captured using Neurolog (Digitimer, Welwyn, UK), Grass (Grass Instrument Co, Quincy MA) and custom-made amplifiers, and a Power 1401 signal acquisition unit with Spike2 software (Cambridge Electronic Design, Cambridge, UK). Analysis was performed in Spike2. Off-line signal conditioning included: the “HumRemove” script to remove mains-related noise (particularly for brainstem units), smoothing (50 ms time-constant for nasal temperature signals), the peak detection feature of Spike2 provided heartrate from the BP signal and respiratory rate from the nasal temperature signal.

Statistical analyses used SPSS version 23 (IBM, New York). Results are expressed as mean ± SD unless otherwise stated. Data were tested for normality, and non-parametric tests used where appropriate.

RESULTS

Cardiorespiratory changes during seizure activity

Following intrahippocampal KA, spontaneous electrographic activity attenuates and is replaced by seizure activity (rhythmic synchronous field potentials in hippocampal and/or cortical recording electrodes; Fig. 2). Seizure activity develops in all animals, starting 1.5-8 min after the start of the injection, and is associated with pronounced cardiorespiratory changes characterised, in all but one animal, by hypertension, tachycardia and hyperpnoea (Fig. 2). Mean arterial pressure increases from 83.9±10.4 to 138.3±19.6 mm Hg (paired t-test; p<0.001), heartrate rises from 342.1±48.5 to 486.8±38.2 beats min⁻¹ (paired t-test; p<0.001), respiratory rate more than doubles from 97.5±30.0 to 226.0±91.2 breaths min⁻¹ (related-samples Wilcoxon Signed Rank Test; p<0.001). Time course of cardiorespiratory responses varies between rats, but all follow a similar pattern. Time from start of injection until onset is: BP 204.9±149.1 s, heartrate 500.3±324.1 s, and respiratory rate 402.8±301.2 s. Most rats develop a prominent, usually bilateral, exophthalmos, and rapid vibrissal movements. Other motor signs of seizures observed when KA is administered to conscious rats, are absent.

Augmented breaths
Augmented breaths (sighs) comprise prolonged inspiration and increased tidal volume followed by a respiratory pause and several seconds of faster breathing (Fig. 3).\textsuperscript{26}

For quantification, we define an augmented breath as an excursion of the nasal air temperature signal $\geq 2x$ baseline amplitude. Before KA injection augmented breaths are infrequent: 30% of rats make none; incidence in the remainder is 0.34±0.21 min$^{-1}$. In all but one of the rats in this latter group, augmented breaths cease for 10.2±8.4 min following KA injection, before resuming at a significantly higher rate (2.2±1.2 min$^{-1}$; p<0.001, paired t-test). In the rats without augmented breaths before KA injection, they appear in all but one rat between 6 and 65 min (median 17) after the injection start, continuing at 1.81±1.5 min$^{-1}$. As tachypnoea strengthens with seizure progression, the number of augmented breaths increases substantially as a fraction of all breaths (Fig. 3).

**Transient apnoeas.**

The most common respiratory disturbances are transient apnoeas, which developed in all but two rats. Transient apnoeas are characterised by abrupt cessation of nasal air temperature oscillations, followed a variable time later by an abrupt resumption (Fig. 4). We use 5-s as a minimum duration for measuring apnoeas; shorter apnoeic periods also occur, sometimes in clusters. If no or few apnoeas develop by 2 h after intrahippocampal KA (in 2 rats), we give a second injection to promote apnoeas. Peak cardiorespiratory changes occurred before the second injection in both cases. The latency to first apnoea from the start of the preceding (or only) KA injection is 4-149 min, mean 63.3±44.4 min. The one rat with no apnoeas until the second injection has a latency of 125 min from the first injection, and increases the mean latency to 69.0±44.2 min. We analyse 267 apnoeas from 23 rats. The number analysed varies considerably between rats (2-39 per rat, 11.4±10.8); duration of each ranges between 5-15 s (10.6±7.1 s).

**Obstructive apnoeas.**

Despite inter-animal variability in frequency and timing, the majority of transient apnoeas displayed features consistent with airway obstruction (Fig. 4A).

*Activity of neurons in the pre-Bötzinger region.* Pre-Bötzinger neurons show ongoing phasic activity time-locked to nasal temperature oscillations. Firing typically precedes onset of inspiration (decrease in nasal air temperature) by 140-170 ms (Fig. 4). In some cases of low signal/noise ratio we measure root-mean-square amplitude of the brainstem recording to
detect multi-unit activity. We record phasic respiratory-related units from the pre-Bötzinger region during 61 apnoeas in 8 rats. When nasal airflow ceases, the units continue to fire rhythmically but at a significantly lower frequency than for eupnoea. Burst rate during apnoeas decreases from 119.3±25.1 to 73.6±18.4 min⁻¹, p<0.0005, paired t-test), a 37.8±10.2% reduction in frequency of oscillations of the Bötzinger respiratory rhythm generator. In 14 apnoeas lasting >10s (14.7±3.75 s) the frequency of oscillations in unit firing during the first 5s and last 5s do not differ significantly (71.0±11.8 min⁻¹ vs. 68.3±15.2 min⁻¹; p>0.05, paired t-test) suggesting that the network oscillator switches to a new state at the onset of apnoea rather than slowing down gradually as the rat struggled ineffectively to breath. Once breathing re-starts, unit firing returns abruptly to the rate time-locked to the nasal signal.

Respiratory effort during apnoeas. Visual observation during periods of arrested nasal airflow clearly showed the rats continued to make respiratory effort, evidenced by movements of the thorax and abdomen. In 11 rats the signal recorded from the chest leads contained in addition to ECG, an EMG component time-locked to the inspiratory phase of the nasal air temperature signal (Fig. 1; Fig. 4A). By extracting this component, we monitor inspiratory EMG activity during apnoeas. In cases of simultaneous recording of EMG and pre-Bötzinger unit activity, their frequencies correspond closely. As with unit activity, respiratory EMG continued at a significantly lower frequency during arrested nasal airflow. Recordings of 94 apnoeas reveal the frequency of inspiratory EMG activity decreases from 124.4±44.8 to 75.9±21.9 min⁻¹; p<0.0005, paired t-test, confirming continued but slower respiratory effort when nasal airflow had ceased.

BP and heartrate. During apnoeas BP become unstable and oscillations develop at the same frequency as the continuing inspiratory effort, likely reflecting changes in venous return due to large fluctuations in intrathoracic pressure as the rat strived, ineffectively, to breath. These observations are consistent with cessation of nasal air flow due to airway obstruction.

A minority of transient apnoeas share features with obstructive apnoeas outlined above, but with an abrupt, >50% reduction in amplitude, rather than complete cessation, of the nasal airflow signal (42 episodes in 3 rats and one each in two others). These partial apnoeas last 5-12 s (7.1±2.9 s). Like the complete apnoeas described above, onset and offset are abrupt and unpredictable. Although effective respiration continued, the frequency of the nasal temperature signal reduces from 186.4±64.1 min⁻¹ to 100.6±33.9 min⁻¹. Oscillations in the BP
signal are at the same frequency as respiration. One rat with simultaneous recordings of both pre-Bötzinger region units and inspiratory muscle EMG extracted from the ECG signal, the frequency of both decreases in line with the nasal airflow signal.

**Central apnoeas**

Four transient apnoeas from 3 rats lack evidence of continued respiratory effort during cessation of nasal air flow (Fig. 4C, lasting 21.0±9.9 s), suggesting failure of central respiratory drive mechanisms. In these cases, rhythmic EMG and BP oscillations cease during the apnoea. With continuing apnoea, heartrate slows by 144.5±102.9 beats min\(^{-1}\) and BP falls by 20.1±11.4 mm Hg. On 3 occasions obstructive apnoeas precede a central apnoea, separated by a single breath (Fig. S2).

**Oral secretions.**

In several early experiments we noted an accumulation of liquid in the mouth towards the end of the experiment. In 12 rats we collect overflowing liquid, which was found in 11 (weight 30-300 µg). The liquid proved to be alkaline (pH 9), consistent with rat saliva.\(^{27}\)

**Spontaneous deaths**

Twenty-two rats die spontaneously after injecting KA. We terminate the experiment if either the time under anaesthesia exceeds 7.5 hours or 4 hours elapses after KA injection (8 rats). Twelve rats die of obstructive apnoea, 119.5±46.0 min after injection of KA (Fig. 5A, Fig. 6A, Fig. S1B). The onset of terminal apnoea does not appear to differ from the preceding transient apnoeas (3-39). After nasal airflow flow ceases, respiratory effort (from brainstem activity, EMG and/or visual observation) continues for ≤43 s before BP and heartrate begin a sharp terminal decline. Median delay from last breath to the start of the rapid drop in heartrate is 15 s, (interquartile range 9-18 s; Fig. S1). Active inspiratory efforts in the absence of nasal airflow suggest that the cause of death is obstructive apnoea. In 11/12 rats recording of pre-Bötzinger neuronal activity continues through the final apnoea. In 9 an intense desynchronised electrical discharge begins 17.9±8.6 s from the cessation of nasal airflow, increasing from 15 to 150 Hz (median increase 140 Hz, interquartile range 56-243 Hz; Fig. 5A, Fig. S1).

The remaining 10 lethal apnoeas are not obstructive: evidence of respiratory drive stops at the last breath (from pre-Bötzinger activity in 4 cases, BP oscillations in 8, and/or EMG in 8), suggesting a central mechanism. In 3/10 rats the primary response to intrahippocampal KA is
progressive slowing of respiration culminating in death within 34 min. In the remaining 7, intrahippocampal KA initially produces tachypnoea punctuated with obstructive apnoeas. Then 2-13 min after respiratory rate returns to baseline, respiration slows progressively until the rat dies, a total of 48.0±33.2 min after KA injection, excluding one outlier of 225 min (Fig. 5B, Fig. 6B). These rats lack the large desynchronised electrical hyperactivity of the brainstem associated with lethal obstructive apnoeas (median firing increase 0 Hz, interquartile range –6 to 0 Hz Fig. 6B, Fig. S1A). Cardiorespiratory responses to KA do not differ between rats dying from central or obstructive apnoea, with the exception that central cases started with faster baseline heartrate than obstructive cases (356±26 vs 313±43 bpm; t-test p<0.05; Supplemental Fig. S3).

**Cardiac function during terminal apnoea.**

BP and heartrate deteriorate following the collapse of respiration. Heartrate decreases rapidly 10-20s after the last breath (Fig. 5, Fig. 6, Fig. S1C-E). Obstructive and central terminal apnoeas do not differ in extent, latency or duration of the rapid decrease in heartrate (Fig. S1 C-E). They only differ in the late brainstem discharge and in the nature of the lethal apnoea.

**Brainstem DC changes.**

DC pre-Bötzinger recordings in 5 rats reveal no consistent changes in DC signal during transient apnoeic periods. Prolonged negative DC shifts are linked to spreading depolarisation. We assess the temporal relationship of DC shifts in pre-Bötzinger potential, defined by time course slower than 20s, with respiratory arrest and ECG changes. In all cases a terminal DC shift of −2.5±1.2 mV follows the final effective breath after 16.5±13.3 s, whether obstructive or central (Fig. 6 A, B). In the case of obstructive apnoeas, a burst of neuronal firing precedes the negative shift. The relationship of the rapid decrease of heartrate with brainstem bursts and DC shifts is more variable. In 2/5 cases (1 obstructive, 1 central) the heartrate drop precedes pre-Bötzinger DC shifts; in the remaining 3/5 (2 obstructive, 1 central), the heartrate drop follows DC shifts.

**DISCUSSION**

Status epilepticus evoked by intrahippocampal KA causes pronounced changes in cardiorespiratory function. As seizures develop, BP, heartrate and respiration rate increase substantially. Tachypnoea is interrupted periodically by transient apnoeas. Of 22 rats that die
following KA injection, 12 (55%) die from obstructive apnoea, failing to breath despite continuing, ineffective, centrally-driven respiratory efforts. In the remaining rats, death also results from respiratory failure but without evidence of efforts to breath; 3 die following progressive slowing of respiration after KA injection, 7 experience tachypnoea before progressive slowing and death.

**Cardiovascular changes.**

The tachycardia and hypertension following intrahippocampal KA resembles previous reports on systemic convulsants in anaesthetised rats and cats, which implicate pronounced sympathactivation alongside near-maximal increases in parasympathetic activity. Others report bradycardia with arrhythmia. This discrepancy could depend on relative levels sympathetic and parasympathetic activity and/or on starting heartrate. Other confounds include: strain differences in susceptibility, sex, type of anaesthetic, convulsant and route of administration. Unlike focal injections into a restricted area of the brain, systemic administration of convulsants may include widespread non-seizure-related responses to actions at multiple cardiorespiratory centres.

**Respiration.**

KA injection into ventral hippocampus causes a substantial increase in respiratory rate, consistent with previous findings from SE induced acutely by systemic agents in anaesthetised rats, cats and piglets. In contrast, slowing of respiration is reported following focal ventral hippocampal microinjection of another excitatory amino acid (DLH). However, that effect is short-lasting (<20min) and selective for CA1 pyramidal layer. Time-limited (1-2 min) seizures induced by brief (2-s) electrical stimulation of dorsal hippocampus also slow respiration, attributed to alterations in serotonergic neuron activity in the raphe nuclei. The contrast with the substantial long-lasting increase in respiratory rate we find likely is due to the prolonged seizures induced by larger-volume injections of KA. Urethane anaesthesia raises basal respiratory rate in both mice and rats. We report that status and recurrent seizures substantially increase respiration rate from the baseline established under urethane. It is conceivable that the cumulative effects of urethane and seizures drives tachypnoea to levels that trigger fatal apnoeas, however deaths occur after the tachypnoea passed its peak, so we conclude that this is unlikely. As with all acute experiments under anaesthesia we need to remember that anaesthetics affect brain and
systemic physiology, and that conclusions ultimately need to be tested in unanaesthetised preparations.

Microinjection of DLH into CA1 suppresses augmented breaths for several minutes, as we report here for KA. However, the DLH study does not report the subsequent long-lasting increase in frequency of augmented breaths found in our experiments. Differences most likely result from different patterns of activity imposed on brainstem respiratory centres by seizure- and non-seizure-associated activation of the ventral hippocampus.

**Transient apnoeas.**

Prolonged seizures induced by KA, both intrahippocampal and systemic, lead to transient apnoeas of similar duration and appearance. Their incidence is considerably greater following intrahippocampal administration, and almost all are obstructive: airflow ceases, brainstem respiratory neurons continue firing rhythmically, matched by respiratory muscle activity and intrathoracic pressure oscillations large enough to modulate BP. In marked contrast, transient apnoeas following systemic KA occur with an open glottis, leaving failure of central respiratory drive as the only plausible mechanism. The reason for this difference is unclear; we can exclude differences in rat strain and sex, and in recording methods because we also find transient central apnoeas when we inject KA systemically (R.B.Budde, D.J.Pederson, E.N.Biggs, J.G.R. Jefferys, P.P.Irazoqui, submitted). Four transient apnoeas following intrahippocampal KA (in 3 rats) are central (Fig. 4C): rhythmic activity ceases of both respiratory units and respiratory muscles, and BP oscillations fail to appear. They cause transient progressive bradycardia and decline in BP.

During transient obstructive apnoeas, the pre-Bötzinger respiratory rhythm resets to a lower frequency. This effect is not secondary to occlusion of the airway since a similar slowing of respiration occurs during partial apnoeas, with reduced rather than absent airflow. The lower frequency remains constant during each apnoea, suggesting a state change in the respiratory rhythm generator that coincides with the cessation of effective respiration. This could be a feature of local networks in the pre- Bötzinger complex; alternatively, control of frequency could switch to other parts of the wider brainstem respiratory rhythm generator. A key question is how obstruction of the airway is associated with respiratory rhythm. Laryngeal motor neurons are located in nucleus ambiguus and receive direct inputs from hippocampus. During apnoeas the abnormal activity from the forebrain could activate
laryngeal pre-motor neurons in parallel with switching the respiratory rhythm network to a lower frequency.

**Lethal apnoeas.**

The majority of rats in our study die from a lethal apnoea after prolonged seizures. Most apnoeas are obstructive, on the basis of continuation of respiratory effort, but a substantial minority are central. Systemic KA also leads to lethal apnoeas, but always obstructive, and associated with laryngospasm resulting from increased descending activity in the recurrent laryngeal nerve.

**Hypotheses for lethal apnoeas**

*Gastric acid reflux.* Previously we reported that systemic administration of KA caused gastric acid reflux, which could provoke laryngospasm. However, KA application to brainstem structures is reported to stimulate production of gastric acid, raising the possibility that this effect is independent of seizures. The salivation we report here does not support gastric acid reflux, but cannot exclude the mechanism. The rapid onset and termination of the brief transient obstructive apnoeas also are hard to reconcile with laryngospasm triggered by oesophageal acid.

*Brainstem spreading depolarization (SD)* is proposed as a mechanism for cardiorespiratory failure. Mice with two epilepsy-related genetic modifications develop brainstem SD following induced seizures, as does a familial hemiplegic migraine mutation. Another mutation, associated with sudden cardiac death and SUDEP, reduces SD threshold in both neocortex and brainstem. Our DC recordings from the brainstem of wild-type rats show the DC shift in pre-Bötzinger always follows the cessation of airflow at the onset of terminal apnoea, whether obstructive or central, which is inconsistent with SD causing apnoea. Precise timing is crucial, and needs to relate airflow (not only respiratory effort) to SD or other neural pathophysiology. Anaesthesia in mice delays the lethal apnoea and SD, but does not change the threshold, amplitude and propagation rate of cortical spreading depression, from which we conclude that while the timing of apnoea and spreading depolarisation may be slowed by anaesthesia, the fundamental pathophysiology is likely preserved.

Obstructive terminal apnoeas are associated with brainstem hyperactivity that could be consistent with the onset of SD. The absence of this hyperactivity from terminal central apnoeas shows it is not required for SD. In both cases the most likely cause of DC shifts in extracellular fields is K⁺ efflux resulting from hypoxia once respiration has ceased. The
timing of cardiac decline is less clear. Terminal cardiac decline/collapse always follows respiratory arrest, but the most rapid decrease in heart rate can either precede or follow the onset of the DC shift in pre-Bötzinger. We cannot exclude SD or a similar event originating in dorsal medullary cardiovascular control centres contributing to cardiac failure: the slow propagation velocity of SD requires additional DC recordings in cardiovascular brainstem structures to address that question. However, it also is possible that DC shift and cardiac rundown are united by dependence on hypoxia.

In seeking alternative mechanisms for lethal apnoeas we need to consider obstructive and central apnoeas separately. Obstructive apnoeas are likely due to closure of the larynx, potentially due to hyperactivity of laryngeal motor neurons in nucleus ambiguus, perhaps driven by descending drive from seizing forebrain structures. In contrast, central apnoeas may be due to suppression of the brainstem, possibly analogous to post-ictal EEG suppression. The absence of brainstem hyperactivity preceding SD following central terminal apnoeas is consistent with reduced excitability.

Conclusions

Prolonged seizure activity induces dramatic cardiorespiratory disturbances, which can lead to sudden death due to failure of respiration. Deaths during acute SE in animal models under anaesthesia is a model of death from status rather than from SUDEP. However, they do open a window on how seizure activity impacts on the respiratory control network. The choice of model is important. Whilst both systemic and focal administration of epileptogenic agents produce the same lethal consequences, cessation of respiration, the underlying mechanisms differ. In particular, direct actions of the stimulant on brainstem control centres may be a confound during seizure activity evoked by systemic administration of epileptogenic agents. Nevertheless, all studies point to apnoea as a likely cause of death in SUDEP and highlight the need for further studies in chronic epilepsy models.

Ethical publication

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.

Conflicts of Interest

Authors declare that they have no conflicts of interest.


Supplemental Material

**Supplemental Fig. S1**
Brainstem discharge and heart rate collapse following final breath. **A.** Increased maximal brainstem firing rate 10-20 s after final breath or attempt to breath as a multiple of maximal firing rate during the previous 10 s (change: $p<0.001$ Mann-Whitney U test). Firing rate is measured by rate of crossings of a threshold voltage. **B.** Obstructive fatal apnoea showing continued rhythmic activity of brainstem respiratory neurons after final breath, followed by large neuronal discharge (shown on r.m.s., raw signal and firing rate). ECG shows visible slowing, quantified in the heart rate trace below. The remainder of this figure concerns the most abrupt section of the heart rate drop, which is clearly visible in the raw ECG trace. **C.** The rapid drop in heart rate does not differ significantly between rats dying from obstructive (red lines) and central (dashed blue lines) apnoeas. Each line represents one rat ($p=0.014$; HR before drop is significantly lower for central apnoeas, $p\leq0.003$, but HR after is not, $p=0.14$). **D.** Latency to onset of rapid HR drop does not differ between obstructive and central terminal apnoeas ($p=0.18$, Mann-Whitney U test). **E.** Duration of rapid HR drop does not differ between obstructive and central apnoeas. Statistics are cited in the main text ($p=0.70$, Mann-Whitney U test).

**Supplemental Fig. S2**
Sequential obstructive and central transient apnoeas. Prolonged (26-s) apnoea split by a single breath with a preceding obstructive apnoea lasting 15 s and a subsequent central apnoea lasting 10.5 s. Respiratory brainstorm activity is visible during the obstructive apnoea but not during the central apnoea. Blood pressure oscillates with attempts to breath during obstructive apnoea, but declines smoothly during central apnoea, and also at the very start of the whole event.

**Supplemental Fig. S3**
Physiological changes preceding deaths by apnoea that are obstructive (boxes labelled “o”) and central (“c”). Seizure-related increases in BP ($\Delta$ BP) and respiration rate ($\Delta$ RR) did not differ between the two groups (t tests $P=0.32$ and 0.8, respectively), but heart rate did ($\Delta$ HR; t-test $P<0.005$). All 3 physiological measures approach their maximal values during seizures, including heart rate, so maximal heart rate did not differ between the two groups ($P=0.12$) but the baseline heart rate preceding KA injection did ($P<0.04$). The relevance, and robustness, of this observation remains to be determined.