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Forebrain control of breathing and sudden death in epilepsy

Edited by
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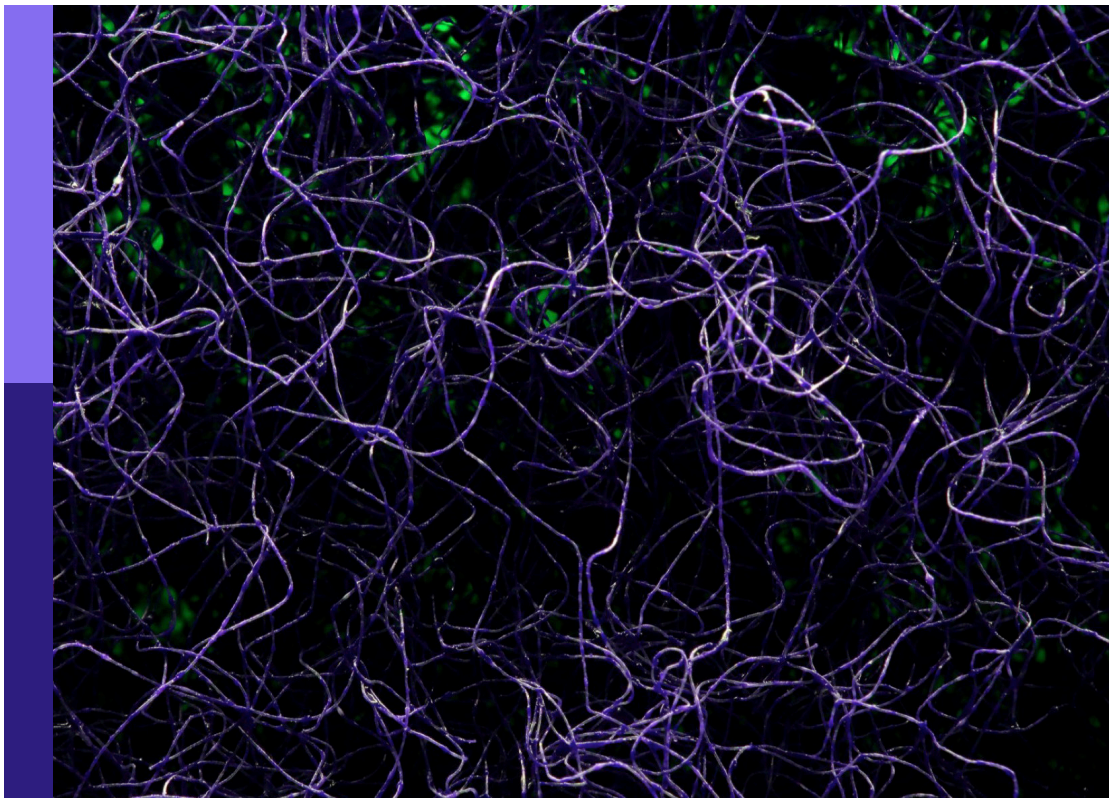

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Forebrain control of breathing and sudden death in epilepsy

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Table of contents

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Editorial: Forebrain control of breathing and sudden death in epilepsy

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KEYWORDS

forebrain, control of breathing, sudden death in epilepsy, SUDEP, epilepsy, respiration, seizure

Editorial on the Research Topic
Forebrain control of breathing and sudden death in epilepsy

Sudden Unexpected Death in Epilepsy (SUDEP) is the unexpected death of a person with epilepsy who was otherwise healthy and no other cause of death was found (Nashef et al., 2012). SUDEP is the second leading neurological cause of lost years of life, behind stroke, and is the leading cause of death among persons with drug-resistant epilepsy (Thurman et al., 2014). Both clinical and basic research studies have implicated respiratory deficits as a major contributor to SUDEP (Devinsky et al., 2016). For example, a study of patients who died while in epilepsy monitoring units found that all SUDEP cases exhibited post-seizure cardiorespiratory abnormalities, with terminal apneas preceding terminal asystole (Ryvlin et al., 2013). However, our current understanding of the causes of respiratory abnormalities in persons with epilepsy that may lead to SUDEP is incomplete.

The forebrain is the most common site of seizures and contributes to the regulation of breathing through connections to brainstem respiratory regions and/or spinal circuits that control respiratory motor neurons. Schottelkotte and Crone review the forebrain regions that have been implicated in the control of breathing. They describe functional evidence of a role in breathing for forebrain structures that include the cerebral cortex, hippocampus, amygdala, thalamus, and hypothalamus. A better understanding of the role of each forebrain region in the control of breathing may improve our understanding of how respiratory failure might occur in cases of SUDEP.

SUDEP is thought to occur primarily during sleep (Buchanan et al., 2021). Joyal et al. review the relationships between sleep state/time of day, seizures, and respiratory function and how they could contribute independently to the risk of SUDEP. Other factors, such as neurotransmitter levels, airway obstruction and arousal responses, may also contribute. It is important to note that increased monitoring during sleep using devices or having another person sleep in the same room can decrease SUDEP risk (Harden et al., 2017).

Several hypotheses have been proposed to explain how epilepsy could disrupt the control of breathing. Mulkey and Milla review potential mechanisms by which seizure activity in the forebrain could propagate to the brainstem to affect breathing. They describe how seizures could activate descending inhibitory drive to brainstem respiratory nuclei or how spreading depolarizations within the brainstem could impair breathing. A critical role for astrocytes as well as epilepsy related mutations in this process is discussed. Bauer et al. review *in vivo* and *ex vivo* techniques to further study the potential mechanisms of SUDEP in animal models. They describe how synaptic or non-synaptic (i.e., spreading depolarizations) mechanisms could disrupt brainstem regions important for breathing and/or cardiac function in epilepsy. Original research by Wenker et al. refute the hypothesis that cortical neurons directly drive apnea in the Scn8a mouse model of epilepsy. They found that silencing forebrain neurons could reduce seizure threshold but did not prevent apneas during the tonic phase of seizures. They propose that brainstem circuitry most likely drives tonic phase apneas that are thought to contribute to SUDEP. Different mechanisms (or combinations of risk factors) may be engaged in different people, necessitating additional research using a variety of approaches and animal models.

There are currently no established biomarkers to identify individuals that are vulnerable to SUDEP. Potential predictors of SUDEP under investigation include impaired chemosensory responses, depressed arousal/resuscitation responses, altered brain activity, abnormal respiratory patterns, or altered neurotransmitter release. For example, prior studies have shown that some epilepsy patients have a decreased ability to respond to increased levels of carbon dioxide in the blood, which correlates with longer episodes of respiratory depression following a seizure (Sainju et al., 2019). Altered chemosensory reflexes could impair auto-resuscitation following a seizure, increasing SUDEP risk. Hampson et al. use functional magnetic resonance imaging (fMRI) to examine changes in brain activity during increased CO_2 exposure in persons with epilepsy compared to healthy controls. They show enhanced activation of the dorsal raphe (which contains CO_2 sensitive serotonergic neurons) as well as altered functional connectivity between the brainstem and adjacent subcortical areas in persons with epilepsy. These results suggest that the respiratory system may be less able to handle respiratory challenges in persons with epilepsy. Ciumas et al. review the current methods to use fMRI to investigate central respiratory control in human patients. The authors discuss the challenges associated with studying the brainstem nuclei involved in breathing and summarize the various methods that can be used to overcome these challenges. They review examples of studies that have used fMRI to study breathing in various conditions, including epilepsy. Functional imaging is a promising tool that may be useful for identifying individuals that are most at risk for SUDEP as well as probing the mechanisms leading to SUDEP.

There are currently no therapeutic approaches to prevent SUDEP and research in this area presents many challenges, including difficulty assessing which patients are at risk, insufficient understanding of the underlying mechanisms, a lack of animal models to recapitulate all aspects of SUDEP, and the diversity of patients at risk for SUDEP. Bauer et al. and Mulkey and Milla describe the current state of research aiming to prevent cardiorespiratory abnormalities that could lead to SUDEP. These include methods to stimulate (or inhibit) specific brain regions implicated in SUDEP as well as methods to prevent initiation or propagation of spreading depolarizations. Two original research articles describe potential novel treatment targets: Collard et al. work indicates that the neurotransmitter Galanin holds promise as a potential future treatment because either systemic or central nervous system delivery of galanin analogs could prevent seizure induced respiratory arrest in mouse electroshock seizure models. Fukushi et al. show that inhibition of microglia activation with minocycline delayed seizures induced by hypoxia and reduced the incidence of post-seizure respiratory arrest.

Epilepsy patients may undergo a vicious cycle whereby seizures impair respiratory responses and impaired breathing increases the incidence or severity of seizures. The complicated interactions between epilepsy and breathing control indicate that collaborations between researchers and clinicians investigating epilepsy and experts on the control of breathing could advance our understanding of SUDEP and accelerate prevention.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

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ORIGINAL RESEARCH
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Functional MRI Correlates of Carbon Dioxide Chemosensing in Persons With Epilepsy

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Objectives: Sudden unexpected death in epilepsy (SUDEP) is a catastrophic epilepsy outcome for which there are no reliable premortem imaging biomarkers of risk. Percival respiratory depression is seen in monitored SUDEP and near SUDEP cases, and abnormal chemosensing of raised blood carbon dioxide (CO_2) is thought to contribute. Damage to brainstem respiratory control and chemosensing structures has been demonstrated in structural imaging and neuropathological studies of SUDEP. We hypothesized that functional MRI (fMRI) correlates of abnormal chemosensing are detectable in brainstems of persons with epilepsy (PWE) and are different from healthy controls (HC).

Methods: We analyzed fMRI BOLD activation and brain connectivity in 10 PWE and 10 age- and sex-matched HCs during precisely metered iso-oxic, hypercapnic breathing challenges. Segmented brainstem responses were of particular interest, along with characterization of functional connectivity metrics between these structures. Regional BOLD activations during hypercapnic challenges were convolved with hemodynamic responses, and the resulting activation maps were passed on to group-level analyses. For the functional connectivity analysis, significant clusters from BOLD results were used as seeds. Each individual seed time-series activation map was extracted for bivariate correlation coefficient analyses to study changes in brain connectivity between PWE and HCs.

Results: (1) Greater brainstem BOLD activations in PWE were observed compared to HC during hypercapnic challenges in several structures with respiratory/chemosensing properties. Group comparison between PWE vs. HC showed significantly greater activation in the dorsal raphe among PWE ($p < 0.05$) compared to HCs. (2) PWE had significantly greater seed-seed connectivity and recruited more structures during hypercapnia compared to HC.

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Significance: The results of this study show that BOLD responses to hypercapnia in human brainstem are detectable and different in PWE compared to HC. Increased dorsal raphe BOLD activation in PWE and increased seed-seed connectivity between brainstem and adjacent subcortical areas may indicate abnormal chemosensing in these individuals. Imaging investigation of brainstem respiratory centers involved in respiratory regulation in PWE is an important step toward identifying suspected dysfunction of brainstem breathing control that culminates in SUDEP and deserve further study as potential imaging SUDEP biomarkers.

Keywords: sudden unexplained death in epilepsy (SUDEP), epilepsy, functional MRI (fMRI), functional connectivity, hypercapnia

KEY POINTS

- Functional MRI can detect abnormal chemosensing in brainstem structures.
- Dorsal raphe a region rich in serotonergic neurons plays a major role in responding to changes in carbon dioxide chemosensing.
- Functional MRI can serve as a biomarker for SUDEP risk among patients with epilepsy.

INTRODUCTION

Sudden unexpected death in epilepsy (SUDEP) affects up to 1% of persons with medically refractory epilepsy (1, 2). It is a catastrophic outcome of epilepsy, most common in young patients who are otherwise healthy. Despite its major impact on life expectancy in refractory epilepsy, no reliable SUDEP biomarkers currently exist and thus represent a critical unmet need for targeted SUDEP intervention.

Observational studies point to the failure of recovery from peri-ictal respiratory depression as a main concern (3, 4). Structural and/or functional compromise of brainstem breathing control networks appears likely. Structural brain MRI of subjects who subsequently succumbed to SUDEP typically indicates volume loss in key autonomic and breathing control sites (periaqueductal gray, raphe nuclei, and medial posterior thalamus) (5, 6). In support of these observations, a series of SUDEP postmortem cases have shown significant reductions in pacemaker-like somatostatin and neurokinin-1 receptor-positive neurons in the ventrolateral medulla, suggesting that both structural and functional brainstem abnormalities exist in patients with SUDEP (7). These findings indicate epilepsy-mediated pathology in brainstem respiratory neuronal groups that promote vulnerability to SUDEP.

Severe and prolonged increases in end-tidal CO_2 ($ETCO_2$) occur with seizures(8). In a recent study of PWE, decreased interictal hypercapnic ventilatory responses (HCVR) to CO_2 were correlated with higher and more prolonged peri-ictal hypercapnia (9). HCVRs seem to vary greatly in PWE, and thus, alternative measures of capturing brainstem dysfunction are attractive (9). One imaging study done in healthy human volunteers showed activation of the thalamus, the inferior ventral and dorsal rostral pons, and dorsal and lateral medulla in response to CO_2 (10), indicating feasibility in PWE.

Premortem identification of compromised structural and functional respiratory control would allow targeted intervention and prevention of SUDEP risk. The primary objective of this work was to identify the activation of brainstem respiratory centers. We hypothesized that blood oxygenation level-dependent (BOLD) responses to increase CO_2 (hypercapnic challenges) are observable in humans and are different in PWE compared to HC. This could potentially lead to the development of premortem risk markers for SUDEP.

METHODS

We investigated brain connectivity alterations in response to hypercapnic challenges in PWE and HC. We were particularly interested in segmenting brainstem responses to hypercapnic challenges and characterizing functional connectivity metrics of these structures.

Participants

The study was approved by the University of Texas Health Sciences Center Houston Institutional Review Board, and participants provided written informed consent. Study patients were recruited from the Adult Epilepsy Monitoring Unit and University of Texas Health Epilepsy clinic. We included patients with a diagnosis of epilepsy, aged 18–60 years, who had not undergone resective brain surgery. Exclusion criteria were as follows: use of antidepressant medications, history of cardiac disease, respiratory disease, claustrophobia, metallic implants or devices (e.g., implantable cardioverter-defibrillator [ICD], pacemaker, embolic coils, aneurysm clips), or other material potentially hazardous in the MRI environment), bodyweight < 275 pounds (125 kg—scanner restrictions), stereoelectroencephalography (SEEG) implantation in the preceding 6 months, pregnancy, history of stroke, diagnosis of psychiatric disease, airway or chest deformities interfering with breathing, mechanical ventilatory or circulatory support, and renal failure.

Subjects were asked to refrain from consuming food or beverages with vasoactive effects, e.g., coffee, tea, and herbal remedies, at least 12 h prior to the fMRI scan. The participant's

systolic, diastolic, and mean blood pressure and peripheral oxygen saturation (SpO_2) were measured before and after the breathing challenge fMRI session. A multidimensional dyspnea profile (MDP) survey (11) was also completed by each participant at the end of the study to gauge overall unpleasantness from the breathing challenge.

Functional MRI Data Acquisition and Analysis

Functional MRI scans were performed on a 3T scanner (Ingenia; Philips Medical Systems) using a 16-channel SENSE head coil. An anatomical overlay scan was acquired using a 3D T1 weighted high-resolution structural scan for normalization using the following parameters (TR/TE: 9.79/4.5 ms, FA = 6, FOV = 256 mm × 256 mm × 176 mm, matrix size 320 × 320 matrix with 220 slices and 0.80 mm in-plane resolution). Whole-brain blood oxygenation level-dependent (BOLD) functional scans were acquired using a T2*-weighted echo-planar sequence using the following parameters: TR/TE 2,400/30 ms, flip angle = 85°, FOV = 224 mm, 48 transverse slices, thickness 3.5 mm × 3.5 mm^2 in-plane resolution. The fMRI protocol consisted of 346 volumes (duration: 13 min 50.4 s) covering the whole brain from the top of the cortex to the base of the brain stem.

Head motion was restricted using foam pads and a forehead strap. Comprehensive training and familiarization with the breathing challenge (dummy runs) were completed prior to MRI to reduce any anxiety with the challenge and to encourage completion of the task.

Breathing Challenge

For the fMRI breathing challenge, we used the well-established physiological research RespirAct™ (RA) device (12, 13). The RA device can deliver precise vasoactive stimuli (CO_2) during MRI for whole-brain mapping of the cerebral blood flow responses. It controls alveolar ventilation, enabling targeted reproducible changes in arterial CO_2 levels ($PaCO_2$), such that CO_2 targets set on RA are identical to measure arterial CO_2 (12, 13). This enabled precise and reproducible "step" increases in CO_2 up to 10 mmHg above resting levels in 1 to 2 breaths for the measurement of BOLD responses to CO_2. Using this device, each participant underwent a 14-min block-design scan, consisting of 2-min blocks of breathing challenges interleaved with rest. The respiratory rate, end tidal CO_2 ($EtCO_2$), and end tidal O_2 (EtO_2) were continuously recorded throughout the scan. Gas supply to mask and breathing circuit was done through a programmable computer-controlled gas delivery system containing a non-rebreathing valve that directs blended gas to an inspiratory gas reservoir and patient. The breathing challenge protocol, previously established in cerebrovascular studies (12), involved two iso-oxic patterns of CO_2 modulation: The first comprised square wave increases in $EtCO_2$ by 10 mmHg from individual baseline value for 120 s, followed by 120 s of baseline. The second sequence was a slow ramp increase of $EtCO_2$ by 15 mmHg from individual baseline for 120 s followed by baseline (120 s). Patients were administered precisely metered CO_2 not exceeding pCO_2 of 50 mmHg, with iso-oxygenation maintained at 110 mmHg at all times. Gas calibrations were performed using an EPA industry-standard calibration gas mixture before the start of each scan. Individual breath-by-breath inspired gas and flow concentrations were pre-calculated before the breathing challenge to establish individual's baseline levels and to set end-tidal gas concentration targets.

Analyses

Data were pre-processed and analyzed using SPM software package version 12 (Statistical Parametric Mapping; Wellcome Department of Cognitive Neurology, London, United Kingdom), as well as the functional connectivity toolbox Conn (Cognitive and Affective Neuroscience Laboratory, Massachusetts Institute of Technology, Cambridge, USA) running under MATLAB 2016a (Mathworks, Sherborn, MA, USA). fMRI data were quality checked for excessive motion. Subject head motion was assessed by evaluating three translations and three rotations motions for each scan. Translational thresholds were set to ± 2 mm, whereas rotational thresholds were limited to ± 1°. Pre-processing steps included slice time correction, CompCor physiological noise correction (14), co-registration (realignment to the first image of the time series), normalization to MNI space (Montreal Neurological Institute), and smoothing with a Gaussian kernel of 6 mm full width at half maximum (FWHM) to compensate for small residual anatomic variations across participants.

BOLD Comparison During Breathing Challenge

A first-level, individual subject analysis was performed using the general linear model implemented in SPM12. Motion parameters from motion correction re-alignment were modeled as regressors of no interest. Breathing challenge blocks (step + ramp response) were convolved with the canonical hemodynamic response function, and resulting activation maps were then passed up to group-level analyses comparing the differences in activation among the patient and healthy participant cohorts using age as a covariate of no interest. Using the available probabilistic brainstem segmentation atlas (15, 16), we created a custom explicit brainstem plus binary mask to study BOLD activation changes in the brainstem hypothalamus, and posterior thalamus structures during both the step and ramp breathing challenge (shown in **Figure 1C**). This mask served to spatially remove confounds from physiological noise found in areas adjacent to the brainstem. Second-order group analysis was performed to compare the breathing challenge-related BOLD changes between PWE and HC groups. The minimum threshold for all analysis results was set at p-value < 0.05 family-wise-error (FWE) cluster-corrected significance (17).

Functional Connectivity During Breathing Challenge

We investigated the breathing challenge-related changes in brain connectivity in the patient groups and healthy controls. Seed regions were identified from the significant group comparison BOLD results from both PWE and HCs. To this effect, regions of interest corresponding to each significant cluster consisted of a sphere (3 mm radius) around the cluster's most significant voxel (shown in **Table 1**); larger activation clusters were further subdivided into sub-regions based on the location of peak voxels, resulting in a set of seed regions. Individual structural CSF and

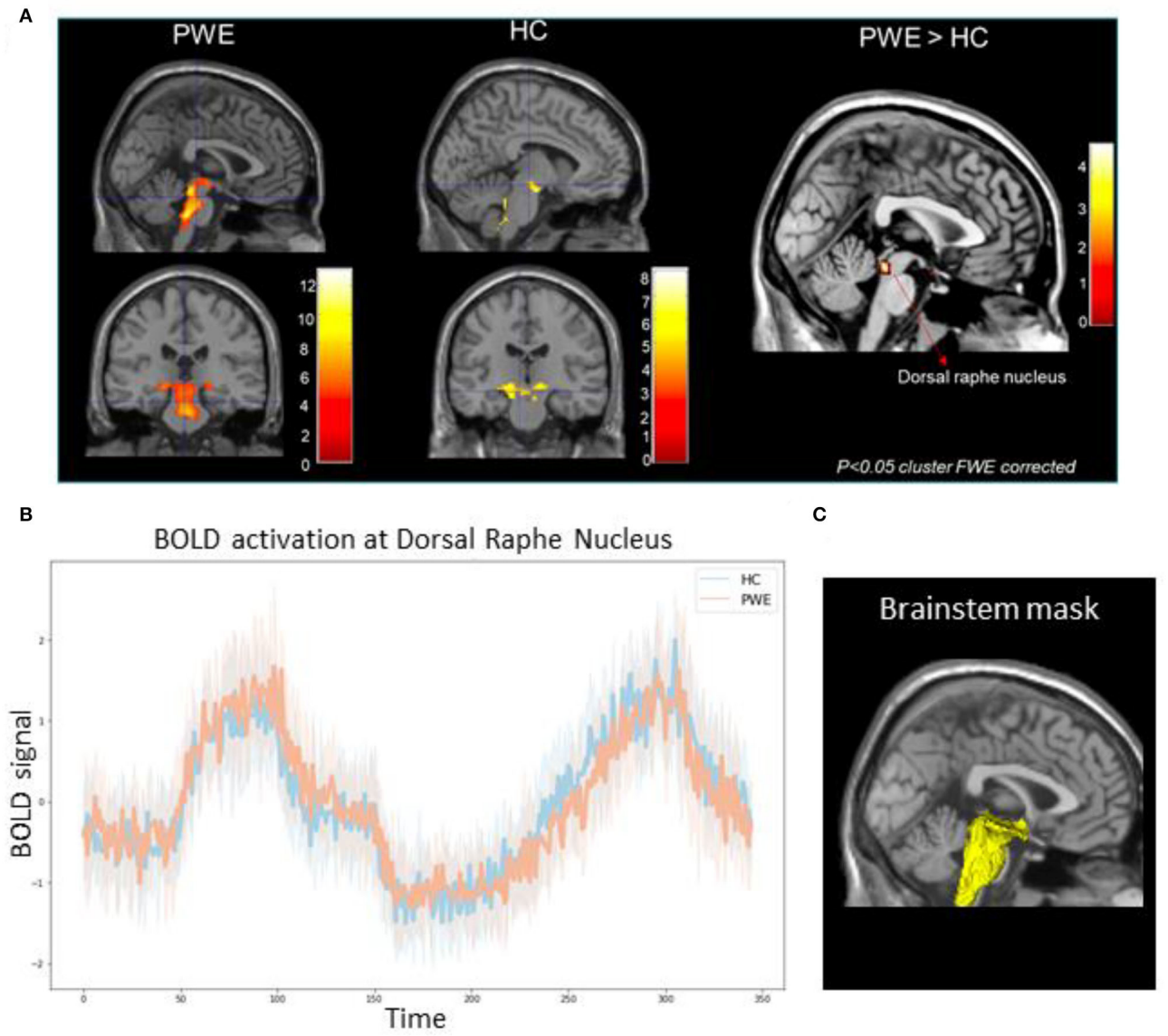

FIGURE 1 | (A) Group-level brainstem BOLD activation differences among 10 patients (PWE) and 10 healthy controls (HC) during CO_2 challenge showed greater activations among PWE at the dorsal raphe nucleus ($p_{FWE} < 0.05$) compared to HCs. Color bars represent Z-score activation values. **(B)** Line plot represents mean BOLD activation time series extraction from all PWE (orange) and HCs (blue) at the dorsal raphe nucleus in response to the step and ramp challenge. Shaded hue areas represent corresponding individual confidence intervals. **(C)** 3D reconstruction of the explicit brainstem plus binary mask (yellow) used in the analysis overlaid over standard template T1 slice.

motion parameters were entered into the analysis as covariates of no interest. A band-pass filter (frequency window: 0.008–0.09 Hz) was applied to remove linear drifts and high-frequency noise from the data. For each subject, regional mean BOLD time series was estimated for each seed by averaging the time series of all voxels. The bivariate correlation coefficient was used to measure the level of linear association between the BOLD time series of every seed region pair (18). The resulting one-sample connectivity maps were compared among PWE and HC groups, using a multivariate statistic parametric (MVPA) omnibus test for cluster-level interference (19), adding age as a covariate of no interest (20). The resulting cluster maps were threshold at $p < 0.05$ FDR/FWE cluster correction for multiple comparisons.

RESULTS

A total of 12 persons with epilepsy (PWE) and 10 healthy controls were enrolled in the study and qualified for the fMRI scan. Out of 12 PWE, one patient could not complete the breathing task in the scanner, and one was excluded due to excessive head motion. Therefore, 10 PWE and 10 HCs completed the study and met inclusion/exclusion criteria standards. The mean age among

PWE was 35.9 ± 14.2 years, HC was 33.4 ± 7.3, and there was no significant difference in age across groups ($p = 0.63$).

Demographic and Clinical Characteristics

There was no significant difference in participants' age, sex, BMI, and baseline BP distributions between the groups. In addition, the multidimensional dyspnea questionnaire completed post-scan did not show any significant difference in unpleasant or discomfort experienced by the patients compared to healthy controls (**Table 2**). A total of 9/10 (90%) PWE were known to have at least one generalized tonic-clonic seizure per year. None were known to have peri-ictal central apnea (refer to the **Supplementary Table 1** for PWE characteristics).

BOLD Activation Patterns

We found significantly greater brainstem BOLD activations in PWE compared to HC during breathing challenges. As shown in **Table 1**, in the PWE group, we observed a pattern of activation in the raphe nucleus, locus coeruleus, hypothalamus, rostral pons, posterior thalamus, and periaqueductal gray regions ($p < 0.001$ cluster FWE-corrected). For the HC group, consistent activation of the ventral respiratory group and midbrain was observed. Group comparison showed significantly greater activation in the dorsal raphe nucleus among PWE ($p < 0.05$ cluster FWE-corrected) as shown in **Figures 1A,B**.

Seed-Based Connectivity Analysis

Grouped second-level seed-based functional connectivity analyses showed an increase in brain connectivity between regions of the brainstem plus structures during CO_2 challenge among PWE compared to HCs (**Figure 2**). PWE had significantly greater seed-seed connectivity and recruited more hypothalamus, pulvinar thalamus to brainstem structures during the breathing challenge compared to HC. This implies that an overall greater effort is required among PWE to complete the same breathing challenge done by HC with little effort. Statistical significance is reported at cluster threshold: $p < 0.05$ cluster level p-FDR-corrected, multivariate statistic parametric (MVPA) omnibus test, and connection threshold. Seed regions in the cluster-level interference ring (**Figure 2**) are sorted using hierarchical clustering in Conn toolbar.

TABLE 1 | Demographic, cardiorespiratory, and multidimensional dyspnea profile data; group comparisons and changes.

	PWE ($n = 10$)	HC ($n = 10$)	p-value
Age, mean ± SD	35.9 ± 14.2	33.4 ± 7.3	$P = 0.63$
Sex, N	5 M, 5 F	5 M, 5 F	
BMI, mean ± SD	22.7 ± 4.2	25.6 ± 6.1	$P = 0.23$
Cardiovascular measurements Baseline, mean ± SD			
○ Systolic	121.4 ± 16.4	127.3 ± 12.5	$P = 0.91$
○ Diastolic	70.2 ± 8.9	75.7 ± 7.1	$P = 0.14$
○ Mean arterial pressure	87.3 ± 10.9	92.9 ± 7.93	$P = 0.20$
○ SpO2	97.8 ± 1.5	98.4 ± 1.0	$P = 0.30$
Post-challenge changes, mean ± SD	5.8 ± 12.8	−2.7 ± 10.4	$P = 0.12$
○ Systolic			
○ Diastolic	3.0 ± 7.02	−2.0 ± 8.2	$P = 0.36$
○ Mean arterial pressure	3.9 ± 8.0	−1.0 ± 7.8	$P = 0.18$
○ SpO2	1.4 ± 1.3	0.50 ± 1.3	$P = 0.13$
Multidimensional Dyspnea Profile (MDP)			
Sensation/Response (0–10 scale)			
Unpleasant/discomfort	3.2 ± 2.2	3.7 ± 3.1	$P = 0.68$
Requires effort	3.8 ± 3.1	3.3 ± 3.1	$P = 0.72$
Not getting enough air	2.4 ± 2.2	3.7 ± 2.8	$P = 0.26$
Feel constricted	1.6 ± 1.6	1.6 ± 1.8	$P = 1.00$
Requires mental effort	3.7 ± 3.1	4.3 ± 3.9	$P = 0.71$
Breathing a lot	4.7 ± 3.5	5.4 ± 2.5	$P = 0.62$
Depressed	0.1 ± 0.3	0.6 ± 1.9	$P = 0.42$
Anxious	6.5 ± 8.9	4.2 ± 3.6	$P = 0.46$
Frustrated	1.0 ± 2.2	0.0 ± 0.0	$P = 0.17$
Angry	0.0 ± 0.0	0.0 ± 0.0	–
Afraid	1.5 ± 2.7	2.2 ± 3.7	$P = 0.63$

DISCUSSION

In this functional magnetic resonance imaging (fMRI) case-control study of breathing responses to hypercapnic challenges, we found BOLD activation of respiratory centers, most significantly in the dorsal raphe nucleus, in PWE compared to HC. In addition, we found pathological functional connectivity alterations in brainstem and adjacent subcortical regions known to specifically modulate breathing as previously implicated in SUDEP(5, 7). Carbon dioxide (CO_2) is potently vasoactive (12) and clearly produces distinct BOLD alterations, and our case-controlled approach suggests significant activations in regions with chemosensing properties in PWE. Serotonergic [5-hydroxytryptamine (5-HT)] dorsal raphe neurons are central chemoreceptors that project rostrally to modulate arousal (21), are highly sensitive to pH and CO_2, (22), and are consistent with the observations here.

Brainstem damage is known to occur in PWE (5, 7, 23, 24). Epilepsy is associated with progressive, structural brain changes detectable on MR imaging (25–27), including some which affect cardiorespiratory control sites (24). Volume loss has been seen in key autonomic and breathing control sites (periaqueductal gray, raphe nuclei, and medial posterior thalamus) (5). In support, a series of SUDEP postmortem cases have shown significant alterations in the ventrolateral medulla and in the medullary raphe nucleus, essential regions for human central control of respiration, suggesting compromised respiratory network hubs. Reductions in somatostatin neurons (SST) and neurokinin-1 receptor (NK1R) in the ventrolateral medulla and reduction of 5-HT transporter in the raphe nucleus were seen in brains of patients who died of SUDEP compared to controls (sudden death patients without epilepsy) (7). Altered medullary neuromodulatory systems, including 5-HT and galaninergic

TABLE 2 | Breathing challenge fMRI results.

Region	MNI coordinates			Cluster size (K_E)	*p*-value (cluster-corrected)	Z score
	X	Y	Z			
Analysis 1: Breathing challenge: PWE						
Dorsal raphe nucleus	0	−30	−14	55	$P_{FWE} < 0.001$	4.43
Locus ceruleus	4	−34	−26	72	$P_{FWE} < 0.001$	4.92
Lateral hypothalamus	−4	−8	−8	146	$P_{FWE} < 0.001$	5.07
Rostral pons	0	−26	−28	80	$P_{FWE} < 0.001$	4.22
Posterior thalamus	8	−16	−4	56	$P_{FWE} < 0.001$	4.55
Periaqueductal gray	0	−26	−6	55	$P_{FWE} = 0.001$	3.44
Analysis 2: Breathing challenge: HC						
Ventral respiratory group	12	−44	−44	104	$P_{FWE} < 0.05$	4.10
Substantia nigra	6	−12	−16	26	$P_{FWE} < 0.05$	3.94
Analysis 3: PWE vs. HC						
Dorsal raphe nucleus	0	−32	−16	37	$P_{FWE} < 0.05$	3.73

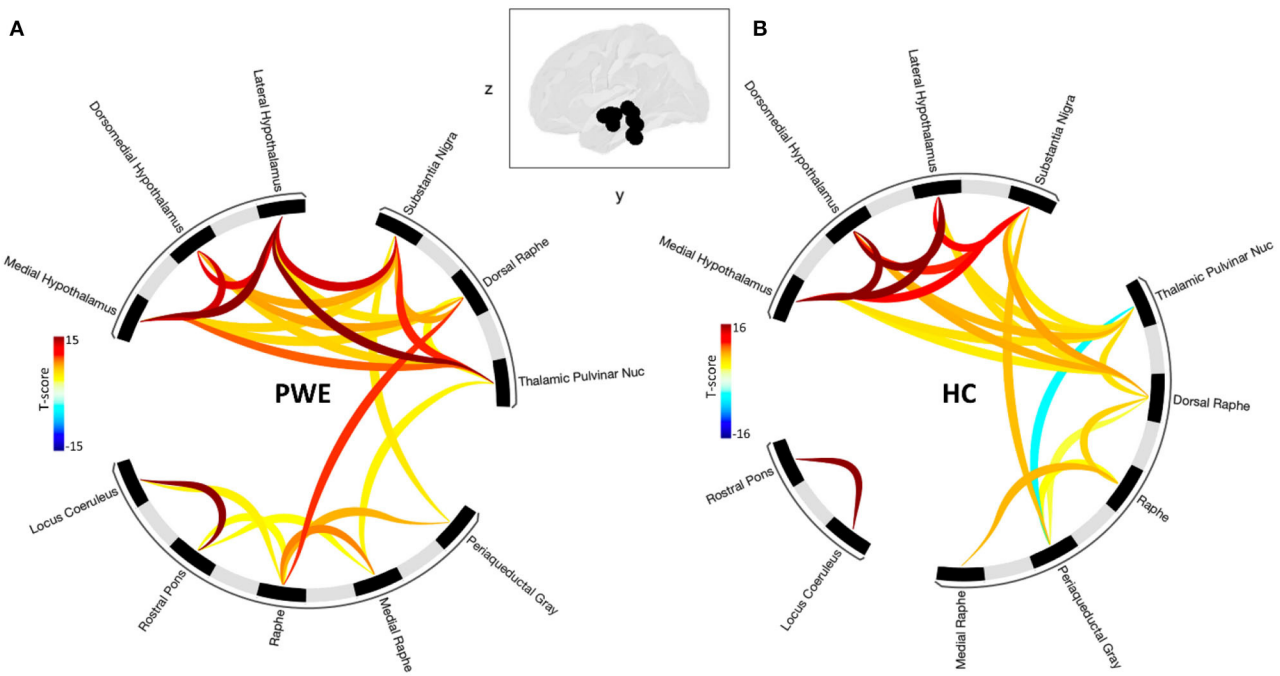

FIGURE 2 | Shows seed-seed functional network connectivity results as cluster-level interference ring. **(A)** The PWE group was characterized by a higher number of connections and higher mean connectivity strength compared to **(B)** healthy controls. Glass brain image represent seed regions used in the analysis. Dark to red connectivity lines signify stronger positive connectivity, lighter to blue connectivity lines denote decreased or negative connectivity. Functional network connectivity cluster threshold: $p<0.05$ cluster-level p-FDR-corrected (MVPA omnibus test); connection threshold.

systems, indicate that both structural and functional brainstem abnormalities exist in SUDEP patients. Alterations in pre-BotC NK1R and SST neurons have also been reported in other sudden death syndromes (28), an entity with similarities to SUDEP.

There are few human functional imaging studies of respiratory control. To our knowledge, all done in healthy subjects. One fMRI study used CO_2 inhalation in healthy volunteers; BOLD signal changes were seen in dorsal rostral pons, inferior ventral pons, dorsal and lateral medulla, thalamus, and putamen(10). Another such study of voluntary respiration (hyperpnea) revealed increased BOLD responses in the brainstem (dorsal medulla) (29).

Brainstem dysfunction in respiratory homeostasis is likely underpinned by 5-HT (24, 30). 5-HT is a key chemosensing

neurotransmitter that is central to brainstem hypercapnic and arousal mechanisms and strongly implicated in SUDEP (24). Reduced peri-ictal firing of raphe 5-HT neurons during cardiorespiratory dysfunction has been demonstrated in animals (31). Reduced 5-HT can cause post-ictal respiratory arrest and death in SUDEP models, preventable with selective-serotonin reuptake inhibitor (SRI) pretreatment (32, 33). In humans, PWE taking SRIs had shortened peri-ictal apnea (24). Peri-ictal breathing dysfunction includes post-convulsive central apnea and prolonged ictal central apnea, both of which elevate pCO_2 (8). Altered 5-HT tone has been reported in high-risk patients who suffer such peri-ictal breathing difficulties (34). Impaired CO_2 chemosensing and failed arousal may underlie SUDEP mechanisms, and premortem identification of abnormal chemosensing using hypercapnic ventilatory responses [HCVR (increase in minute ventilation induced by an increase in end-tidal CO_2)] has been posited as a potential biomarker(24). HCVR slopes have been shown to be inversely associated with post-ictal hypercapnia duration and magnitude (24). However, large variability in HCVR in PWE (24) and HC (35) poses a challenge for risk stratification that may be overcome by the fMRI approach shown here.

We found fMRI evidence of abnormal chemosensing in the form of a striking pattern of hypercapnia-related BOLD activations in PWE compared to HC, most marked in the dorsal raphe nuclei, a chemosensing region known to be richly innervated by 5-HT neurons. Hypercapnic activation enhances minute ventilation for blood gas stabilization and is critical for respiratory homeostasis (36, 37). The respiratory drive is tightly regulated by CO_2 concentration, and increased pCO_2 is a powerful arousal stimulus (38). Both may be relevant to SUDEP. CO_2-enriched CSF rouses wild-type mice but fails to rouse 5-HT neuron-deficient mice (39). In our study, this abnormally enhanced activation compared to HC suggests abnormal, enhanced recruitment of chemosensing neurons in respiratory hemostasis for restoration of normocarbia, indicating a system under significant "stress." In contrast, grouped analysis of HC showed less activation, suggesting efficient homeostasis to hypercapnic challenges. Consistent with the BOLD activations described here, we found evidence for abnormal circuitry with increased connectivity between multiple sites with chemosensing properties in PWE compared to HC. Pathologically increased connectivity within what may be called the breathing network may indicate a tendency to impaired recovery from generalized tonic-clonic seizure (GTCS)-induced breathing compromise. Altered networking among autonomic and breathing-related brain areas in patients with a high risk of SUDEP has been described in two previous resting-state fMRI studies (23, 40).

Further, one-sample *t*-test group-level BOLD analysis in PWE showed a number of additional brain regions known to participate in breathing control, including lateral hypothalamus, periaqueductal gray (PAG), posterior thalamus, rostral pons, and locus ceruleus (41–43). Activation of the lateral hypothalamus, a chemosensing region, supports preclinical, within-subject neuroplastic reports of enhanced orexinergic influence on cardiorespiration and autonomic networks prior to SUDEP (44). Volume loss in the periaqueductal gray and medial posterior (pulvinar) thalamus has been described in PWE, and both structures are implicated in respiratory control (5, 45). These network abnormalities may reflect pathomechanisms that increase the risk for breathing dysfunction, particularly in circumstances under which autonomic and respiratory processes are challenged, such as during and after GTCS.

The results of this study suggest distinct differences between PWE and HC. These findings are encouraging and raise the possibility that fMRI biomarkers for patients at high risk of SUDEP may be feasible and are worthy of further study. In particular, stratification of potential BOLD activation extents and locations depending on known presence/absence of GTCS, GTCS frequency, and electroclinical features, such as peri-ictal apnea, O_2 desaturations, and seizure durations, are the important next steps.

There are several notable limitations here, including small sample size and the lack of correlation between ventilatory parameters with BOLD findings. Thus, there is no stratification of BOLD in PWE into those with or without documented breathing dysfunction since not all patients had epilepsy monitoring unit assessments for observation of habitual peri-ictal breathing compromise. Furthermore, better powered studies designed to examine the correlation of known SUDEP risk factors (GTCS, age at onset, and duration of epilepsy) and electroclinical seizure features, such as prolonged ictal central apnea and post-convulsive central apnea, are necessary for further characterization of these potential premortem SUDEP imaging biomarkers. To maximize brainstem resolution, fMRI was limited to a narrow field of view focus limited to the brainstem. Most brainstem nuclei are in mm resolution, and the imaging protocol of our preliminary study was limited by the resolution of the BOLD MRI sequence; dedicated high resolution EPI sequence is needed to improve signal to noise ratio at the brain stem. Further expansion of this work to include cortical structures with multimodal polygraphy (cardiorespiratory and blood pressure monitoring) during MRI is currently underway.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Texas Health Sciences Center Houston Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JoH, NL, and SL contributed to conception and design of the study. MR and JaH organized the study database.

JoH performed the imaging, statistical analysis, and wrote the first draft of the manuscript. NL, KS, TS, LL, and SL wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.896204/full#supplementary-material

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Galanin analogs prevent mortality from seizure-induced respiratory arrest in mice

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Objective: Sudden Unexpected Death in Epilepsy (SUDEP) accounts for 20% of mortality in those with recurrent seizures. While risk factors, monitoring systems, and standard practices are in place, the pathophysiology of SUDEP is still not well understood. Better knowledge of SUDEP and its potential mechanisms of action is crucial to reducing risk in this patient population and developing potential treatment options. Clinical studies and animal models of SUDEP suggest that diminished post-ictal respiratory control may be the dominant mechanism contributing to mortality. Recently, it was demonstrated that the depletion of the neuropeptide galanin in the amygdala occurs in human SUDEP. The amygdala plays a key role in the central integration of respiratory signaling; the depletion of galanin may represent a critical change that predisposes individuals to SUDEP.

Materials and methods: To evaluate the impact of enhancing galaninergic signaling to potentially protect against SUDEP, we studied seizure-induced respiratory arrest (S-IRA) following central (intracerebroventricular, intra-amygdala) and systemic (intraperitoneal, subcutaneous) administration of galanin analogs. Seizure naïve and seizure experienced (fully kindled) mice were tested.

Results: Central and systemically administered galanin analogs protect against S-IRA in naïve C57Bl/6J mice. Differential efficacy between receptor subtype-selective analogs varied based on the route of administration. Sub-chronic systemic administration at doses that reduced 6 Hz seizures also protected against S-IRA. Acute treatment benefits also extended to fully kindled mice experiencing tonic extension.

Significance: These data demonstrate that galanin analogs may be protective against post-ictal respiratory collapse.

KEYWORDS

animal models, respiratory arrest, SUDEP, neuropeptide, epilepsy

Highlights

- Central and systemic galanin analogs prevent seizure-induced respiratory arrest.
- Efficacy was observed in three separate mouse strains under various experimental conditions.
- Sub-chronic administration demonstrated galanin analog protection against respiratory arrest.
- Acute systemic administration also conferred protection against respiratory arrest following tonic extension in fully kindled mice.
- Galanin analogs may represent a novel potential therapy in SUDEP-susceptible individuals.

Introduction

Epilepsy can lead to a variety of serious complications and is associated with a reduction in life expectancy. One of the leading causes of mortality in those with recurrent seizures is an event known as Sudden Unexpected Death in Epilepsy (SUDEP) (Devinsky et al., 2016; Dlouhy et al., 2016; DeGiorgio et al., 2017; Jones and Thomas, 2017; Michalak et al., 2017). A pivotal clinical study demonstrated that post-seizure (post-ictal) apnea precedes asystole (Ryvlin et al., 2013), suggesting that respiratory control may be a major mechanism in SUDEP. Further, clinical observations and mouse models suggest that respiratory neurocircuitry is altered in individuals susceptible to early mortality (Seyal et al., 2010; Mueller et al., 2014; Lacuey et al., 2018; Kuo et al., 2019; Vilella et al., 2019a). Death after seizures may result from a combination of poor respiratory responses and failure in normal arousal mechanisms that promote breathing (Tomson et al., 2008; Tolstykh and Cavazos, 2013; Devinsky et al., 2016; Dlouhy et al., 2016; Vilella et al., 2019a,b).

Generalized tonic-clonic seizures (GTCS) are a major risk factor for SUDEP and may lead to hypoxia, hypercapnia, failed arousal mechanisms, and thus an inability to recover breathing following seizures (Monte et al., 2007; Nashef et al., 2007; Devinsky et al., 2016; Dlouhy et al., 2016). A GTCS can spread to other brain regions, including those involved in breathing. Several forebrain and brainstem nuclei may regulate breathing following seizures. One important area of interest is implicated in post-ictal respiration is the amygdala. Apnea results when seizures spread to the amygdala, and amygdala lesions reduce seizure-induced respiratory arrest (S-IRA) in mice (Marincovich et al., 2019; Nobis et al., 2019; Rhone et al., 2020). Moreover, the amygdala has direct connections with brainstem breathing control sites (Saha et al., 2000; Dong and Swanson, 2006). The amygdala is enriched in neuropeptides that modulate neuronal activity and can be depleted following seizures (Somani et al., 2020). Interestingly, depletion of the neuropeptide galanin in the amygdala occurs in human SUDEP (Somani et al., 2020). Galanin is an anticonvulsant neuropeptide activated under conditions of enhanced neural activity (Lang et al., 2015) and plays a critical role in responses to respiratory stressors (Stornetta et al., 2009; Shi et al., 2017). Further, galanin is robustly upregulated after various insults such as nerve injury and seizures (Mazarati et al., 2000; Liu and Hokfelt, 2002; Lundstrom et al., 2005). Similarly, galanin is increased in the amygdala following seizures (Christiansen and Woldbye, 2010). Therefore, the amygdala is a critical relay point between seizures and downstream breathing control following seizures, and the depletion of galanin may represent a critical pathophysiologic change predisposing some individuals to SUDEP. It may also be that enhancement of galanin signaling in the amygdala protects against respiratory collapse following seizures.

There remains no evidence-based treatment to prevent SUDEP. The mainstay of management has been addressing modifiable risk factors such as ensuring medication adherence, but with few results (Tomson et al., 2008; Lhatoo et al., 2015; Devinsky et al., 2016; Dlouhy et al., 2016). Novel therapies may therefore prove beneficial if they offer benefits targeted explicitly to SUDEP risk, in addition to any potential benefits of reducing seizure burden. Therefore, the purpose of this study was to use rodent models to demonstrate how galanin analogs may prevent mortality in mice experiencing S-IRA. Respiratory arrest, arising following tonic extension (TE) elicited in mice, has been used as a model of SUDEP and recapitulates apnea following GTCS (Purnell et al., 2017; Kruse et al., 2019). Further, this model offers the opportunity to screen compounds with acute and chronic effects at preventing S-IRA. These studies described herein will therefore demonstrate the potential utility of galanin analogs in restoring respiration after seizures in naïve and seizure-experienced mice.

Materials and methods

Animals

Three different mouse strains were used: male C57BI/6J (5–6 weeks old, Jackson Laboratory, Bar Harbor, ME, United States), male CD-1 (5–7 weeks old, Charles River Laboratories, Kingston, NY, United States), and male CF-1 (5–7 weeks old, Charles River Lab, Kingston, NY, United States). Animals were allowed free access to food and water, except during testing periods. Prior to testing, animals were allowed 1 week to acclimate to housing conditions. All mice were housed in plastic cages in rooms with controlled humidity, ventilation, and lighting (12 h on–12 h off). The animals were housed and fed in a manner consistent with the recommendations in the "Guide for Care and Use of Laboratory Animals" (National Research Council). Housing, handling, and testing was performed in accordance with Public Health Service policy

guidelines and a protocol approved by the Institutional Animal Care and Use Committee of the University of Utah.

Compound preparation/administration

A total of 810–2 (Gal_2-preffering) and 505–5 (Gal_1-preferring) (molecular weights 2,124 and 2,113 g/mol, respectively) (White et al., 2009; Bialer et al., 2013, 2015, 2017; White, 2017) was synthesized by PolyPeptide Laboratories (San Diego, CA United States). A total of 810–2 was dissolved in a vehicle (VEH) solution of 2% (central) or 20% (systemic) hydroxy propyl beta cyclodextrin (HPβCD; Sigma, St. Louis, MO, United States) and 3.75% D-mannitol in acetate buffer [0.1 M acetic acid (from glacial acetic acid stock; Sigma, St. Louis, MO, United States); 0.1 M sodium acetate (Sigma, St. Louis, MO, United States), pH 4.5]. A total of 505–5 was dissolved in a VEH solution of 1% Tween 20 (Sigma, St. Louis, MO, United States) in 0.9% NaCl. Compounds were administered by intraperitoneal (IP), intracerebroventricular (ICV; free-hand injection), or intra-amygdala (IA; 1–2 weeks post unilateral amygdala cannulation). For ICV and IA administration of 810–2, the vehicle solution was 2% HPβCD in water. Separate experiments were also conducted for administration of galanin analogs by subcutaneous (SC) sub-chronic (14-day) administration. A total of 810–2 and 505–5 were prepared in 50:50 dimethylsulfoxide (DMSO): water at concentrations of 67.2 and 16.8 mg/ml (respectively), and placed in implantable mini-pumps (Alzet, model 1002). For SC administration in pumps, larger concentrations (e.g., 16.8, 67.2 mg/ml) are beyond solubility limitations using the HPβCD-acetate buffer vehicle (used for IP, ICV, and IA). Therefore, the peptides were solubilized in DMSO: H_2O and only a small total volume (∼100 μl) was used for each pump). By contrast, volumes injected for intraperitoneal administration are much greater and the larger relative amounts of DMSO would produce untoward effects.

Surgical implantation of unilateral amygdala cannulas

Procedures were similar to those described previously for implantation of unilateral cannulas for drug administration (West et al., 2022). Buprenorphine (0.01–0.2 mg/kg) was administered 1 h prior to surgery. Isoflurane (2–5% in O_2) was used for anesthesia, and mice were placed in a stereotaxic apparatus. A Dremel drill was used to drill a single hole over the right hemisphere followed by placement of a 22-gauge cannula above the dura mater (AP −1.2, ML 3.3). This guide cannula was glued in place using dental acrylic. Antibiotic ointment was applied to the surface around the head cap and penicillin (60000 units, SC) was administered. Mice were then allowed to recover in their home cages (singly housed) for ∼1 week.

Surgical implantation of osmotic minipumps

Alzet minipumps were prepared and immersed in saline overnight. Under isoflurane anesthesia (2–5% in O_2), as described above, a small incision was made between the scapulae (shaved prior to incision) followed by clearance of subcutaneous fascia with forceps. Minipumps were placed in the SC space and the incision sutured with surgical silk. Antibiotic ointment was placed around the incision and mice were allowed to recover. Mice were monitored daily for the duration of the 14-day treatment.

Electrical seizure induction and post-stimulation monitoring

Maximal electroshock seizure model

Maximal electroshock seizure has been used as a model of GTCS for preclinical pharmacology studies routinely for many years (Loscher, 2017). Furthermore, TE following MES stimulation is followed by apnea and death in some mouse strains and has been used as a model of S-IRA (Purnell et al., 2017). Prior to testing, tetracaine (0.5% in saline) was applied to the corneas. MES seizures were induced using a 50 mA current (0.2 s duration) *via* corneal electrode (Barton et al., 2001, 2003). This stimulation intensity produces TE in nearly all mice tested. Following stimulation, mice were observed for the presence of TE and the resumption of post-ictal breathing. Mice not displaying TE were considered protected.

6 Hz seizure induction

This seizure assay has been used as a model of preclinical pharmacology studies in mice (Barton et al., 2001) and was used in these studies to confirm efficacy observed following treatment with galanin analogs (Bulaj et al., 2008; White et al., 2009; Metcalf et al., 2017a). Seizures were induced using the 32 mA stimulus intensity (3 s, 6 Hz, 32 mA) *via* corneal electrodes. Prior to testing, tetracaine (0.5% in saline) was applied to the corneas. Seizure induction is associated with characteristic behaviors including jaw and forelimb clonus with or without loss of righting and rear limb clonus. Animals not displaying any of these behaviors were considered protected.

Corneal kindling

The mouse corneal kindling model was used in CF-1 and C57Bl/6J mice. Mice receive twice daily corneal stimulation (60 Hz, 3 s, 5 days/week) (3 mA–CF-1; 1.5 mA–C57Bl/6). Seizures are scored using the Racine scale (Racine, 1972)

and progress during kindling from mild motor behaviors (e.g., jaw clonus) to generalized seizures (bilateral forelimb clonus, rearing and falling). When five consecutive stage five (generalized seizure with loss of righting reflex) seizures are observed, animals are considered fully kindled. Drug administration studies were performed on fully kindled mice, where MES was applied after drug treatment. TE following MES was evaluated similarly to that described above for naïve animals.

Respiratory monitoring

CF-1 mice subjected to MES were also monitored for cardiorespiratory activity before and following seizures using a MouseOx (Starr Life Sciences) monitoring system. While other strains of mice (e.g., CD-1, C57Bl/6) experience a high mortality following TE, CF-1 mice survive this stimulation. This mouse strain was included for respiratory evaluation so that recordings could be obtained prior to and following seizures. On the day prior to testing, mice were anesthetized with 2–5% isoflurane and the neck region shaved with a surgical razor. On the following day, prior to seizure induction, mice were acclimated to pulse oximetry collars for ∼15 min followed by a baseline recording session (5 min). A plastic sham collar (not connected to monitoring hardware) was placed initially, followed by oximetry collars and baseline recording sessions. After the baseline recording, mice were subjected to MES stimulation followed by a post-stimulation recording session (5 min). Oximetry data were extracted as text files and transferred to Microsoft Excel databases for data analysis. A 30-s recording epoch was selected from each recording session and heart rate, respiratory rate, and oxygen saturation (SpO_2) values averaged over this period.

Intraperitoneal administration

A total of 810–2 and 505–5 were administered to mice 1 h prior to MES stimulation This time point was selected based on previous studies demonstrating peak efficacy for these analogs in the mouse 6 Hz assay 1 h following IP administration (Bulaj et al., 2008; White et al., 2009). A total of 810–2 was administered at doses of 8, and 16 mg/kg IP (N = 10-24 per group), doses were selected based on previous *in vivo* efficacy studies for this compound (Bialer et al., 2015; Metcalf et al., 2017b). A total of 505–5 was administered at doses 2, and 4 mg/kg IP (N = 9–24 per group). Doses for each compound were selected based on previous *in vivo* efficacy studies (White et al., 2009; Bialer et al., 2013, 2015; Metcalf et al., 2017b). A separate group of mice were treated with VEH (N = 37), IP administration, 1 h pre-treatment time.

Central administration

Mice received a single administration centrally of 810–2, 505–5, or VEH. A 5 μl injection volume was used for all central injection studies, and mice only received one injection before being tested and euthanized. Doses of 1–4 nmol were used for intracerebroventricular (ICV) administration whereas a larger dose range was used for IA treatment. For ICV administration a 10 μl Hamilton syringe with a 25-gauge needle was used for administration. Mice were placed under gentle restraint and a free-hand injection was made through the skull surface and into the lateral ventricular space. By contrast, for IA injection studies, mice were implanted 1–2 weeks prior with cannulae centered unilaterally over the amygdala and an injection needle was used to administer compounds over a 30 s period. Initial low doses of 810–2 were selected (0.02–2 nmol) to evaluate tolerability to injection of peptides in this brain region. Following a maximal dose (4.7 nmol) was used, comparable to that used for ICV studies. Treatments occurred 15 min prior to testing for all central injections. For the purposes of these studies, validation of ICV or IA injection was not included. We have previously used ICV free-hand injections for central administration of neuropeptides (Lee et al., 2009; Green et al., 2011; Platt et al., 2012). Similarly, the methods described for IA administration utilize a guide cannula and the same surgical procedures, coordinates, and injection techniques used in our lab as those described for central administration of kainic acid to the amygdala (West et al., 2022).

Statistical analysis

The data was presented as means ± standard error. A comparison between two means was performed using a Student's t-test, and multiple comparisons were made using a one-way ANOVA followed by a Newman-Keuls or a Dunnett's test for *a posteriori* analysis of the difference between group means. A Fisher's exact test was used to compare numbers of mice protected and/or surviving between groups. Survival analyses were analyzed using a log-rank (Mantel-Cox) test. Results where $P < 0.05$ was considered significant.

Results

Galanin analogs reduce seizure-induced respiratory arrest in mice: Differential effects of route of administration and analog

Galanin analogs 810–2 and 505–5 were administered to C57Bl/6J mice using different routes of administration, followed

by MES stimulation for evaluation of S-IRA. First, each analog was administered 1 h prior to testing using IP administration: 810–2 8,16 mg/kg, 505–5 (2, 4 mg/kg) vs. VEH. All mice tested demonstrated a characteristic TE following MES (Tedeschi et al., 1956; Loscher, 2011, 2017). Typically, breathing is absent or diminished during TE in all mice, and many mice die as a result, particularly for CD-1 and C57Bl/6J strains. Following corneal MES stimulation, all mice experienced tonic extension. As shown in **Table 1**, VEH treatment was associated with high mortality (62%, 23/37 mice died) following TE. Although systemic administration of 810–2 did not prevent TE, the analog decreased mortality following TE at a dose of 16 mg/kg (25%, 5/20 died; $P < 0.05$, Fisher's exact test). By contrast, 505–5 did not prevent TE or reduce mortality at the doses tested. As higher doses of each analog are associated with untoward effects (sedation, lethargy), additional doses were not included. CD-1 mice respond similarly to testing, and both 505–5 and 810–2 significantly improve mortality following TE in these mice (505–5, 4 mg/kg IP, $P < 0.05$ vs. VEH, Fisher's exact test; 810–2, 16 mg/kg, $P < 0.05$ vs. VEH, Fisher's exact test; **Supplementary Table 1**).

Following systemic administration studies, ICV administration was performed. Previously, doses of 1–2 nmol (ICV) demonstrated efficacy against 6 Hz seizures for galanin analogs (Bulaj et al., 2008). Therefore, doses in this range were initially used for these studies. Similar to that observed following systemic administration, ICV VEH was associated with a high mortality rate (81%, 13/16) following TE. While the 810–2 did not prevent TE, and was without significant effect on mortality using the doses tested, 505–5 reduced mortality from S-IRA at the highest dose tested (4 nmol; 4/11, $^{*}P < 0.05$ vs. VEH, Fisher's exact test) without preventing TE. Next, using unilateral IA cannulas, galanin analogs were administered by direct injection in freely behaving mice. Initially, a dose of VEH was administered and showed a high mortality rate (65%; 11/17). Following several low doses of 810–2 were administered (0.02–0.2 nmol) to determine whether direct injection would produce untoward effects. As these concentrations were well-tolerated, a higher concentration (4.7 nmol) was administered for both 505–5 and 810–2. A total of 810–2 significantly reduced mortality (26%, 6/23; $P < 0.05$ vs. VEH, Fisher's exact test) following TE, whereas 505–5 was not significantly effective (43%; 9/21). None of the doses tested prevented TE.

TABLE 1 Systemic and central administration of galanin analogs: effect on (S-IRA) in C57B1/6J mice.

Compound	Mortality (# died/N) (%)		
	Systemic (IP)	Central (ICV)	Central (IA)
VEH	23/37 (62%)	13/16 (81%)	11/17 (65%)
505–5	2 mg/kg: 9/20 (45%) 4 mg/kg: 8/20 (40%)	1 nmol: 11/16 (69%) 2 nmol: 4/8 (50%) 4 nmol: 4/11* (36%)	4.7 nmol: 9/21 (43%)
810–2	8 mg/kg: 11/19 (58%) 16 mg/kg: 5/20* (25%)	1 nmol: 6/11 (55%) 2 nmol: (10/16) (63%) 4 nmol (11/16) (69%)	0.02 nmol: 6/7 (86%) 0.1 nmol: 8/8 (100%) 0.2 nmol: 9/9 (100%) 4.7 nmol: 6/23* (26%)

$^{*}P < 0.05$, Fisher's exact test vs. VEH. Routes of administration: IP (intraperitoneal), ICV (intracerebroventricular), IA (intra-amygdala). All mice tested experienced tonic extension.

The Gal_2-preferring analog 810–2 prevents mortality from seizure-induced respiratory arrest following sub-chronic subcutaneous administration

We sought to determine whether sub-chronic (14 days) systemic administration would produce similar efficacy against S-IRA. These galanin analogs have a short half-life (1–2 h) (Metcalf et al., 2017a), thereby requiring repeated (multiple times/day) injections, implantable minipumps were used to provide a more consistent compound exposure. Therefore, SC minipumps were prepared to administer daily doses of 4 or 16 mg/kg of 505–5 and 810–2, respectively. Galanin receptors are G-protein coupled transmembrane proteins and develop tolerance to repeated agonist exposure warranted verification of efficacy (Flynn and White, 2015; Lang et al., 2015). Because these analogs are effective in the mouse 6 Hz seizure model (Bulaj et al., 2008; White et al., 2009; Metcalf et al., 2017a), a single stimulus was administered at the end of the study on the day prior to MES testing. As shown in **Table 2** for the 6 Hz assay, 505–5 and 810–2 both reduced the number of seizures, with 505–5 producing superior efficacy (21/26 protected, $P < 0.001$ vs. VEH), but 810–2 also proving efficacious (6/11 protected, $P < 0.05$ vs. VEH). On the following day, MES was performed and it was observed that a majority of VEH-treated mice

TABLE 2 Reduced S-IRA following SC administration of galanin analogs in C57Bl/6J mice.

Groups	Dose (mg/kg/day)	6 Hz efficacy (# protected/N)	Mortality (# died/N) (%)
VEH	0	2/16	12/16 (75%)
505–5	4	21/26***	18/26 (69%)
810–2	16	6/11*	2/11** (18%)

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. VEH, Fisher's exact test. A total of 6 Hz testing occurred on the day prior to MES testing and mortality assessment following S-IRA. All mice tested experienced tonic extension.

died following TE (75%, 12/16). Also, while 505–5 did not improve mortality from S-IRA (69% mortality, 18/26), 810–2 significantly reduced S-IRA (18%, 2/11, $P < 0.01$ vs. VEH) (Table 2). In the MES assay, neither 810–2 nor 505–5 prevented TE at the doses tested.

Evaluation of post-ictal hypoxia, respiration, and heart rate following maximal electroshock seizure seizures

To study effects on postictal respiration following MES, we evaluated SpO_2, respiratory rate, and heart rate in CF-1 mice. Notably, this strain of mice survives TE despite having a period of apnea following seizures. VEH-treated CF-1 mice subjected to MES-induced TE experience a brief period (10–20 s) of apnea and decreased SpO_2, which coincides with tachypnea (see Figure 1). There were no major changes in heart rate observed.

Following a baseline MouseOx analysis, galanin analogs were administered by IP injection 1 h prior to testing. VEH, 810–2 (dose range 4–16 mg/kg), and 505–5 (dose range 1–4 mg/kg), were administered prior to a single MES stimulation and 5 min observation using MouseOx. A total of 810–2 was without major effect on post-ictal hypoxia (SpO_2), tachypnea (respiratory rate), or heart rate (Figures 2A–C). By contrast, 505–5 worsened hypoxia at 2 mg/kg (Figure 2D)

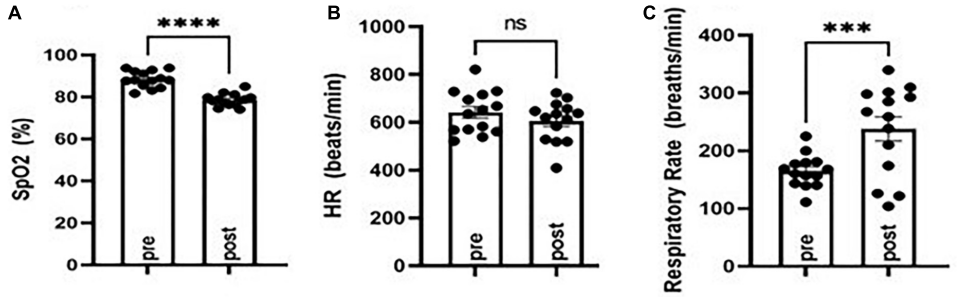

FIGURE 1
Evaluation of oxygen saturation (SpO_2), respiratory rate, and heart rate before (pre) and following (post) MES-induced tonic extension in CF-1 mice. SpO_2 was diminished **(A)** concomitant with an increased respiratory rate **(B)** and no major changes in heart rate **(C)**. ***$P < 0.001$, ****$P < 0.0001$ compared to pre, Student's *t*-test. $N = 14$.

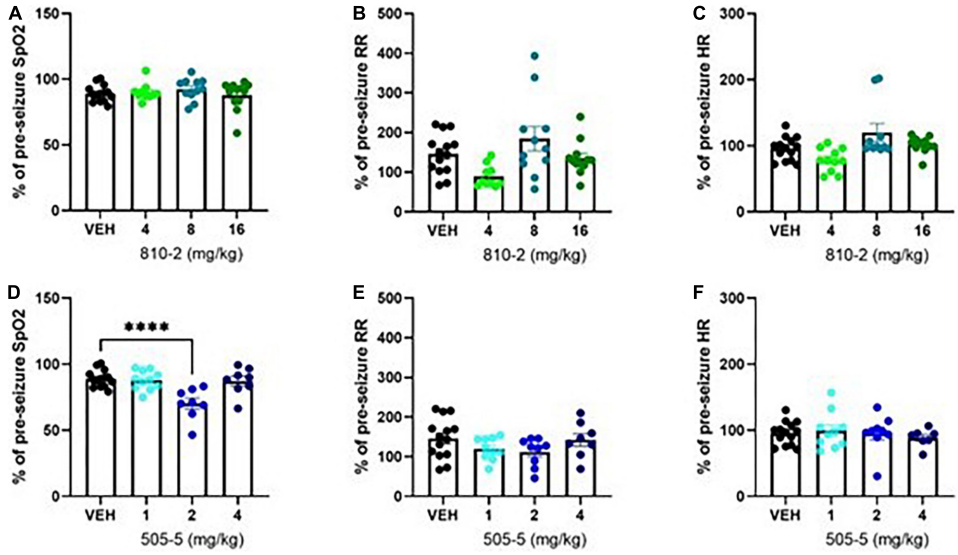

FIGURE 2
Evaluation of cardiorespiratory parameters following tonic extension in CF-1 mice. Data are presented as a percentage of baseline (pre-seizure) values. VEH-treated values are the same as shown in Figure 1, except expressed as a percent of baseline. A total of 810–2 **(A–C)** and 505–5 **(D–F)** were administered by IP injection 1 h prior to tonic extension and testing (MouseOx). Oxygenation [SpO_2; **(A,D)**], respiratory rate [RR; **(B,E)**], and heart rate [HR; **(C,F)**] were assessed for all animals. $N = 8–14$. ****$P < 0.0001$ (One-way ANOVA, Sidak's multiple comparison *post-hoc* test).

but was without major effect on respiratory rate or heart rate (Figures 2E,F).

Galanin analogs reduce seizure-induced respiratory arrest in fully kindled mice

Evaluation of corneal kindling as a potential model of sudden unexpected death in epilepsy

We obtained kindling records from the NIH Epilepsy Therapy Screening Program for several cohorts of CF-1 mice subjected to the corneal kindling paradigm. While this mouse strain is resistant to mortality when experiencing TE under naïve conditions, fully or partially kindled CF-1 mice often die following TE. To determine whether kindling state (fully kindled vs. partially kindled) affected S-IRA, we evaluated multiple cohorts of kindling data. Data presented here are from naïve kindled mice not treated with any investigational compounds. We observed that fully kindled mice have a high mortality rate, particularly when TE occurs after acquiring full kindled status (Supplementary Figure 1).

In separate studies, a cohort of CF-1 mice were kindled and subjected to baseline and post-ictal (MES assay) cardiorespiratory evaluation (MouseOx). We also observed that although SpO_2 was similarly reduced in age-matched control (CONT) and fully kindled (KIND) mice following TE, respiration did not significantly increase in KIND mice, though this change was present in mice that were partially kindled (PART; i.e., received daily stimulations but failed to acquire full kindled status) (Supplementary Figure 2). Interestingly, heart rate was decreased in KIND mice following TE, whereas it was unchanged in other groups.

Evaluation of the effect of galanin analogs on seizure-induced respiratory arrest in fully kindled mice

As a comparator to studies in naïve C57Bl/6J mice, a cohort of mice from this strain was fully kindled and subjected to treatment with galanin analogs using MES-induced TE. Initially, a group of untreated kindled mice was subjected to MES-induced TE and it was observed that a majority (6/9 animals died following TE). Similarly, VEH-treated kindled mice also demonstrate a high mortality after TE (11/16 died) (Table 3). A total of 505–5 improved mortality following TE in kindled mice (4/17 died) (Table 3). While 810–2 had a similar magnitude of effect, the difference was not significant compared to VEH (Table 3).

Table 4 includes a summary of major results observed in these studies. In seizure-naïve mice, systemic (IP, SC mini-pumps) and central (ICV, IA) administration reduced S-IRA, likely due to a Gal_2 mechanism. Central (ICV) administration, by contrast, appeared to reduce S-IRA predominantly *via* Gal_1. In seizure experienced animals, the reduction in S-IRA may be due to both Gal_1 and Gal_2 (comparable degree of effect for both analogs).

Discussion

SUDEP is a major cause of mortality in epilepsy, and better understanding of mechanisms of death may aid in the development of novel therapies to prevent or reduce the likelihood of death following seizures. Recent studies in human SUDEP have suggested a potential role for the neuropeptide galanin in the amygdala. Therefore, we sought to further evaluate the role of galanin in a mouse model of S-IRA under various conditions. Our principal findings include (1) galanin analogs reduced S-IRA in naïve mice following systemic and central administration, (2) 810–2 prevented mortality following sub-chronic administration, (3) protective effects of systemic galanin analogs were confirmed in fully kindled mice.

Poor adherence to treatment or pharmacoresistance may increase the occurrence of GTCS in patients with various forms of epilepsy. Further, GTCS are a critical risk factor for SUDEP. While genetic and acquired epilepsy models may recapitulate seizures and mortality in epilepsy, drug screening in these models may be challenging due to the need for prolonged administration and monitoring to determine whether treatment reduces seizure burden, TE, and mortality. This kind of study may be critical in demonstrating translational relevance for novel therapies, but throughput may be low. However, acute treatment and testing models may be more amenable to drug screening and help identify lead candidates for more rigorous

TABLE 3 Reduced S-IRA following a single administration of galanin analogs in fully kindled C57Bl/6J mice.

Groups	Dose (mg/kg)	Mortality (# died/N) (%)
VEH	0	11/16 (69%)
505–5	2	4/17* (24%)
810–2	16	2/8 (25%)

All mice tested experienced tonic extension. *$P < 0.05$ vs. VEH; Fisher's exact test.

TABLE 4 Summary of major findings for the effect of galanin analogs on S-IRA.

Receptor effect	IP	ICV	IA	SC (pump)
	Seizure-naïve			
Gal_1	±	+	−	−
Gal_2	+	−	+	+
	Seizure-experienced			
Gal_1	+	NE	NE	NE
Gal_2	±	NE	NE	NE

Gal_1-preferring analog (505–5), Gal_2-preferring analog (810–2). ND (not evaluated). (+) significant reduction in S-IRA, (–) no differences observed.

testing. Therefore, we used our previous knowledge of galanin analogs in S-IRA to better understand potential benefits of galanin-based therapies on reducing the likelihood or onset of SUDEP. Systemic administration of receptor subtype preferring analogs suggested that targeting of Gal_2 may be beneficial, as 810–2 demonstrated superior reduction in S-IRA over 505–5. Of note, the systemic doses used for each compound were not raised beyond those used in the study, as higher doses are associated with untoward effects (sedation and motor impairment, data not shown). Therefore, future endeavors with galanin analogs may seek to optimize administration through combinatorial pharmacology. For example, recent work has clearly demonstrated a potential benefit for serotonergic therapies in reducing the incidence of SUDEP in animal models (Richerson and Buchanan, 2011; Purnell et al., 2017; Buchanan, 2019; Kruse et al., 2019). While beyond the scope of this study, combined treatment with serotonin- and galanin-based therapies may yield an added benefit.

We sought to extend our systemic administration studies by using central injection to specifically target regions where galanin may exert protective effects against S-IRA. Galanin is expressed throughout the forebrain neurocircuitry, including several sites that affect cardiorespiratory and autonomic function. ICV administration studies suggest an important role for Gal_1 over Gal_2 in reducing S-IRA. Conversely, Gal_2 played a more prominent role when galanin analogs were administered directly into the amygdala. The amygdala has emerged as an important regulatory center for the interaction of seizures with respiratory control. Apnea results when seizures spread to the amygdala and amygdala lesions reduce S-IRA in mice (Marincovich et al., 2019; Nobis et al., 2019; Rhone et al., 2020). Additionally, the amygdala has reciprocal connections with brainstem respiratory centers (Saha et al., 2000; Dong and Swanson, 2006). Importantly, galanin is depleted in the amygdala in human SUDEP (Somani et al., 2020), which suggests that supplemental galanin in this region may reduce the incidence of SUDEP. The reason behind these discrepancies in our ICV and IA data are unclear but may involve multiple neural circuits affecting respiration after seizures. Furthermore, it is important to recognize that IA administration was centered over the basolateral amygdala but may have also spread to the central amygdala. The central amygdala may play an important role in post-ictal breathing by suppressing respiration (Nobis et al., 2018, 2019; Marincovich et al., 2019; Rhone et al., 2020). Future work may include application of galanin analogs in a more subregion-specific and receptor subtype-specific manner.

Tonic extension is lethal in a variety of mouse strains. We evaluated TE following MES stimulation in C57Bl/6J, CD-1, and CF-1 mice. Both C57Bl/6J and CD-1 mice experience similar mortality following MES stimulation. Given this observation, improved mortality following treatment with galanin analogs was the most important outcome observed in these strains. Further, we observed differential responses to galanin compounds: 810–2 (Gal_2-preferring) was effective in both strains in reducing S-IRA whereas 505–5 (Gal_1-preferring) was only effective in CD-1 mice. The mechanism for this strain-dependent discrepancy is unknown and may be due to different galanin receptor expression in brainstem respiratory centers between the two species. Future studies may further explore these potential differences. CF-1 mice generally survive TE and therefore this strain was used to study respiration following seizures. Despite the tendency to survive MES stimulation, we observed that this mouse strain demonstrates respiratory distress (diminished SpO_2, tachypnea) following TE. Thus, TE is a major respiratory stressor, yielding periods of apnea that may be insurmountable for some mouse strains. We hypothesized that galanin compounds may prevent mortality by preventing post-ictal hypoxia and therefore we evaluated oxygenation, heart rate, and respiratory rate in CF-1 mice treated prior to MES stimulation. Neither galanin compound prevented post-ictal hypoxia at any dose tested. Further, 505–5 demonstrated an exacerbation of this response (2 mg/kg). Therefore, the protective effects of galanin compounds in S-IRA may not be due to a direct effect on preventing the cardiorespiratory response to apnea.

G-protein coupled receptors may undergo internalization following stimulation with agonists (Calebiro and Godbole, 2018). To explore the use of galanin compounds as potential therapeutic agents, we performed sub-chronic administration using implanted minipumps pre-filled with either 810–2 or 505–5. To confirm antiseizure efficacy at the doses selected, we first performed 6 Hz seizure testing and observed that both 810–2 and 505–5 retained efficacy in this assay. On the following day, mortality following TE was assessed, and we observed that only 810–2 was effective in reducing mortality. The effect of 810–2 in this assay is consistent with the acute (single administration, IP) effect of this compound and confirms that systemic administration of Gal_2-preferring compounds exerts a protective effect against S-IRA.

Corneal kindling has long been used as an assay for studying epileptogenesis, epilepsy pathology, and antiseizure drug pharmacology. We observe that in CF-1 mice, a portion of animals die during and following kindling acquisition. We obtained several historical data cohorts from the ETSP (NIH, NINDS) Contract Site (University of Utah) and reviewed kindling histories for the presence of TE after daily kindling stimulation. We reviewed data from naïve kindled mice and we surmised that while naïve CF-1 mice may be resistant to death following TE, this may shift in a kindled state. We identified several animals that died after having experienced one or more bouts of TE. Deaths observed in this cohort did not necessarily occur immediately following TE. Further, these data are consistent with clinical observations that the presence of GTCS are a major risk factor for SUDEP. To better understand

the effect of TE on kindled mice, we subjected kindled CF-1 mice to pulse oximetry recordings before and after MES stimulation. We observed that oxygenation drops following TE in kindled mice in a similar manner to age-matched control mice. Interestingly, tachypnea observed in control mice was not observed to the same extent in kindled mice, though heart rate was diminished in these animals. Reduced post-ictal tachypnea among kindled mice may contribute to an increased risk for mortality in some animals.

To extend our studies of galanin analogs as protective of S-IRA in naïve mice, we also evaluated these analogs in fully kindled C57Bl/6J mice. In contrast to observations in naïve mice, where 505–5 was ineffective in reducing S-IRA, 505–5 reduced mortality (2 mg/kg) following TE. These data suggest a potential shift in receptor expression in brain respiratory centers toward Gal_1 sensitivity (e.g., receptor upregulation or downregulation of Gal_2), though this was not examined in the present study. Both Gal_1 and Gal_2 are expressed in the amygdala (Moller et al., 1999; Lundstrom et al., 2005; Christiansen and Woldbye, 2010; Webling et al., 2012; Li et al., 2017). While galanin receptor changes may occur in epilepsy (e.g., in hippocampus), the relative changes of each of these receptors in the amygdala are unknown. Future studies will examine the expression of each galanin receptor subtype in kindled mice.

Our previous work identified 505–5, 810–2, and other analogs as anticonvulsant neuropeptides in the mouse 6 Hz, Frings audiogenic, and corneal kindling models (Bulaj et al., 2008; White et al., 2009; Bialer et al., 2010, 2013, 2015). It was also observed during initial studies with these analogs that they were ineffective in preventing TE following MES stimulation. These studies, as well as several others examining galanin in seizure models (Mazarati et al., 1992, 1998; Saar et al., 2002; Lundstrom et al., 2005; Mazarati and Lu, 2005; Mitsukawa et al., 2008), provide a strong rationale for the therapeutic potential for galanin analogs as antiseizure medications. Herein we have extended these findings by demonstrating that galanin analogs can prevent S-IRA following TE in naïve and seizure experienced animals.

Limitations of the study

It is noteworthy that only males were used in screening for the reduction of S-IRA in these studies. Although seizure thresholds for TE are similar between males and females (data not shown), it is possible that the pharmacological response and/or prevention of mortality may be different in female animals. Future studies may extend these observations to more etiologically relevant animals models consisting of spontaneous seizures, wherein both male and female animals can be studied. Central injections were used for galanin analogs based on previously established protocols for ICV (Lee et al., 2009; Green et al., 2011; Platt et al., 2012) and IA (West et al., 2022) injections. However, verification of injection location was not performed in the current studies. It is possible that improper ICV injection and local diffusion rather than ventricular distribution may account for the effects observed. Further, IA injection not properly administered may have affected surrounding regions that also control respiration (Homma and Masaoka, 2008; Evans, 2010; Trevizan-Bau et al., 2021). Because central injection parameters were not verified explicitly, it is possible that off-target effects arising from galanin analog administration may have contributed to the outcomes observed. The precise mechanism whereby galanin agonism may prevent S-IRA has yet to be determined. While galanin may play an important modulatory role on synaptic transmission in key respiratory control centers such as the amygdala, the means whereby galanin may protect against S-IRA is unclear. Future studies may include the use of a neuronal activation marker (e.g., Fos) following treatment and stimulation to determine the effect of galanin analogs on post-ictal activation of respiratory cell populations.

In summary, we have observed that galanin compounds protect against S-IRA following TE in naïve and kindled mice. This was observed after systemic and central administration, and responses varied depending on route, location, strain, seizure history, and receptor subtype preference. While these studies suggest the potential translational benefit for galanin in SUDEP prevention, additional work is needed to differentiate the utility of these analogs in SUDEP-susceptible individuals.

Data availability statement

The datasets presented in this article are readily available and can be accessed by contacting CM (cameron.s.metcalf@utah.edu).

Ethics statement

This animal study was reviewed and approved by the Institutional Animal Care and Use Committee, University of Utah.

Author contributions

RC designed and conducted the experiments, analyzed the data, and prepared the manuscript. MA and ED designed and conducted the experiments and analyzed the data. KR and CC conducted the experiments. CM designed and conducted the experiments, analyzed the data, and prepared the manuscript,

oversaw all work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

CM was a former full-time employee of NeuroAdjuvants, Inc., a biotechnology company seeking to develop galanin-based neuropeptides for CNS conditions. CM holds two patents for galanin-based therapies for CNS conditions (University of Utah).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The role of sleep state and time of day in modulating breathing in epilepsy: implications for sudden unexpected death in epilepsy

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Sudden unexpected death in epilepsy (SUDEP) is the leading cause of death among patients with refractory epilepsy. While the exact etiology of SUDEP is unknown, mounting evidence implicates respiratory dysfunction as a precipitating factor in cases of seizure-induced death. Dysregulation of breathing can occur in epilepsy patients during and after seizures as well as interictally, with many epilepsy patients exhibiting sleep-disordered breathing (SDB), such as obstructive sleep apnea (OSA). The majority of SUDEP cases occur during the night, with the victim found prone in or near a bed. As breathing is modulated in both a time-of-day and sleep state-dependent manner, it is relevant to examine the added burden of nocturnal seizures on respiratory function. This review explores the current state of understanding of the relationship between respiratory function, sleep state and time of day, and epilepsy. We highlight sleep as a particularly vulnerable period for individuals with epilepsy and press that this topic warrants further investigation in order to develop therapeutic interventions to mitigate the risk of SUDEP.

KEYWORDS

epilepsy, SUDEP, sleep, circadian, breathing

Introduction

Epilepsy is one of the most common neurological disorders. One in 26 Americans will develop epilepsy during their lifetime (Kotsopoulos et al., 2002 ; Hesdorffer et al., 2011). Despite its prevalence, approximately 35% of patients will not achieve seizure freedom with medical treatment (Kwan and Brodie, 2000; Chen et al., 2018). Though there has been continued expansion in the availability of anti-seizure medications (ASM), patients who exhibit an inadequate response to initial ASM treatment are likely to have medically refractory epilepsy (Kwan and Brodie, 2000). The leading cause of death among these individuals with poor seizure control is sudden unexpected death in epilepsy or SUDEP (Devinsky et al., 2016). SUDEP is defined as the "sudden, unexpected, witnessed or unwitnessed, nontraumatic and nondrowning death in patients with epilepsy, with or

without evidence for a seizure and excluding documented status epilepticus, in which postmortem examination does not reveal a toxicologic or anatomic cause of death" (Nashef et al., 1998). While by definition SUDEP does not have to follow a seizure, there is strong evidence to suggest it is a seizure-related phenomenon, with its agonal mechanisms beginning during or in the immediate aftermath of a seizure (Nashef et al., 1998; Nilsson et al., 1999; Surges et al., 2009; Surges and Sander, 2012; Bozorgi and Lhatoo, 2013). There is a slight predominance of SUDEP cases in males compared to females (Tennis et al., 1995; Nilsson et al., 1999; Shankar et al., 2013).

Despite the tremendous burden of SUDEP, its underlying pathological mechanisms are poorly understood. However, evidence is accumulating that implicates seizure-related respiratory failure as a major factor in this deadly phenomenon (Ryvlin et al., 2013; Buchanan et al., 2014; Kim et al., 2018; Dhaibar et al., 2019). In SUDEP cases that were captured in epilepsy monitoring units (EMU), terminal apnea preceded terminal asystole in every case (Ryvlin et al., 2013). Further, mechanical ventilation has been found to greatly reduce seizure-induced mortality, both in human patients and animal models (Tupal and Faingold, 2006; Ryvlin et al., 2013; Buchanan et al., 2014). Thus, further investigation into respiratory dysfunction in epilepsy is critical to untangle the underlying mechanisms of SUDEP, as well as to assist clinicians in developing respiratory-focused interventions.

Another consistent observation is that SUDEP cases predominantly occur during the night (Nobili et al., 2011; Lamberts et al., 2012; Sveinsson et al., 2018). Around 95% of SUDEP cases occur inside the victim's residence, with the majority of victims found in or near a bed in a prone position (Opeskin and Berkovic, 2003; Zhuo et al., 2012; Ali et al., 2017; Sveinsson et al., 2018). Despite occurring so close to home, the vast majority of these cases are unwitnessed (Lamberts et al., 2012; Zhuo et al., 2012; Rugg-Gunn et al., 2016; Purnell et al., 2018). Patients who die of SUDEP are twice as likely to have a history of nocturnal seizures, and thus the presence of nocturnal seizures are considered a risk factor for SUDEP (Lamberts et al., 2012; Shankar et al., 2013; Sveinsson et al., 2018; Van Der Lende et al., 2018). Seizures and epileptiform discharges occur more frequently during non-rapid eye movement (NREM) sleep in both human patients and animal models (Bazil and Walczak, 1997; Malow et al., 1998). Sleep state can influence the frequency, severity, and duration of seizures (Bazil and Walczak, 1997; Ng and Pavlova, 2013). Seizures occurring during sleep tend to be longer and are more likely to evolve into focal and bilateral tonic-clonic seizures (Bazil and Walczak, 1997).

As humans tend to consolidate their sleep during the night, many investigations of and conclusions about SUDEP risk factors conflate sleep-state and nighttime as one in the same. In reality, sleep and circadian rhythmicity can independently alter physiological processes, including respiratory and cardiac function (Snyder et al., 1964; Browne et al., 1983; Spengler et al., 2000; Mortola, 2004; Buchanan, 2013). The major influence of sleep and circadian timing on respiration makes this a salient point of examination when considering SUDEP pathophysiology. The aim of this review is to examine the distinct influences of sleep and circadian rhythms on respiration both in a healthy brain and in patients with epilepsy (**Figure 1**). We hope to not only highlight the factors that make nocturnal seizures more deadly, but to better differentiate between sleep-state and time-of-day influences on breathing, so that clinicians can develop specific preventative strategies for fatal seizure-induced respiratory dysfunction.

Influence of sleep on breathing

It has long been appreciated that breathing is regulated in a sleep state-dependent manner (Snyder et al., 1964; Spengler et al., 2000; Haxhiu et al., 2003; Mortola, 2004; Malik et al., 2012; Buchanan, 2013). Inspiratory drive is lower during NREM sleep and lowest during rapid-eye movement (REM) sleep, with tidal volume (V_T) being reduced to 73% of its level during wakefulness (Douglas et al., 1982a; **Figure 2A**). Within NREM sleep, the nadir of minute ventilation (V_E) occurs during NREM stage 3 (N3) sleep—although this is likely driven by the reduction in V_T. This results in an end-tidal carbon dioxide ($ETCO_2$) concentration that is 1–2 torr higher than waking levels (Krieger, 2005). This drop in V_T and V_E is likely due to decreased chemosensitivity during the onset of sleep (Bulow, 1963; Douglas et al., 1982b,c). During sleep there is a decrease in the respiratory response to hypercapnia (Reed and Kellogg, 1958; Birchfield et al., 1959; Cherniack, 1981; Douglas et al., 1982c; Berthon-Jones and Sullivan, 1984; **Figure 3A**) as well as hypoxia (Berthon-Jones and Sullivan, 1982; Douglas et al., 1982b; Malik et al., 2012). Like inspiratory drive, there is an even larger decrease in the hypoxia-induced respiratory drive during REM compared to NREM sleep (Berthon-Jones and Sullivan, 1984; Malik et al., 2012). There are sex-specific differences in the response to hypercapnia, with males exhibiting a 50% decrease in the hypercapnic ventilatory response (HCVR) compared to wakefulness, while females exhibit a reduced HCVR during wakefulness compared to males but have less apparent reductions in the response during sleep (Berthon-Jones and Sullivan, 1984). Progesterone has been found to stimulate breathing during sleep, including increasing hypoxic and hypercapnic respiratory responses (Javaheri and Guerra, 1990; Saaresranta et al., 1999). Progesterone oscillates in a circadian fashion, with its zenith at around midnight (Junkermann et al., 1982; Gharib et al., 2018). No sex-specific differences in respiratory responses to hypoxia have been identified (Malik et al., 2012).

Breathing during NREM sleep has a more regular pattern compared to breathing during wakefulness, without altering mean breathing frequency (Malik et al., 2012). Conversely,

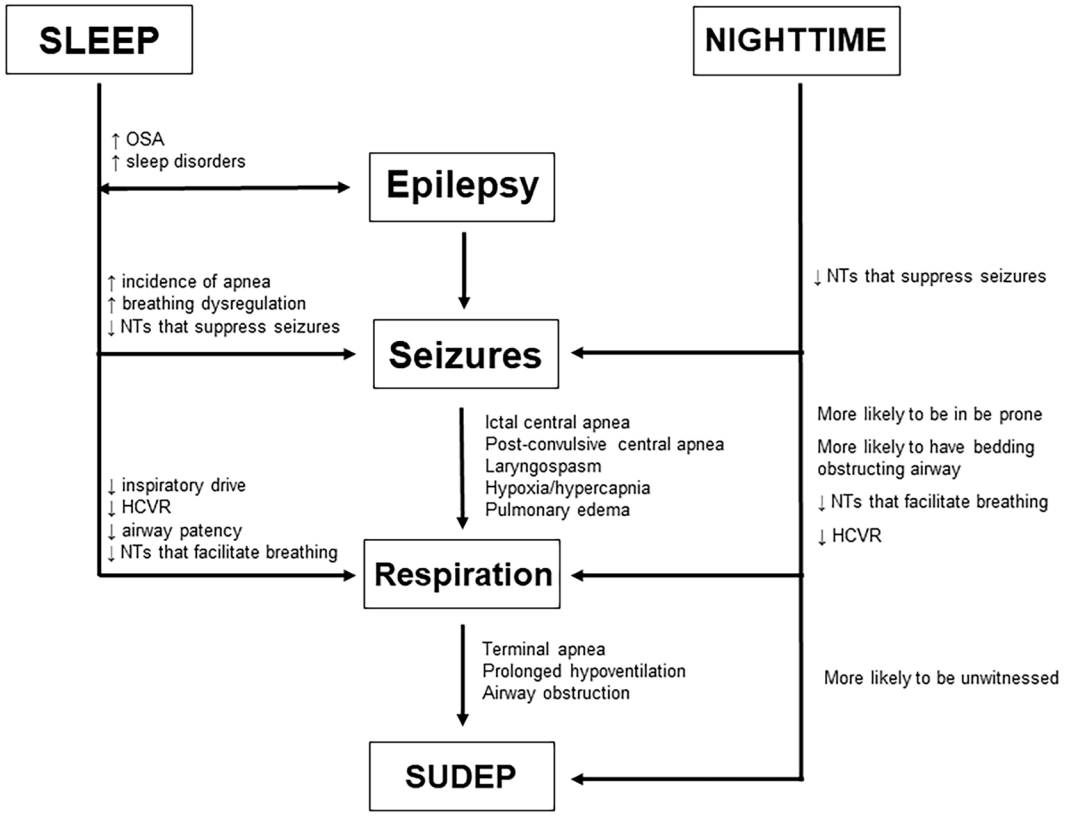

FIGURE 1
Potential risk factors associated with seizures emerging from sleep vs. nocturnal seizures and how they may facilitate SUDEP by modulating epilepsy, seizures, and respiration as well as seizure-induced death itself.

during REM sleep there is more variability in respiratory patterns, including increased frequency, decreased regularity, and brief periods of central apnea (Aserinsky and Kleitman, 1953; Cherniack, 1981; Malik et al., 2012). There is some evidence that indicates this irregular breathing is a response to cortical inputs that reflect the content of the individual's dream (Oudiette et al., 2018). Periodic breathing, which is characterized as clusters of breaths separated by intervals of central apnea or near apnea, also sometimes occurs during sleep. Although previously thought to arise from a severe neurological or cardiovascular condition, it now found that periodic breathing can occur in healthy individuals, especially during hypoxia (Berssenbrugge et al., 1983; Cherniack, 1999; Ainslie et al., 2013). During intervals of periodic breathing, cyclic changes in ventilation as well as the partial pressures of carbon dioxide (CO_2) and oxygen (O_2) can trigger oscillations in heart rate, blood pressure, autonomic nervous system activity, and upper-airway resistance. This may create a feedback loop whereby these oscillations in turn affect ventilation and increase the length and symmetry of these periodic breathing cycles (Cherniack, 1999). Males tend to exhibit periodic breathing in response to hypoxia more frequently than females (Pramsohler et al., 2019). Breathing patterns are heavily dependent on the pre-Bötzinger complex (pre-BötC; Smith et al., 1991; Buchanan, 2013; Del Negro et al., 2018; Muñoz-Ortiz et al., 2019). When neurons expressing neurokinin-1 receptors (NK1R) in the pre-BötC complex were bilaterally ablated in adult rats there was a progressive and irreversible disruption in breathing stability, which initially occurred only during sleep, but eventually led to ataxic breathing during wakefulness as well (Mckay and Feldman, 2008). When these pre-BötC NK1R-expressing neurons were unilaterally ablated, there was a disruption in respiratory pattern and increase in the frequency of central sleep apnea and hypopneas solely during sleep, particularly during REM sleep, which never developed during wakefulness (Mckay and Feldman, 2008).

During sleep there is a reduction in upper airway patency and an increase in respiratory resistance. This is caused by a preferential reduction in tone of laryngeal and pharyngeal muscles that help to maintain the structure of the upper airway (Cherniack, 1981; Haxhiu et al., 1987; Buchanan, 2013; Kubin, 2016). This reduced patency and can be especially problematic during REM sleep, when breathing is particularly unstable (Cherniack, 1981). Upper airway tone is controlled by inputs

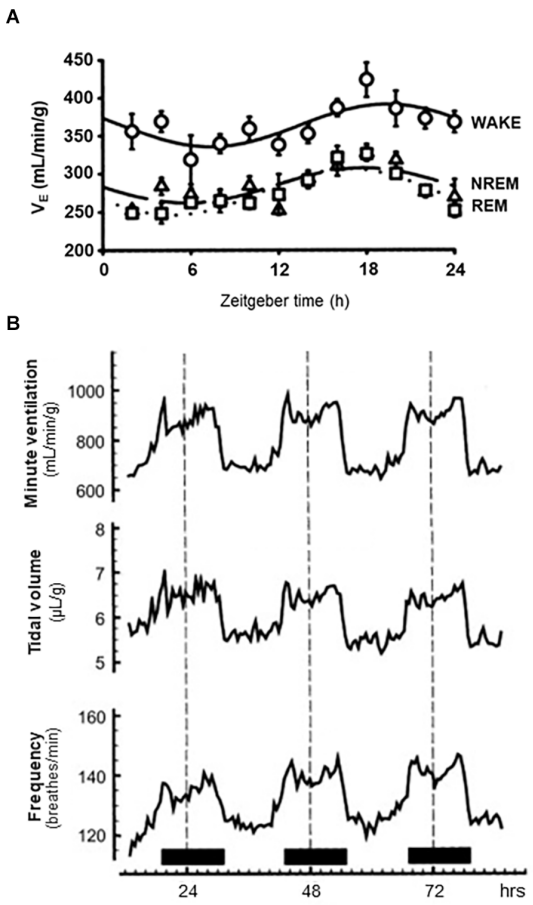

FIGURE 2
Circadian and sleep state-dependent effects on ventilation. **(A)** 72-h traces of average minute ventilation (top), tidal volume (middle) and breathing frequency (bottom) in adult male rats housed under a 12:12 h light:dark cycle and receiving room air (21% O_2, balance N_2). Solid horizontal bars at the bottom indicate periods where lights were off. **(B)** 24-h trace of average minute ventilation in rats during wake, non-rapid eye movement (NREM) sleep, and rapid-eye movement (REM) sleep as indicated. All animals housed in a 12:12 h light:dark cycle. **(A)** Redrawn with permission from Seifert and Mortola (2002). **(B)** Redrawn with permission from Stephenson et al. (2001).

from trigeminal (CN V), facial (CN VII), and hypoglossal (CN XII) motor neurons (Buchanan, 2013). The genioglossus muscle, which is innervated by hypoglossal motor neurons, is the largest and most extensively studied of the airway dilator muscles. It has been suggested that decreased serotonergic and noradrenergic inputs to hypoglossal motor neurons during REM sleep causes atonia of the genioglossus (Fenik et al., 2005). The genioglossus and other muscles of the upper airway require both tonic and phasic inspiratory activation in order to protect against collapse (Kubin, 2016). When the tone of these airway-dilating muscles can no longer oppose the negative inspiratory pressure, the result is obstructive sleep apnea (OSA), which features recurrent episodes of hypopneas and apneas (Remmers et al., 1978; Kubin, 2016). While these obstructive apneas only occur during sleep, frequent sleep apnea and hypoventilation can result in breathing abnormalities during wakefulness (Simonds, 1994).

Changes in several non-centrally mediated respiratory mechanisms are also associated with the onset of sleep. During NREM sleep, the activity of the intercostal muscles is increased compared to wakefulness (Malik et al., 2012). This may be indicative of increased contribution of the chest wall to respiration in order to compensate for decreased central inspiratory drive. During REM sleep, there is a loss of tonic activity in the intercostals and diaphragm (Tusiewicz et al., 1977; Bryan and Muller, 1980; Malik et al., 2012). Chest wall compliance is also increased during this time, and, in conjunction with decreased intercostal tone, can cause paradoxical collapse of the chest during inspiration (Malik et al., 2012). Lastly, the pulmonary stretch receptor reflex and irritant receptor reflex are suppressed during sleep—thus, coughing in response to apnea only occurs after arousal (Douglas, 2000). In summary, sleep is a period where many facets of breathing are suppressed—thus rendering it a particularly vulnerable period for further insults to the respiratory system.

Circadian influences on breathing

Early studies of time-of-day variability in mammalian (adult rat) breathing physiology revealed time-of-day differences in breathing; however, the effect was limited to CO_2 production and the mean inspiratory air flow (Peever and Stephenson, 1997). Under hypercapnic conditions, breathing frequency and V_E also appeared to be time-of-day dependent. Unfortunately, these studies only involved two time-points, limiting the resolution of a daily rhythm, which may have been masked by higher frequency ultradian variation in breathing (Stupfel and Pletan, 1983; Stupfel et al., 1985).

The first clear evidence that respiratory function demonstrated daily oscillations came from Seifert et al. (2000). Adult rats were housed in 10 L barometric chambers with carefully controlled in-flow and out-flow of gas, allowing for measurement of breathing physiology over the course of several days. Clear time-of-day differences in frequency, V_T and V_E were observed. The highest levels were observed during the dark phase, coinciding with elevated temperature and activity. These findings were further expanded in a later study, demonstrating that O_2 consumption (a measure of metabolic activity), inspiratory time, and expiratory time also varied across the day (Seifert and Mortola, 2002; **Figure 2B**). Interestingly, controlling for level of activity did not eliminate the effect of time of day on V_E, V_T, frequency of breathing, or O_2 consumption. The authors conclude that the daily variability observed in breathing (specifically ventilation) is likely driven by other physiological variables oscillating throughout the day, such as temperature and oxygen consumption.

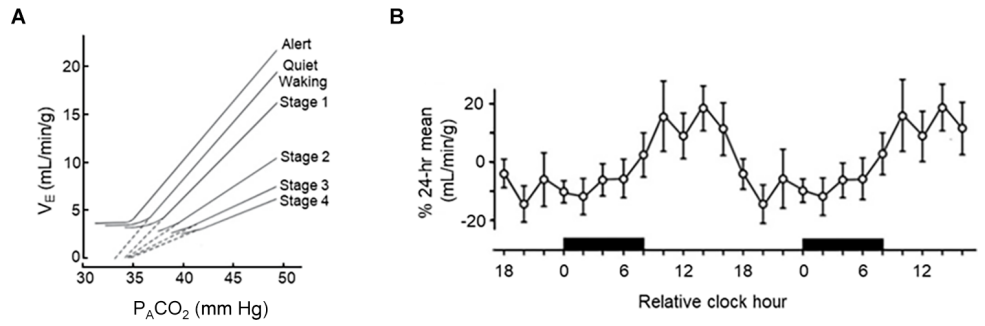

FIGURE 3
Circadian and sleep state-dependent effects on the hypercapnic ventilatory response (HCVR). **(A)** 48-h trace of circadian variations in HCVR in adult humans. **(B)** Sleep state-dependent differences in HCVR in adult males. **(A)** Redrawn with permission from Spengler et al. (2000). **(B)** Redrawn with permission from Bulow (1963).

Using similar methods to Seifert et al., 2000, long-term respiratory monitoring in non-human primates has also been performed (Iizuka et al., 2010). Whole body plethysmography was performed in 11 unrestrained, unanesthetized male cynomolgus monkeys. Like findings from adult rats, multiple respiratory parameters, including respiratory rate, V_T, and V_E were shown to vary depending on the time of day. However, recordings were only obtained hourly, and it is unclear if sleep-state was controlled for.

Time-of-day variability in a number of respiratory parameters has also been demonstrated in humans (Spengler and Shea, 2000; Spengler et al., 2000). In a carefully controlled laboratory setting, which included the removal of external time cues (except for lighting), a constant environmental temperature, controlled dietary intake, and carefully controlled sleep schedules. Temporal variation in rectal temperature and plasma cortisol were used as endogenous circadian markers. $ETCO_2$, O_2 consumption, and CO_2 production were all shown to oscillate throughout the 24 h day, with highest levels in the morning. Interestingly, there was also time-of-day variability in HCVR magnitude, a finding previously demonstrated in awake, adult rats (Peever and Stephenson, 1997; **Figure 3B**), suggesting respiration-influencing chemosensitivity may also be under circadian regulation. Sensitivity to isocapnic hypoxic challenge has some evidence of time-of-day dependence; however, the effect is far less pronounced (Siekierka et al., 2007).

While the studies described above in rats, monkeys, and humans demonstrate temporal variation in breathing physiology, they did not control for the rhythmic effect of light. Therefore, whether this variability is due to the effect of an external time cue or that of an endogenous circadian rhythm cannot be concluded. The first study of time-of-day dependent on breathing physiology that accounted for the influence of light was performed in garter snakes (Hicks and Riedesel, 1983). Animals were housed in either a 14:10 light-dark cycle or constant darkness environment. Under these conditions, it was revealed that time-of-day variability in oxygen consumption, breathing frequency, V_T, and V_E persisted in constant darkness, suggesting endogenous regulation of these breathing parameters.

This time-of-day dependent variation in breathing has been shown to be endogenously circadian in mice and mediated by the body's central circadian pacemaker, the suprachiasmatic nucleus (Purnell and Buchanan, 2020). C57BL/6J mice were housed in either 12:12 light-dark or constant darkness environments, and running wheels were used to assess active phase onset for the determination of individual free-running locomotive rhythms. As sleep-state has been shown to influence breathing (as described in detail earlier above), measurements of breathing were only performed while the animals were awake. Time-of-day variability in the frequency of breathing and V_E, but not V_T, was observed. Both frequency and V_E were highest during the dark phase of the day. This time-of-day rhythm was shown to be circadian, as these two rhythms persisted when animals were housed in constant darkness. Electrolytic lesioning of the suprachiasmatic nucleus eliminated these breathing rhythms, suggesting that the circadian variation in breathing was controlled by the suprachiasmatic nucleus.

Although the suprachiasmatic nucleus is frequently referred to as the master circadian oscillator, nuclei outside of the suprachiasmatic nucleus and peripheral tissues may contain autonomous circadian clocks (Mohawk et al., 2012). Such peripheral clocks have been described in brainstem and spinal cord neurons involved in the coordination and output necessary to maintain normal respiration. Through the measurement of molecular clock gene transcripts, such as *Clock*, *Bmal1*, and *Per1/2*, researchers have identified robust molecular clock gene rhythms in the nucleus tractus solitarius (Kaneko et al., 2009; Chrobok et al., 2020), phrenic motor nucleus (Kelly et al., 2020), and laryngeal, tracheal, bronchial, and lung tissues within the airway (Bando et al., 2007). Bando et al. (2007) demonstrated that the peripheral clock of the airway tissues could be rendered arrhythmic following electrolytic lesioning of the suprachiasmatic nucleus. Similarly, genetically arrhythmic *Clock 1/Clock2* knock-out mice did not

demonstrate peripheral rhythmicity of clock gene expression in airway tissues. In conclusion, circadian phase exerts its own powerful influences on breathing, irrespective of vigilance state.

Effect of seizures/epilepsy on breathing

Some patients with epilepsy experience breathing abnormalities at baseline which may be further compromised during a seizure. Sainju et al. found a blunted hypercapnic ventilatory response in a subset of patients with epilepsy, placing them at greater risk for peri-ictal hypoventilation (Sainju et al., 2019). Patients with Dravet syndrome (DS) similarly exhibit a decreased ventilatory response to CO_2 (Kim et al., 2018). Several animal models of epilepsy present with respiratory dysregulation, even in the absence of a seizure. The *Kcna1*-null mutant model exhibits progressive respiratory dysfunction with age (Simeone et al., 2018). Like their human counterparts, the $Scn1a^{R1407X/+}$ human knock-in mouse model of DS has a diminished ventilatory response to CO_2, as well as baseline hypoventilation and apnea (Kuo et al., 2019). A similar loss of the hypercapnic ventilatory response has been found in animals that have undergone amygdala kindling (Totola et al., 2019). Hajek and Buchanan (2016) found that mice with increased respiratory rate variability at baseline are more likely to die following a maximal electroshock (MES) seizure. These findings support the idea that interictal respiratory dysfunction may serve as a biomarker for those at greater risk for SUDEP.

Seizures themselves can cause profound alterations in respiration, including coughing, apnea, hyperventilation, bronchial spasms, increased pulmonary vascular pressure, laryngospasm, and pulmonary edema (Bayne and Simon, 1981; Kennedy et al., 2015; Nakase et al., 2016; Rugg-Gunn et al., 2016). Seizures appear to cause varying degrees of respiratory dysregulation depending on seizure type and origin (Bateman et al., 2008; Blum, 2009). Longer duration of seizures is associated with a greater degree of dysfunction, particularly in regard to hypercapnia, pulmonary pressure, and pulmonary edema (Bayne and Simon, 1981; Bateman et al., 2008; Seyal et al., 2010; Kennedy et al., 2015).

Hypoventilation during a seizure may occur due to airway obstruction or dysregulation of the brain's respiratory centers and usually results in hypercapnia and hypoxemia (Rugg-Gunn et al., 2016). Dravet syndrome patients in particular demonstrate peri-ictal hypoventilation, which precedes the onset of bradycardia (Kim et al., 2018). Hypoventilation can lead to secondary cardiac failure, especially during seizures where oxygen saturation (SaO_2) drops below 90% (Seyal et al., 2011). A cause of some ictal hypoventilation is central apnea. Ictal central apnea (ICA) is a relatively frequent occurrence during seizures, especially ones with bihemispheric involvement (Nashef et al., 1996; Rugg-Gunn et al., 2016). ICA occurs exclusively in focal epilepsy, emerging during 33–50% of focal seizures (Lacuey et al., 2018; Vilella et al., 2019; Tio et al., 2020). ICA can precede electrographic seizure activity as well as clinical seizure onset by up to 7–10 s (Nishimura et al., 2015; Tio et al., 2020). These apneas tend to be brief and do not substantially impact O_2 saturation (Bateman et al., 2008). A multivariate analysis indicated that contralateral seizure spread and seizure duration mutually contribute to increased $ETCO_2$ that follows ICA (Seyal et al., 2010). Several animal models of epilepsy and SUDEP exhibit ICA, including $Scn1a^{R1407X/+}$ mice, in which mechanical ventilation can prevent fatal seizure-induced respiratory arrest (Kim et al., 2018). Additionally, a model of status epilepticus induced in sheep features ICA and hypoventilation in 100% of the animals, with some resulting in death (Johnston et al., 1997). Post-convulsive central apnea (PCCA), in contrast, occurs in both focal and generalized epilepsies, suggesting a separate pathophysiology from ICA (Vilella et al., 2019). PCCA is less common than ICA—occurring during only 18% of generalized seizures. However, PCCA may be much more dangerous than ICA. PCCA is associated with a longer recovery time from hypoxemia, and it is considered by some to be a biomarker for SUDEP (Jin et al., 2017; Vilella et al., 2019).

Seizures may impair a person's ability to autoresuscitate after central apnea. Autoresuscitation is a spontaneous protective cardiorespiratory phenomenon which promotes the recovery of normal breathing and heart rate after primary apnea by initiating a gasping reflex (Adolph, 1969; Guntheroth and Kawabori, 1975). Failure to autoresuscitate has been documented in infant deaths that were eventually classified as sudden infant death syndrome (SIDS; Meny et al., 1994; Sridhar et al., 2003). There are numerous parallels between SIDS and SUDEP, including normal autopsy, prone position, predominance during the nighttime, predicted respiratory mechanism, and evidence of serotonergic system dysfunction (Richerson and Buchanan, 2011; Buchanan, 2019).

Obstructive apnea, or laryngospasm, is another seizure-associated phenomenon that can result in death (Stewart, 2018). DBA/2 mice, which display lethal audiogenic seizures, have a significantly reduced mortality rate following seizures after being implanted with a tracheal T-tube as a surrogate airway (Irizarry et al., 2020). Seizures induced *via* kainic acid in rats have been documented to cause partial or complete glottic closure and subsequent death (Nakase et al., 2016; Budde et al., 2018; Jefferys et al., 2019). It has been postulated that fatal obstructive apnea is a consequence of bronchial spasms or hypotonia of the muscles involved in respiration (Stöllberger and Finsterer, 2004). Nakase et al. proposed that ictal laryngospasm is caused by the spread of a seizure *via* autonomic medullary motor regions to the laryngeal branches of the vagus nerve (Nakase et al., 2016).

Spreading depolarization (SD) may be one of the underlying mechanisms behind cardiorespiratory failure in SUDEP. In

Cacna1a^{S218L} mutant mice, which carry a gain of function mutation in the $Ca_v2.1$ voltage-gated calcium channel, brainstem SD occurs during all spontaneous fatal seizures, as well as a subset of nonfatal seizures (Jansen et al., 2019). Additionally, seizure-related SD in the ventrolateral medulla is correlated with the incidence of respiratory suppression (Jansen et al., 2019). Chemically induced seizures in *Kcna1* and *Scn1a* mutant mice cause a wave of SD in the dorsal medulla, which may temporarily silence the cells that would serve to reoxygenate the brain following a seizure (Aiba and Noebels, 2015). This depolarizing blockade may cause a positive feedback loop in which the brain cannot reoxygenate following a seizure during which oxygen saturation has dropped dramatically, potentially leading to complete cardiorespiratory arrest (Aiba and Noebels, 2015).

Numerous other potential mechanisms underlying ictal respiratory dysfunction and failure have been proposed. A leading hypothesis is that seizures activate inhibitory subcortical projections to brainstem respiratory centers (Dlouhy et al., 2015; Lacuey et al., 2017). It has also been found that central apnea occurs in human patients when seizures spread to the amygdala (Dlouhy et al., 2015; Rhone et al., 2020). Similarly, stimulation of the amygdala as well as the hippocampus produces central apnea that patients are not aware of (Dlouhy et al., 2015; Lacuey et al., 2017; Nobis et al., 2018), and they are able to voluntarily initiate inspiration when prompted (Dlouhy et al., 2015). Further studies revealed that stimulation of the basal amygdala in particular (including the basomedial and basolateral nuclei) was particularly likely to cause apnea, while stimulation of more lateral regions produced fewer apneas (Rhone et al., 2020). In DBA/1 mice, unilateral lesions to the amygdala was sufficient to suppress seizure-induced respiratory arrest (S-IRA) (Marincovich et al., 2021). This suggests that apnea is due to the loss of involuntary ventilatory drive rather than an issue with the respiratory motor output pathways or musculature.

Despite the implications of both respiratory and cardiac dysfunction contributing to SUDEP, recent evidence has surfaced suggesting respiratory failure precedes cardiac failure during instances of seizure-induced death. In 2013, a multi-center MORTality in EMUs Study (MORTEMUS) of SUDEP incidents in EMUs found that all recorded cases of SUDEP featured terminal respiratory arrest prior to terminal asystole (Ryvlin et al., 2013). Similar results have been found in the *Kcna1*-null mouse model (Dhaibar et al., 2019) and in an MES model (Buchanan et al., 2014). Another indicator of respiratory failure's pivotal role in SUDEP is that mechanical ventilation, if administered immediately, can greatly reduce mortality in both human patients and animal models (Tupal and Faingold, 2006; Ryvlin et al., 2013; Buchanan et al., 2014). In a similar vein, oxygenation prior to seizure induction can prevent fatal audiogenic seizures in several strains of audiogenic mice, without impacting seizure incidence or severity (Venit et al., 2004). To summarize, seizures cause profound alterations in breathing which may directly contribute to seizure-induced death.

Sleep and circadian effects of seizures/epilepsy on breathing

Approximately 10–15% of epilepsy patients have seizures solely or primarily during sleep (Grigg-Damberger and Foldvary-Schaefer, 2021). Seizures occurring during sleep tend to be longer and are more likely to evolve into focal and bilateral tonic-clonic seizures (Bazil and Walczak, 1997). As mentioned above, longer convulsive seizures are associated with an increased degree of respiratory dysfunction (Bayne and Simon, 1981; Bateman et al., 2008; Seyal et al., 2010; Kennedy et al., 2015). Seizures emerging from sleep are also more likely to be associated with the presence of post-ictal generalized EEG suppression (PGES) and greater oxygen desaturation (Latreille et al., 2017). A clinical study in 20 patients with epilepsy found that 44% of nocturnal seizures are associated with ICA, and although the difference did not reach significance, a smaller fraction of wake-related seizures were accompanied by ICA (28%; Latreille et al., 2017). MES seizures in mice that are induced during NREM sleep are also associated with greater respiratory dysfunction than those induced during wakefulness (Hajek and Buchanan, 2016). When factoring in time of day, MES seizures that were induced during the day, the rodent inactive phase, resulted in a greater degree of postictal respiratory and EEG suppression than those induced during the nighttime. This effect was even greater when the seizures were induced during this time while the animal was in NREM sleep (Purnell et al., 2017). When DBA/1 mouse model of audiogenic seizures were exposed to an audiogenic stimulus during the day, the ensuing seizures resulted in death during 21.7% of trials. Conversely, seizures induced during the night resulted in seizure-induced death in 46.7% of trials (Purnell et al., 2021b; **Figure 4A**). The same study used mice living in constant darkness to access circadian influence on seizure-induced death in the MES mouse model. They found that during the subjective night there was a decrease in postictal ventilation and an increase in the probability of seizure-induced death without altering seizure severity (Purnell et al., 2021b; **Figure 4B**). $K_v1.1$ potassium channel knockout (KO) mice and *SCN1A*$^{R1407X/+}$ mice, which experience progressive breathing dysregulation (Kim et al., 2018; Kuo et al., 2019; Iyer et al., 2020), also experience seizure-induced death more commonly during the nighttime (**Figures 4C,D**).

Because humans typically sleep during the night, nighttime seizures are often unwitnessed (Lamberts et al., 2012; Zhuo et al., 2012; Rugg-Gunn et al., 2016; Purnell et al., 2018). Lamberts et al. (2012) reported that 86% of SUDEP cases are unwitnessed. It is hypothesized that being unaccompanied during a nocturnal

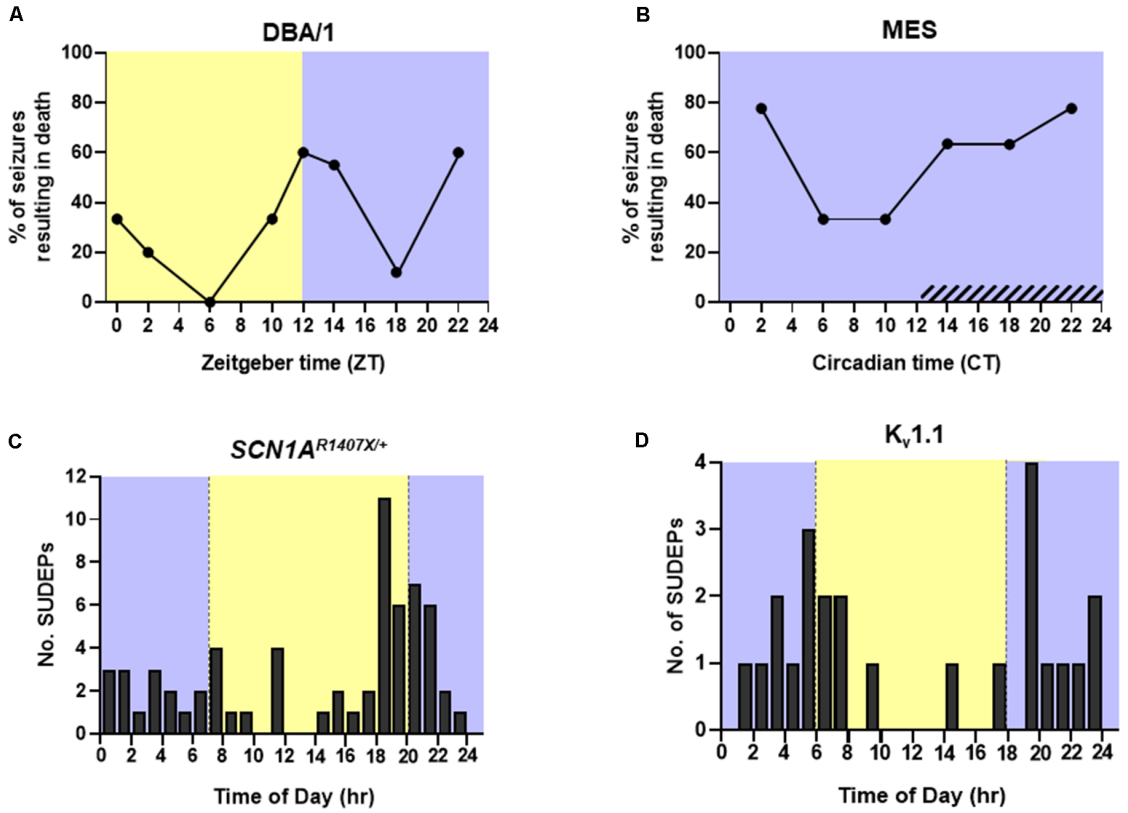

FIGURE 4
Time-of-day and circadian probability of seizure-induced death in mouse models of epilepsy. Temporal distribution of spontaneous seizure-induced death in **(C)** *SCN1A*$^{R1407X/+}$ and **(D)** Kv1.1 knockout mice housed in a 12:12 h light:dark cycle. **(A)** Percentage of audiogenic seizures resulting in death in DBA/1 mice housed in a 12:12 h light:dark cycle. **(B)** Percentage of maximal electroshock (MES) seizures resulting in death in mice housed in constant darkness. Redrawn with permission from **(C)** Teran et al. (2019), **(D)** Moore et al. (2014), **(A,B)** Purnell et al. (2021b).

seizure may carry even more risk than the severity of sleep-related respiratory dysfunction or PGES duration (Peng et al., 2017; Sveinsson et al., 2020). The presence of someone who could intervene and administer lifesaving resuscitative measures may mean the difference between a case of near SUDEP and actual SUDEP (Nashef et al., 1998; Langan et al., 2005; Lamberts et al., 2012). Increasing nocturnal supervision through the use of monitoring devices, checkups, or having another person asleep in the same room is associated with decreased SUDEP risk (Langan et al., 2005; Ryvlin et al., 2006; Harden et al., 2017). The majority of SUDEP victims are found prone in or near a bed (Opeskin and Berkovic, 2003; Sowers et al., 2013; Ali et al., 2017; Sveinsson et al., 2018). Many generalized seizures are followed by a period of PGES where the patient is more likely to be immobile, unresponsive, and require resuscitative measures (Semmelroch et al., 2012; Kuo et al., 2016). If a patient is unresponsive after a seizure that renders them prone, their nose and mouth may become obstructed by bedding. This may result in upper airway occlusion or asphyxiation against the surface the patient is positioned on. Outside of total airway occlusion, ending a seizure in the prone position on bedding may impairing postictal breathing by increasing inspiratory resistance and causing the patient to rebreathe trapped air (Kemp et al., 1994; Tao et al., 2010, 2015; Rugg-Gunn et al., 2016). This would cause an acute rise in CO_2 in the blood, potentially leading to severe acidosis, which would potentiate the postictal immobility and further prolong the respiratory dysfunction until terminal apnea and asystole develop (Peng et al., 2017; Purnell et al., 2018).

Clock genes

Although seizures are frequently thought to be unpredictable phenomenon, patients often display time-of-day-specific timing of seizure onset. In a recent study of patients implanted with responsive neurostimulators, it was shown that nearly 90% of patients with focal epilepsy had circadian timing of seizure onset (Leguia et al., 2021). Interestingly, circadian risk of seizure onset could be clustered into five general times of day, with seizures more likely to occur during the morning, mid-afternoon, evening, early night, or late night.

The circadian influence on seizures may be due in part to the bi-directional relationship of epilepsy and clock genes. Alterations in clock mechanisms increase the susceptibility for epilepsy, while seizures have the potential to disrupt the internal clock (Re et al., 2020). A significantly higher current is required to induce both maximal and generalized seizures in wild type (WT) mice during the dark phase of their diurnal cycle compared to the light phase. This rhythm is abolished in *Bmal1* KO mice, who also exhibit significantly lower seizure thresholds at all times compared to their WT counterparts (Gerstner et al., 2014). Similarly, conditional KO of *Bmal1* in neurons in the dentate gyrus increased the susceptibility to pilocarpine-induced seizures in mice (Wu et al., 2021). Hippocampal BMAL1 expression is reduced overtime in pilocarpine-treated rats as they begin to develop spontaneous seizures—suggesting that BMAL1 also plays a role in epileptogenesis (Matos et al., 2018). Levels of BMAL1 protein have been found to be decreased in the dentate gyrus and CA1 of mice with TLE (Wu et al., 2021). Mutations in the RAR related orphan receptor alpha (*RORA*) gene, which encodes for an activator of *Bmal1* transcription, have been linked to intellectual developmental disorder with or without epilepsy or cerebellar ataxia (IDDECA) (Guissart et al., 2018). Deletion of the gene *Clock* in cortical pyramidal neurons in mice results in epileptiform discharges in excitatory neurons as well as a decreased seizure threshold (Li et al., 2017). Real-time quantitative PCR (qPCR) analysis has revealed a loss in the rhythmic expression of CLOCK and decreased levels of its transcript in a post-status epilepticus rat model (Santos et al., 2015). *Clock* RNA and protein are similarly downregulated in brain tissue resected from patients with TLE (Li et al., 2017). Another oscillating clock gene, *Per1*, is upregulated in the hippocampus following electrical and kainic acid-induced seizures in mice (Eun et al., 2011). One study found an alteration in the rhythmic expression of PER1, PER2, and PER3 in a rat model of pilocarpine-induced seizures (Santos et al., 2015). However, a subsequent study found that an increase in PER1 expression and a decrease in PER2 expression prior to the development of spontaneous seizures, while PER3 expression was unaltered (Matos et al., 2018). To conclude, sleep and circadian phase have direct effects on periictal breathing and potentially the development of epilepsy itself.

Sleep impairment, sleep-disordered breathing (SDB), and epilepsy

Sleep deprivation/sleep disorders

Apart from nocturnal seizures, patients with epilepsy also have a greater prevalence of sleep disorders compared to healthy individuals (Vaughn and D'cruz, 2004). A myriad of studies over the past 30 years have repeatedly found that adults with epilepsy are 2–3 times more likely to have a sleep/wake disorder compared to the general population (Grigg-Damberger and Foldvary-Schaefer, 2021). Patients with temporal lobe epilepsy exhibit reduced sleep efficiency and more arousals compared to those with frontal lobe epilepsy (Crespel et al., 2000). In addition, amygdala kindling decreases REM sleep in experimental animals, and selective REM sleep deprivation accelerates the kindling process (Cohen and Dement, 1970; Tanaka and Naquet, 1975). The *Scn1a*$^{R1407X/+}$ mouse shows impairments in circadian sleep regulation, including a fragmented rhythm of NREM sleep and an elongated circadian period of sleep (Sanchez et al., 2019).

Sleep deprivation caused by sleep disorders of frequent nocturnal seizures can result in sleep deprivation. Sleep deprivation itself can induce seizures and interictal spiking (Mattson et al., 1965; Pratt et al., 1968; Malow et al., 2000b; Konduru et al., 2021). In amygdala kindled cats, acute sleep deprivation reduces seizure and after discharge threshold (Shouse and Sterman, 1982). However, more prolonged sleep deprivation increases their susceptibility to both kindled and penicillin-induced seizures, regardless of sleep state (Shouse, 1988). Additionally, when kindled rats were administered a microinjection of a cholinergic agonist into the pontine reticular formation to enhance REM sleep, the result was a significant increase in the current threshold needed to elicit afterdischarge spiking in the amygdala (Kumar et al., 2007). Sleep deprivation studies in healthy individuals have shown hypertension and increased sympathetic nervous system activity after nights where sleep was less than 5 h (Lusardi et al., 1996; Tochikubo et al., 1996; Gangwisch et al., 2006). Thus, sleep deprivation may not only worsen seizures themselves, but also leave patients more vulnerable to seizure-induced autonomic insults.

SDB

Up to 9–11% of adult patients with epilepsy exhibit SDB (Vendrame et al., 2014; Popkirov et al., 2019). This number jumps up to 40% when looking at children with epilepsy (Kaleyias et al., 2008). A recent case study highlighted a male patient with a history of secondary generalized tonic/clonic seizures who displayed paroxysmal nocturnal breathing. The patient experienced periods of breathing arrest in conjunction with an odd expiratory noise—primarily during REM sleep or the transition between REM and NREM—despite being seizure free for a year (Künstler et al., 2022).

OSA is a relatively common form of SDB, in which the upper airway collapses, preventing ventilation. The ensuing apnea provokes an arousal response which allows for re-positioning and recovery of gas exchange (Butler et al., 2015). The precise occurrence of OSA in people with epilepsy has yet to reach a consensus. Popkirov et al. estimates that 7% of epilepsy patients have mild-to-moderate OSA (Popkirov et al., 2019). A separate polysomnography study postulates that one-third of patients

with medically refractory epilepsy who were candidates for epilepsy surgery have concomitant OSA (Malow et al., 2000b). This is also closer to an estimate produced from a meta-analysis in 2017, which determined the prevalence of mild-to-severe OSA in patients with epilepsy to be 33.4%—2.4 times more likely than healthy comparisons (Lin et al., 2017). This same meta-analysis found that the prevalence of OSA in patients with refractory epilepsy was not greater than the overall prevalence of OSA in patients with epilepsy (Lin et al., 2017). Patients with generalized epilepsy experience more severe OSA than those with focal epilepsy. Both populations reported similar degrees of abnormal daytime sleepiness. Older age, higher body mass index (BMI), and a history of hypertension are also associated with more severe OSA (Scharf et al., 2020). The incidence of OSA apnea in individuals without epilepsy is higher in males than in females (4% in men, 2% in women) (Block et al., 1979; Young et al., 1993). Men are also much more likely to experience O_2 desaturation during apnea compared to women (Block et al., 1979). In patients with epilepsy, males are roughly three times more susceptible to OSA compared to females (Lin et al., 2017).

The length of obstructive apneas tends to increase over the course of a night's sleep (Montserrat et al., 1996; Butler et al., 2015). It is suggested that this is due to a blunting of the CO_2 arousal response over the course of the night, leading to longer periods of hypercapnia before arousal occurs (Montserrat et al., 1996). It is possible that this increase in OSA in epilepsy patients is due to an inherent blunting of chemosensitivity in an epileptic brain. Obese adolescents with OSA have an increased HCVR during wakefulness and a blunted HCVR during sleep (Yuan et al., 2012). There are also endogenous circadian components to the prolongation of respiratory events across the night. At circadian phases that correspond to the early morning, the duration of apnea and hypopneas are typically longer, but apnea/hypopnea index (AHI), a measurement of OSA severity, is low. In contrast, during the late afternoon to early evening, event durations were short and AHI was high. Events during REM sleep also tended to be 14% longer than those emerging from NREM sleep (Butler et al., 2015).

Comorbidity of epilepsy with OSA can increase the incidence of arrhythmias and increase the patient's risk for sudden cardiac death (Gami and Somers, 2008; Gami et al., 2013). Patients with OSA experience disruption of the autonomic system during sleep (Adlakha and Shepard, 1998), which may be further imbalanced by seizures. While no direct correlation between OSA and SUDEP has been identified, higher revised SUDEP-7 scores [presence of seizures in the past 12 months—especially generalized tonic clonic seizures (GTCS), longer duration of epilepsy, increased number of ASMs, and lower IQ/more cognitive impairment]—are associated with probable SUDEP (Phabphal et al., 2021). OSA decreases the amount of time a person spends asleep each night, potentially leading to further sleep deprivation. Sleep deprivation is particularly dangerous for those with epilepsy as it can have an epileptogenic effect (Nobili et al., 2011; Popkirov et al., 2019). It follows then that when epilepsy patients with OSA were treated with continuous positive airway pressure (CPAP) they exhibited better seizure control than their untreated peers (Lin et al., 2017).

Vagus nerve stimulation (VNS) is a technique used to treat refractory epilepsy *via* a neurostimulation device. While these devices have been found to lessen seizure frequency and severity, there is a lack of conclusive evidence indicating that VNS lessens SUDEP risk (Annegers et al., 1998; Ryvlin et al., 2018). There is, however; evidence that VNS activation during sleep can induce mild OSA or exacerbate preexisting OSA. VNS activation during sleep is similarly linked to decreased V_T and SaO_2, increased respiratory rate and AHI, and excessive daytime somnolence (Malow et al., 2000a; Holmes et al., 2003; Marzec et al., 2003; Zambrelli et al., 2016; Somboon et al., 2019; Kim et al., 2022). A recent study has also indicated HCVR slope is attenuated in patients with an active VNS (Sainju et al., 2021). Evidence suggests the exacerbation of OSA after VNS is due to reduction of the glottal space or lack of laryngeal–respiratory coordination (Zambrelli et al., 2016). This is notable as patients with refractory epilepsy are at higher risk for SUDEP and are more likely to opt for VNS as a method of seizure control. To summarize, individuals with epilepsy are more likely to experience sleep disorders and SDB, which may directly influence seizure frequency. Moreover, a common treatment for refractory epilepsy appears to aggravate SDB in these patients.

Neurotransmitter mechanisms

While the underlying mechanisms behind the sleep and circadian effects on breathing in epilepsy are still not fully understood, numerous neurotransmitters and signaling molecules have been implicated. For instance, the monoaminergic neurotransmitter serotonin (5-HT) plays an important role in sleep-wake regulation and respiration (Jouvet, 1999; Richerson, 2004; Hodges et al., 2009; Ptak et al., 2009; Hodges and Richerson, 2010; Depuy et al., 2011; Buchanan, 2013; Iwasaki et al., 2018; Smith et al., 2018). It is also heavily implicated in epilepsy and SUDEP pathophysiology (Bagdy et al., 2007; Richerson and Buchanan, 2011; Richerson, 2013; Feng and Faingold, 2017; Li and Buchanan, 2019; Petrucci et al., 2020). Serotonergic tone is modulated in both a sleep state and circadian phase-dependent manner, with the nadir occurring during the nighttime and during sleep (Mcginty and Harper, 1976; Rosenwasser et al., 1985; Agren et al., 1986; Rao et al., 1994; Sakai and Crochet, 2001; Mateos et al., 2009; Sakai, 2011; Purnell et al., 2018). 5-HT neurons in both the midbrain and medullary raphe have been demonstrated to be robustly chemosensitive (Larnicol et al., 1994; Richerson, 1995, 2004; Wang et al., 1998; Severson et al., 2003). It is likely that 5-HT neurons in the

medulla mediate increased respiration in response to a rise in CO_2 whereas midbrain 5-HT neurons mediate non-respiratory responses to CO_2, such as arousal (Richerson, 2004; Buchanan and Richerson, 2010; Buchanan et al., 2015; Kaur et al., 2020). Firing of medullary raphe 5-HT neurons is markedly reduced during the ictal and postictal period, coinciding with severe respiratory depression (Zhan et al., 2016). Further, lower postictal serum 5-HT levels have been associated with postictal central apnea (Murugesan et al., 2019). Numerous studies have demonstrated that pre-treatment with serotonergic agents prior to seizure onset can ameliorate this respiratory dysfunction. The incidence of S-IRA in DBA/2 mice can be reduced through the administration of fluoxetine, a selective 5-HT reuptake inhibitor (SSRI), prior to seizure induction (Tupal and Faingold, 2019). A similar finding was discovered in DBA/1 mice, where fluoxetine was also found to reduce S-IRA without increasing basal ventilation or the ventilatory response to 7% CO_2 (Zeng et al., 2015; Feng and Faingold, 2017). Other serotonergic agents, including fenfluramine, can selectively block S-IRA without influencing convulsive behavior (Feng and Faingold, 2017; Tupal and Faingold, 2019).

Another monoaminergic signaling molecule with links to sleep/wake regulation, respiration, and epilepsy is norepinephrine (NE; Hobson et al., 1975; Aston-Jones and Bloom, 1981; Foote et al., 1983). Plasma concentrations of NE are significantly lower during nocturnal sleep compared to wakefulness (Linsell et al., 1985). Like, 5-HT, NE also exhibits circadian rhythmicity with the lowest concentrations occurring during the night (Morgan et al., 1973; Agren et al., 1986; Cagampang and Inouye, 1994). The NE reuptake inhibitor (NRI) atomoxetine suppresses seizure-induced respiratory arrest following audiogenic seizures in DBA/1 mice (Zhang et al., 2017; Zhao et al., 2017) as well as MES seizures (Kruse et al., 2019). Another NRI, reboxetine, and the dual 5-HT/NE reuptake inhibitor (SNRI), duloxetine, are also able to suppress respiratory arrest following MES seizures (Kruse et al., 2019). More recently, evidence has indicated that selective activation of the noradrenergic α_2 receptor is sufficient to suppress S-IRA in DBA/1 mice (Zhang et al., 2021).

The excitatory neuropeptide orexin is also involved in sleep and arousal and is a wake-promoting substance (Sakurai, 2007; Bonnavion and De Lecea, 2010; Nattie and Li, 2012). Orexin displays a strong diurnal circadian variation. This rhythm has been measured in the cerebral spinal fluid (CSF) and hypothalamus of rats, with an even stronger variation in the CSF of older rats (Yoshida et al., 2001; Desarnaud et al., 2004). This robust circadian rhythm is likely in part due to the dense projections that orexin neurons receive from the suprachiasmatic nucleus (SCN), the brain's main circadian oscillator (Saper et al., 2005). Orexin neurons also contribute to respiratory function, in part due to orexinergic innervation of serotonergic and noradrenergic nuclei (Kuwaki, 2008; Inutsuka and Yamanaka, 2013). Orexin is thought to play a proconvulsant role in epilepsy, although there is some discrepancy regarding the effects of orexins and their antagonists on seizure activity. In *Kcna1*-null mutant mice, the dual orexin receptor antagonist (DORA), almorexant, decreases the incidence of severe seizures, improves O_2 saturation, and increases overall longevity (Roundtree et al., 2016; Iyer et al., 2020).

The inhibitory neuromodulator, adenosine, is released in large quantities during seizures (During and Spencer, 1992; Berman et al., 2000; Van Gompel et al., 2014). This is likely a mechanism of seizure termination (Shen et al., 2010; Purnell et al., 2021a). Unlike, 5-HT, NE, and orexin, adenosine promotes sleep and suppresses wakefulness (Feldberg and Sherwood, 1954; Buday et al., 1961; Haulică et al., 1973; Huber et al., 2004). As such, adenosine levels increase during wakefulness and are depleted during sleep (Porkka-Heiskanen et al., 2000; Bjorness and Greene, 2009). Adenosine has an inhibitory effect on respiration, predominately causing a reduction in frequency and V_T (Eldridge et al., 1984; Lagercrantz et al., 1984; Wessberg et al., 1984). The accumulation and clearance of adenosine is regulated in a circadian manner (Cornélissen et al., 1985; Chagoya De Sánchez et al., 1993; Huston et al., 1996). The adenosine hypothesis of SUDEP was first proposed in 2010, when Shen et al. noted that upregulated adenosine tone in a kainic acid model of epilepsy suppressed seizure activity but paradoxically caused death when seizures did occur (Shen et al., 2010). This hypothesis posits that a surge of adenosine is released during a seizure as a termination mechanism. However, this large increase in extracellular adenosine can result in suppression of breathing which can lead to terminal respiratory failure (Shen et al., 2010; Purnell et al., 2021a).

This is far from a comprehensive list of salient signaling molecules when it comes to respiratory function and SUDEP. However, these neuromodulators are especially of interest in the field of SUDEP and their complex role in sleep-wake regulation, breathing, and seizures makes them excellent candidates for therapeutic intervention.

Conclusions

SUDEP is a complex and devastating phenomenon; the underlying mechanisms of which investigators are just beginning to unravel. The time of day and sleep state in which seizures occur are indisputably factors that can confer further risk to patients with epilepsy. While nighttime and sleep tend to go hand-in-hand, it is crucial that we acknowledge the two are not one in the same and come with their own risk factors from both shared and separate mechanisms. Respiratory failure is a major precipitating factor for seizure-induced death. Monoaminergic neurons, including 5-HT, NE, and orexin, play a crucial role in respiratory function and have seizure-protective properties. Levels of monoaminergic neurons are decreased

during the nighttime and even further reduced during sleep. This may explain why seizures emerging from sleep tend to be longer and cause more severe respiratory dysfunction. Other signaling molecules, such as adenosine, may have an even more complex role in SUDEP pathophysiology—contributing to respiratory dysfunction during the process of terminating seizures. Many SUDEP victims are found in a prone position in bed, suggesting that respiratory distress was amplified by airway obstruction. As patients are also more likely to be unaccompanied at night, the chance of successful intervention is low.

Thus, while sleep and nighttime appear to confer their own risk of SUDEP, the fact that the two tend to occur in conjunction contributes greatly to the "perfect storm" of factors that ultimately leads to seizure-induced death. Nevertheless, it is our hope that this review imparts the notion that sleep state and time of day are factors that should be considered independently while developing preventative strategies to mitigate the severity of respiratory dysfunction brought about by seizures.

Author contributions

KJ and BK drafted the initial document. The final manuscript was edited and approved by KJ, BK, and GB. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Forebrain epileptiform activity is not required for seizure-induced apnea in a mouse model of *Scn8a* epilepsy

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Sudden unexpected death in epilepsy (SUDEP) accounts for the deaths of 8–17% of patients with epilepsy. Although the mechanisms of SUDEP are essentially unknown, one proposed mechanism is respiratory arrest initiated by a convulsive seizure. In mice, we have previously observed that extended apnea occurs during the tonic phase of seizures. Although often survived, tonic seizures became fatal when breathing did not immediately recover postictally. We also found that respiratory muscles were tonically contracted during the apnea, suggesting that muscle contraction could be the cause of apnea. In the present study, we tested the hypothesis that pyramidal neurons of the motor cortex drive motor units during the tonic phase, which produces apnea. Mice harboring the patient-derived N1768D point mutation of an *Scn8a* allele were crossed with transgenic mice such that inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADD) receptors were selectively expressed in excitatory forebrain neurons. We then triggered audiogenic and hippocampal (HC) stimulated seizures under control conditions and when excitatory forebrain neurons were inhibited with the synthetic ligand Clozapine-*N*-Oxide (CNO). We found that inhibition with CNO was sufficient to increase seizure threshold of HC stimulated, but not audiogenic, seizures. In addition, regardless of seizure type, CNO nearly eliminated epileptiform activity that occurred proximal to the tonic phase; however, the seizure behaviors, notably the tonic phase and concomitant apnea, were unchanged. We interpret these results to indicate that while cortical neurons are likely critical for epileptogenesis and seizure initiation, the behavioral manifestations of tonic seizures are generated by neural circuitry in the mid- and/or hindbrain.

KEYWORDS

seizure, tonic phase, SUDEP, brainstem, epilepsy, breathing, apnea

Introduction

Sudden unexpected death in epilepsy (SUDEP) is defined as the sudden, unexpected, non-traumatic, and non-drowning death of a person with epilepsy for which post-mortem examination does not reveal another cause of death (Nashef et al., 2012). SUDEP is the most common cause of death associated with epilepsy, accounting for up to 17% of all epilepsy-related deaths (Terra et al., 2013), and up to 50% for patients with poorly controlled seizures (Devinsky, 2011; Tolstykh and Cavazos, 2013). Furthermore, amongst all neurological disorders SUDEP is second only to stroke in the number of life years lost (Thurman et al., 2014).

Although SUDEP is likely multi-factorial, peri-ictal apnea and breathing dysfunction are believed to be a primary cause of SUDEP. Apnea and oxygen desaturation have been reported in a large percentage of patients during and after convulsive and non-convulsive seizures (Nashef et al., 1996; Bateman et al., 2008; Lacuey et al., 2018a,b; Vilella et al., 2019), and in nine cases of SUDEP with adequate postictal cardiorespiratory monitoring, terminal apnea occurred prior to terminal asystole (Ryvlin et al., 2013). It is believed that most SUDEP cases occur after generalized convulsive seizures (Dasheiff and Dickinson, 1986; Bird et al., 1997; Nilsson et al., 1999; So et al., 2000; Ryvlin et al., 2013). Thus, mouse models of SUDEP include those in which death occurs immediately after convulsive seizures. Indeed, we and others have found that mouse models of epilepsy that experience early mortality die from seizures that produce apnea. This includes transgenic mice with epilepsy-related mutations such as *Scn8a*^{N1768D}, *Scn8a*^{R1872W}, *Cacna1a*^{S218L}, *Lmx1b*$^{f/f/p}$, and *Scn1a*^{R1407X}, in addition to pharmacological and electrically stimulated models of seizure-induced death (Buchanan et al., 2014; Kim et al., 2018; Jansen et al., 2019; Loonen et al., 2019; Wenker et al., 2021).

Although progress toward understanding mechanisms of SUDEP has been made over the last decade, our understanding of seizure-induced apnea at the level of the CNS is rudimentary. Early studies in rats suggest that seizure spread to the brainstem reticular formation is the cause of apnea, particularly during the tonic phase (Faingold, 2012). In *Scn8a* mutant mice, we have observed tonic contraction of the main breathing muscle, the diaphragm, during the tonic phase (Wenker et al., 2021). This tonic contraction of the diaphragm is sufficient to produce the concomitant apnea; thus, determining which neural circuitry is stimulated by seizure activity to produce apnea is of critical significance. The final output of the central nervous system that controls the musculature are upper motor neurons, which reside in the primary motor cortex, as well as several subcortical regions (Purves et al., 2018). While hypersynchronous activity of cortical neurons, including those of the motor cortex, is a hallmark of convulsive seizures, it is unclear whether the activity of these neurons drive the tonic phase and concurrent apnea.

In the present study, we tested the hypothesis that while excitatory forebrain neurons are sufficient for epileptogenesis in mice harboring the N1768D point mutation in an *Scn8a* allele ("D/+"), their hyperactivity is not required for the tonic phase or concomitant apnea. We utilize mice heterozygous for a patient-derived, gain-of-function *Scn8a* mutated allele that have spontaneous, audiogenic, and hippocampal (HC) stimulated seizures. We found that in spontaneous and HC stimulated seizures, where the focus is likely the temporal lobe, cortical ictal activity is initiated prior to seizure behavior, including wild running and tonic phase apnea. However, in audiogenic seizures cortical ictal activity is not initiated until after tonic phase apnea has begun, suggesting that cortical activity does not drive tonic contraction or apnea. To directly test this hypothesis, we used chemogenetics to selectively inhibit forebrain excitatory neurons during both audiogenic and HC stimulated seizures of D/+ mice. Inhibition of forebrain excitatory neurons was sufficient to suppress electrocorticogram (ECoG) ictal activity in the motor cortex but had no effect on the tonic phase or seizure-induced apnea.

Materials and methods

Mouse husbandry and genotyping

All mice were house and cared for in accordance with the Animal Care and Use Committee standards of the University of Virginia. Mice were housed in a temperature and humidity-controlled vivarium with a standard 12-h light/dark cycle with food and water *ad libitum* in accordance with NIH guidelines. Mice harboring the N1768D point mutation in an *Scn8a* allele ("D/+ mice") were used as a model of epilepsy. To test the role of forebrain excitatory neurons in generation of seizure-induced apnea, we used *Emx1*-Cre mice (Jax # 005628) to genetically target these neurons. To confirm *Emx1*-Cre mice generated recombination specifically in the forebrain (**Figure 2**), we crossed them with Ai9 reporter mice (Jax # 007909), that express TdTomato from the Rosa26 locus upon Cre mediate recombination (Madisen et al., 2010). To test the efficacy of neuronal inhibition by the synthetic ligand Clozapine-*N*-Oxide (CNO; **Figure 3**), we crossed *Emx1*-Cre mice with LSL-GiDREADD mice (Jax # 026219), that express GiDREADD receptors from the Rosa26 locus upon Cre mediate recombination (Zhu et al., 2016). The mice for experiments testing the role of forebrain excitatory neurons in seizure behaviors (**Figures 4**, **5**), D/+ mice were crossed with *Emx1*-Cre and floxed-GiDREADD mice. Genotyping was performed by Transnetyx, Inc. (Cordova, TN, United States), with methods of each mouse line as previously described (Wagnon et al., 2015; Bunton-Stasyshyn et al., 2019; Wengert et al., 2021a).

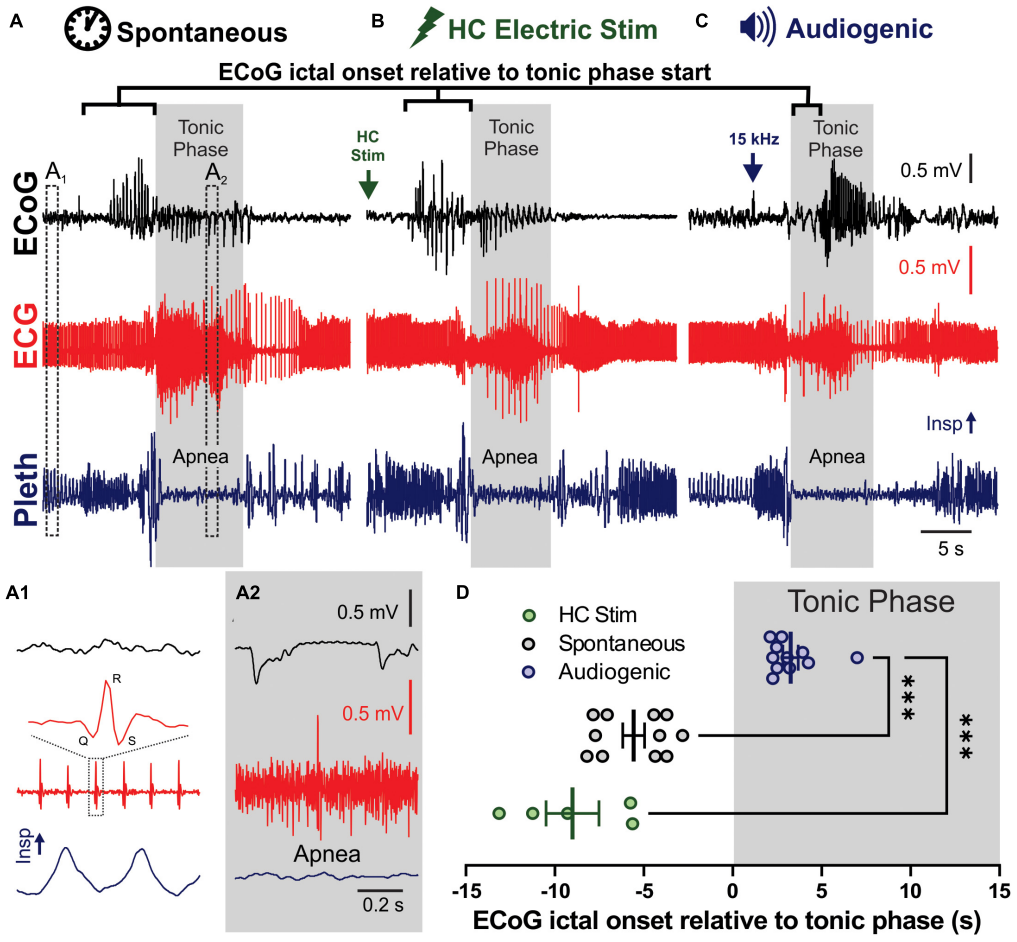

FIGURE 1
Spontaneous, audiogenic, and hippocampal (HC) electrical stimulated seizures in D/+ mice have similar semiology. **(A–C)** Electrocorticogram (ECoG), electrocardiogram (ECG), and breathing (Pleth) were recorded during spontaneous **(A)**, audio induced **(B)**, and HC electrically stimulated **(C)** seizures. Arrows indicates onset of electrical stimulation **(B)** and 15 kHz acoustic stimulation **(C)**. Gray boxes indicated timing of the tonic phase based on gross electromyography (EMG) activity detected in the ECG signal. (A1) Expanded traces of pre-ictal biosignals from A1 in panel (A) that demonstrates individual QRS complexes in the ECG and inspirations in the pleth. (A2) Same signals during the tonic phase, demonstrating EMG noise in ECG and apnea. **(D)** Audiogenic seizures (blue, $n = 11$ seizures recorded from 5 mice), but not HC stimulated (green, $n = 5$ seizures recorded from 3 mice), showed a delayed onset of ECoG ictal activity relative to tonic phase compared to spontaneous seizures (black, $n = 8$ seizures recorded from 3 mice; *** indicates $p < 0.001$, Dunnett's multiple comparison test after significant Kruskal–Wallis, KW statistic = 20.27, $p < 0.0001$).

Surgical preparation

Custom electrocorticogram (ECoG)/electrocardiogram (ECG) headsets (P1 Technologies, Roanoke, VA, United States) were implanted in 6–8-week-old mice using standard aseptic surgical techniques as done previously (Wengert et al., 2021a,b; Wenker et al., 2021, 2022). Anesthesia was induced with 5% and maintained with 0.5–3% isoflurane. Adequacy of anesthesia was assessed by lack of toe-pinch reflex. A midline skin incision was made over the skull and the skull was cleared from connective tissue with 3% peroxide. For mice destined for audiogenic seizures, burr holes were drilled in both the left and right frontal bones to place ECoG leads in the motor cortex at the approximate coordinates of 1 mm rostral, 1 mm lateral, and 1.5 mm ventral to bregma. Bur holes were also drilled in the occipital bone for reference and ground electrodes. Surgery was the same for mice used for HC electrically stimulated seizures, except that a bipolar electrode was implanted into the left hippocampus at coordinates 2 mm caudal, 2 mm lateral, and 2 mm ventral of bregma. The headsets were attached to the skull with dental acrylic (Jet Acrylic; Lang Dental, Wheeling, IL, United States). Two ECG leads were passed subcutaneously to the left abdomen and right shoulder and sutured into place to approximate a lead II arrangement. Mice received post-operative analgesia with ketoprofen (5 mg/kg, i.p.) and 0.9% saline (0.5 ml i.p.) and were allowed to recover a minimum of 5 days prior to experiments.

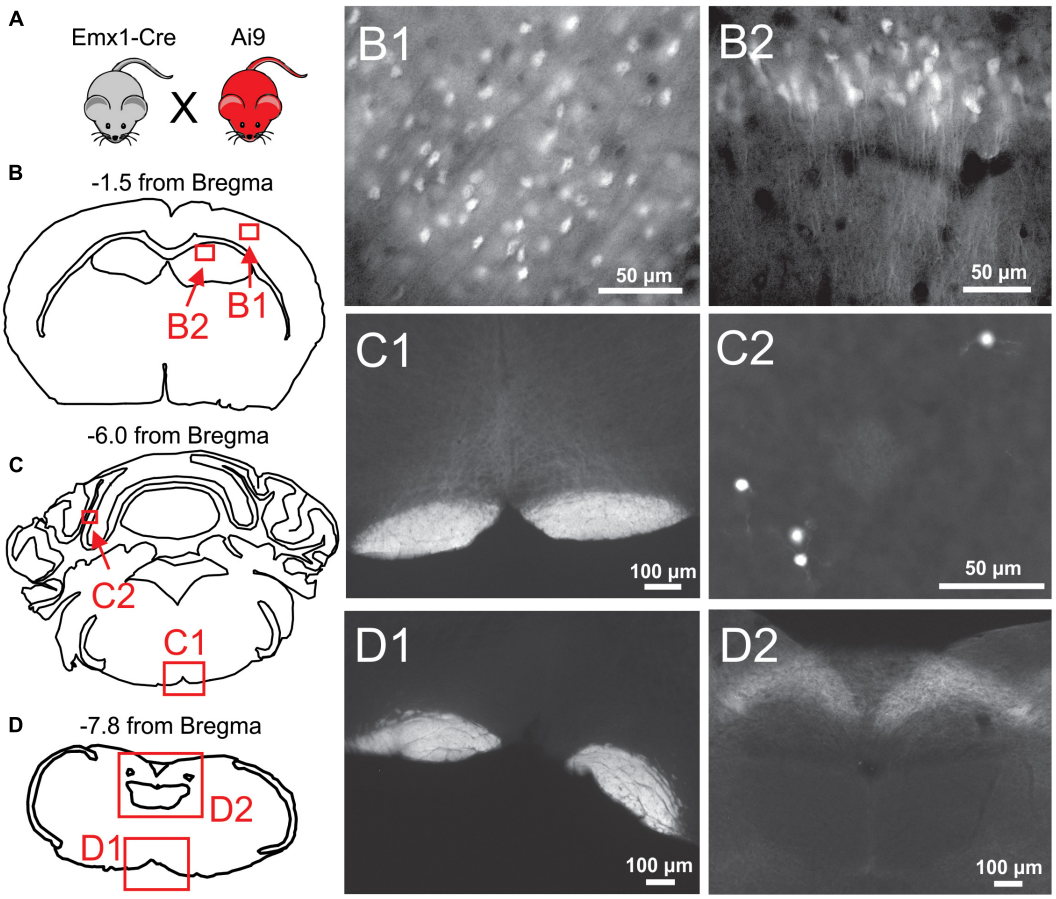

FIGURE 2
Histological examination of *Emx1*-Cre expression in the cortex and brainstem. **(A)** *Emx1*-Cre mice (Jax #005628) were crossed with a Cre-inducible TdTomato mouse line (Ai9 mice; Jax 007909) to produce Ai9$^{Emx1-Cre}$. **(B–D)** Locations of fluorescent images taken from transverse brain sections of an Ai9$^{Emx1-Cre}$ mouse. Recombination, as indicated by TdTomato fluorescence in cell bodies, was observed in Pyramidal cells of Layers 2/3 of the cortex (B1), and the cell layers of the hippocampus, including the CA1 (B2) and dentate gyrus (data not shown). No cell somata were observed in any brainstem sections analyzed (12 analyzed in total, 3 depicted here as examples). As expected, the pyramidal tracts expressed TdTomato in all brainstem sections (examples in panels C1,D1), as these are axons of passage originating from pyramidal neurons. Sparse recombination was observed in cell bodies of the cerebellar granule layer (C2). In addition, what appeared to be terminal fields or diffuse axonal processes routinely were in the nucleus of the solitary tract (NTS; D2).

Recording of electrocorticogram, electrocardiogram, and breathing

Recording of ECoG, ECG, and breathing was performed as previously described (Wengert et al., 2021b; Wenker et al., 2021). Plethysmography chambers were built to comply with requirements for continuous housing described in the Guide for the Care and Use of Laboratory Animals (Council, 2011). The floor of the chambers had approximate dimensions of 4.5 × 4.5 inches (>20 sq. inches) and 7 inches tall. There were ports for air in and air out, and for pressure monitoring. The chamber was supplied with a continuous flow of room air at approximately 400 ml/min *via* supply and exhaust air pumps (MK-1504 Aquarium Air Pump; AQUA Culture) balanced to maintain chamber pressure near atmospheric. Mice had access to a continuous supply of water and food. The surgically implanted headsets were attached to a custom low torque swivel cable, allowing mice to move freely in the chamber. To assess breathing frequency, the pressure of the EMU chamber was measured with an analog pressure transducer (SDP1000-L05; Sensirion, Stafa, Switzerland). ECoG and ECG signals were amplified at 2000 and bandpass filtered between 0.3–100 and 30–300 Hz, respectively, with an analog amplifier (Neurodata Model 12, Grass Instruments Co., West Warwick, RI, United States). Biosignals were digitized with a Powerlab 16/35 and recorded using LabChart 7 software (AD Instruments, Sydney, NSW, Australia) at 1 kS/s. Video acquisition was performed by multiplexing four miniature night vision-enabled cameras and then digitizing the video feed with a Dazzle Video Capture Device (Corel, Inc., Ottawa, ON, Canada) and recording at 30 fps with LabChart 7 software in tandem with biosignals.

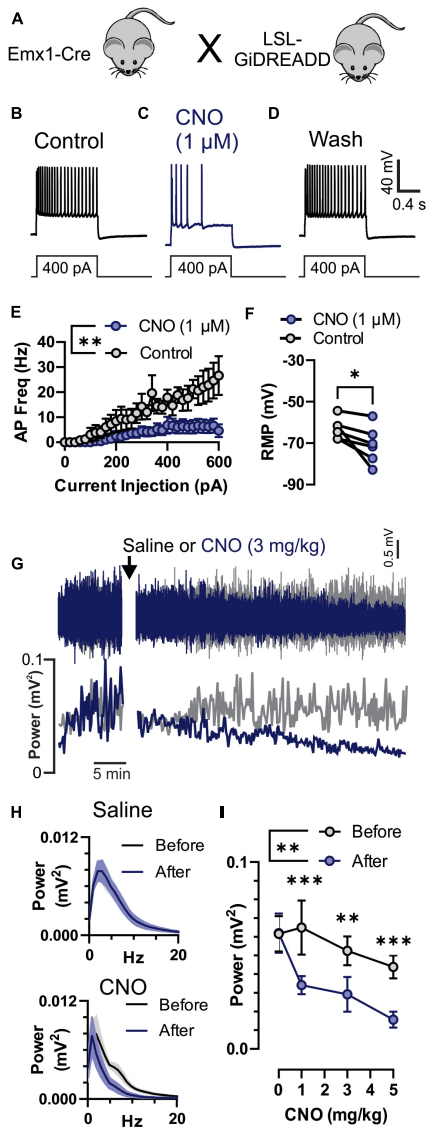

FIGURE 3
Inhibition of neuronal activity by Clozapine-*N*-Oxide (CNO) in GiDREADD$^{Emx1-Cre}$. **(A)** *Emx1*-Cre mice were crossed with a Cre-inducible Gi-DREADD receptor mouse line to produce GiDREADD$^{Emx1-Cre}$ mice. **(B–D)** Current clamp recordings from a layer 5 pyramidal neuron during a 1 s 400 pA square current pulse. Addition of 1 μM CNO to the bath solution resulted in hyperpolarization of the resting membrane potential (RMP) and fewer action potentials (APs) upon current inject (panel **C** compared to panels **B** or **D**). **(E)** APs generated by increasing current pulses from 0 to 600 pA were detectably less in the presence of 1 μM CNO compared to control (n = 5 cells, 3 mice). **(F)** RMP in 1 μM CNO and control (n = 5 cells, 3 mice). **(G)** Raw electrocorticogram (ECoG) signal and spectral power (0.5–20 Hz) from motor cortex before and after i.p. injection of saline (gray trace) or 3 mg/kg CNO (blue trace). **(H)** Average ECoG power spectrum histograms (n = 5 mice) for before (black) and 30–45 min after (blue) saline (top) and 3 mg/kg CNO (bottom) treatments. **(I)** Average ECoG spectral power (0.5–20 Hz; n = 5 mice) before (gray) and 30–45 min after (blue) i.p. injection of 0, 1, 3, and 5 mg/kg CNO. CNO reduced ECoG power at all doses. *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively.

Seizure recording and stimulation

Spontaneous, audiogenic, and HC stimulated seizures were recorded from separate sets of mice.

Three 8–12 week-old D/+ mice were used for chronic recording of spontaneous seizures. The mice were housed in the plethysmography chambers and recorded as described above 24 h a day and provided with water and food *ad libitum* as we have previous described (Wengert et al., 2021b; Wenker et al., 2021).

All stimulated seizures were performed between 10 a.m. and 3 p.m. and mice were allowed at least two recovery days in-between seizure stimulations. All spontaneous and stimulated seizures were behaviorally and electrographically tonic seizures, as previously described for spontaneous seizures in these mice (Wengert et al., 2021b; Wenker et al., 2021), and as depicted in **Figure 1**.

Seven 8–10 week-old D/+ mice were used to induce audiogenic seizures at 15 kHz signal pure tone (~90 dB) generated using Tone Generator software (NCH Software, Inc., Canberra, ACT, Australia), amplified using a Kinter K3118 stereo amplifier (Kinter, Waukegan, IL, United States), and converted to sound using a small 3-watt speaker lowered into the plethysmography chamber. To determine the effect of CNO administration on audiogenic seizure threshold, a subset of four mice were used. On separate days mice were either injected with saline or 5 mg/kg CNO, placed in a cage, and exposed to increasing intensities (48–95 dB) of a 15 kHz tone for 30 s.

Five 8–10-week-old D/+ mice were used for HC stimulated seizures. Mice were placed in the chamber 30–60 min prior to stimulation. Stimulation of seizures was achieved by connecting the two HC leads to an isolated pulse stimulator (Model 2100, A-M Systems, Inc., Sequim, WA, United States) and stimulating 2 s trains of 1 ms biphasic current pulses at 50 Hz. Stimulations were repeated every 60 s with increased current amplitude (20–600 μA, in 20 μA increments) until a seizure was produced. The final current amplitude that produced a seizure was recorded as the after-discharge threshold (ADT) for that stimulation.

In vivo biosignal analysis

In vivo electrophysiological data was analyzed with Spike2 software (Cambridge Electronic Design, Ltd, Cambridge, United Kingdom). Determination of cortical ictal activity and tonic phase timing (**Figure 1**), apnea duration (**Figures 4**, **5**), and ECoG power (**Figures 3–5**) were done by an experimenter blinded to the treatment at the time of analysis. The beginning of cortical ictal activity was defined as the time of the first spike wave discharge that was not due to the movement artifact commonly observed during wild running immediately before the tonic phase. The beginning of the tonic phase was defined as the time when tonic muscle electrical activity

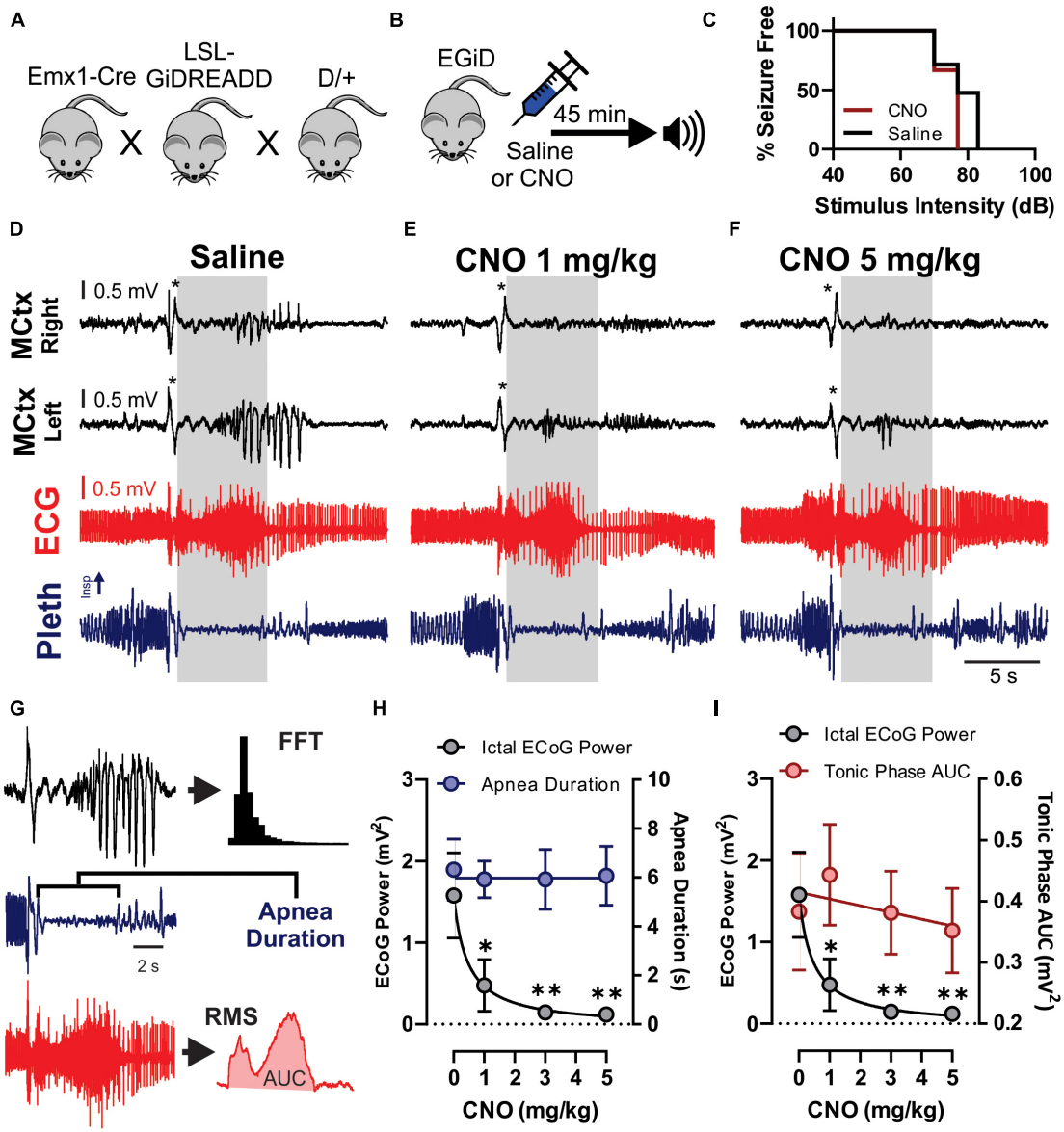

FIGURE 4
Clozapine-*N*-Oxide (CNO) suppresses cortical ictal activity but not apnea associated with audiogenic seizures. **(A)** D/+ mice were crossed with *Emx1*-Cre and floxed-GiDREADD mice to produce D/+ mice engineered with GiDREADD receptors expressed in cortical neurons (EGiD mice). **(B)** Depiction of experimental paradigm where EGiD mice were injected with saline or CNO 45 min prior to stimulation of an audiogenic seizure. **(C)** EGiD mice were exposed to increasing sound intensities until seizures were initiated. There was no detectable change in audiogenic seizure threshold between control and CNO (5 mg/kg) conditions. (D–F) Right and left motor cortex electrocorticogram (ECoG) activity (MCtx), electrocardiogram (ECG), and breathing (pleth) were recorded during audiogenic seizures after injection with saline **(D)**, 1 mg/kg CNO **(E)**, and 5 mg/kg CNO **(F)**. Asterisks denote movement artifact from brief wild running phase that routinely occurs prior to tonic and clonic phases in D/+ mice after audiogenic stimulation (Wengert et al., 2021b). Gray boxes represent the tonic phase. Note: cortical spike wave discharges that occur during seizures are blocked by CNO but not by saline. **(G)** Depiction of analysis of ECoG power (black), apnea duration (blue), and tonic phase area under the curve (AUC, red). **(H,I)** Plots of ictal ECoG power (gray) vs. apnea duration (**G**; blue; $n = 7$ mice) and tonic phase AUC (**H**; red; $n = 6$ mice). * and ** and $p < 0.05$ and 0.01, respectively.

first became apparent during the seizure, and was confirmed in the video as the time when hindlimb extension began. Apnea duration was measured as the time difference between the two detectable breaths on either side of the apnea; breaths were detected based on a rising threshold set by the experimenter that was sufficient to detect all normal breaths preceding the seizure by 30 s, as we have done before (Wengert et al., 2021b; Wenker et al., 2021). ECoG power was performed with Spike2 software's built in Fast Fourier Transformation (FFT) using a Hanning window with

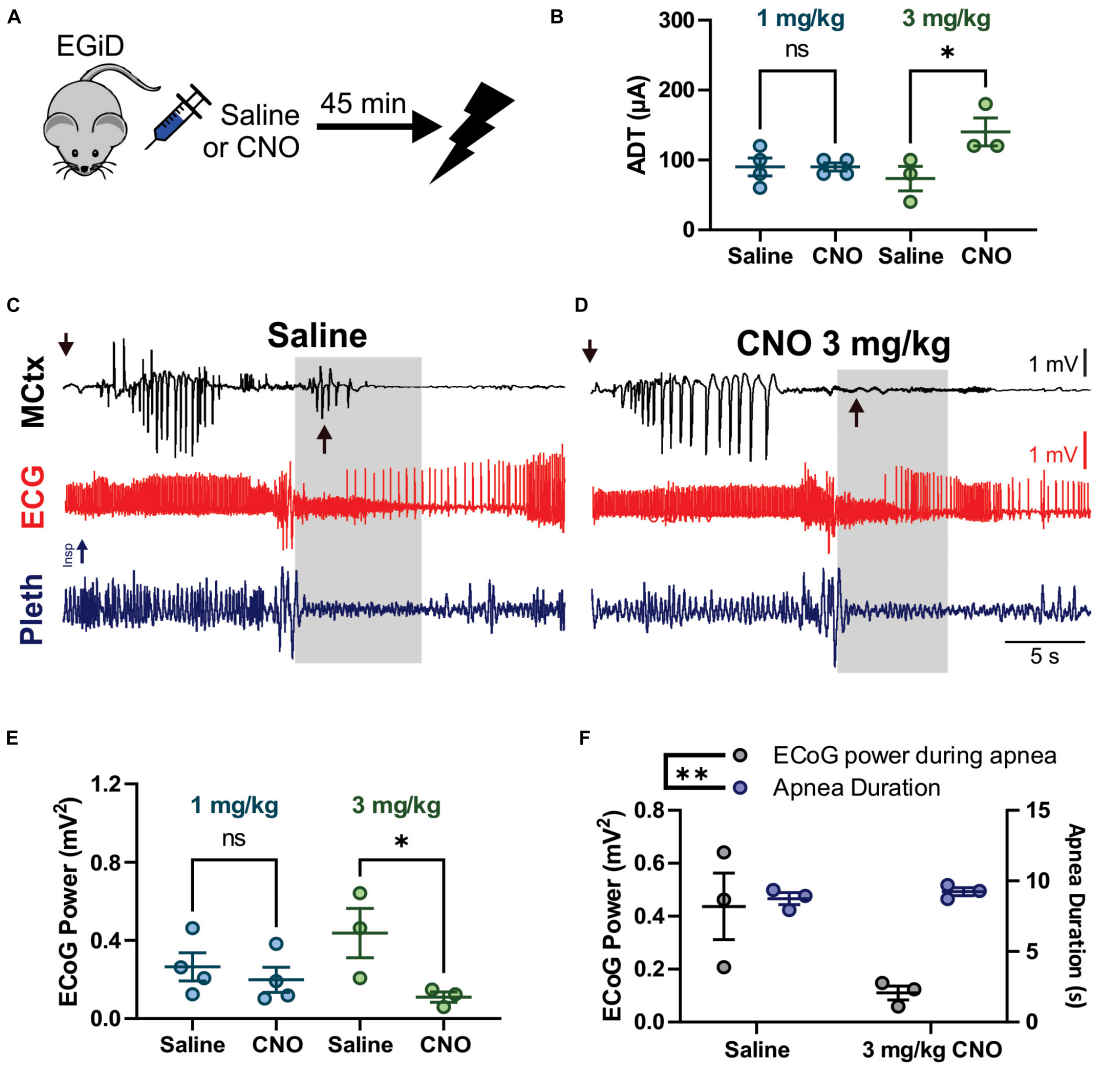

FIGURE 5
Clozapine-*N*-Oxide (CNO) suppresses cortical ictal activity during the tonic phase, increases after-discharge threshold (ADT) levels, but does not affect apnea or early seizure cortical ictal activity for hippocampal (HC) stimulated seizures. **(A)** Depiction of experimental paradigm where EGiD mice were injected with saline or CNO 45 min prior to HC stimulated seizure. **(B)** ADT comparing saline to 1 mg/kg CNO (aqua) and 3 mg/kg CNO (green). **(C,D)** Left motor cortex electrocorticogram (ECoG) activity (MCtx), electrocardiogram (ECG), and breathing (pleth) were recorded during HC stimulated seizures after i.p. injection with saline **(C)** and 3 mg/kg CNO **(D)**. Gray boxes represent the tonic phase. Downward arrows indicate end of HC stimulation. Upward arrows indicate cortical spike wave discharges that occur during the tonic phase. **(E)** Plot of ECoG Power during the tonic phase comparing saline to 1 mg/kg CNO (aqua) and 3 mg/kg CNO (green). **(F)** Plot of ECoG Power (gray) and Apnea Duration (blue) for saline and 3 mg/kg CNO conditions. * and ** and $p < 0.05$ and 0.01, respectively.

a window size of 1024 data points (1.024 s), resulting in a frequency resolution of 0.9766 Hz. Within an individual mouse, the same amount of time was analyzed for seizures under each condition (i.e., saline and CNO dosages). Tonic phase area under the curve (AUC) was also calculated in Spike2 by first deriving root mean square (RMS) of the ECG signal and then measuring the AUC between the beginning and end of the tonic phase (Figure 4F, middle). Reported ECoG power and tonic phase AUC was always expressed as mV^2.

In vitro patch clamp electrophysiological recording

Preparation of acute brain slices for patch-clamp electrophysiology experiments was modified from standard protocols previously described (Wengert et al., 2021a). Mice were anesthetized with isoflurane and decapitated. The brains were rapidly removed and kept in chilled Artificial Cerebrospinal Fluid (ACSF) (0°C) containing the following (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1

$MgCl_2$, 0.5 L-ascorbic acid, 10 glucose, 25 $NaHCO_3$, and 2 Na-pyruvate (osmolarity 310 mOsm). The slices were continuously oxygenated with 95% O_2 and 5% CO_2 throughout the preparation; 300 μm coronal or horizontal brain sections were prepared using a Leica Microsystems VT1200 vibratome. Slices were collected and placed in ACSF warmed to 37°C for ∼30 min and then kept at room temperature for up to 6 h.

Brain slices were placed in a chamber continuously superfused (∼2 ml/min) with continuously oxygenated recording solution. Layer 5 pyramidal neurons were identified based on anatomical location and pyramidal morphology *via* DIC video microscopy using a Carl Zeiss Axioscope microscope. Whole-cell recordings were performed using a Multiclamp 700B amplifier with signals digitized by a Digidata 1322A digitizer. Currents were amplified, low-pass filtered at 2 kHz, and sampled at 100 kHz. Borosilicate electrodes were fabricated using a Brown-Flaming puller (model P1000, Sutter Instruments, Novato, CA, United States) and had pipette resistances between 1.5 and 3.5 mΩ.

Current-clamp recordings of neuronal excitability were collected in ACSF solution identical to that used for preparation of brain slices. The internal solution contained the following (in mM): 120 K-gluconate, 10 NaCl, 2 $MgCl_2$, 0.5 K_2EGTA, 10 HEPES, 4 Na_2ATP, 0.3 NaGTP, pH 7.2 (osmolarity 290 mOsm). Intrinsic excitability was assessed using methods adapted from those previously described (Ottolini et al., 2017; Wengert et al., 2019, 2021a). Action potential (AP) frequency–current relationships were determined using 1 s current injections ranging from 0 to 600 pA. APs were counted as deflections that rose above 0 mV. Resting membrane potential (RMP) was determined from the period prior to current injection. Data was acquired with Clampex software and analyzed with Clampfit software (Molecular Devices, San Jose, CA, United States).

Pharmacology

Clozapine-*N*-Oxide was purchased from Sigma-Aldrich, St. Louis, MO, United States For *in vivo* experiments, CNO was dissolved into sterile saline at a concentration of 0.5 mg/mL, and was sterile filtered prior to injection. Injections were given intraperitoneal (i.p.) in a volume of 50–250 μl per mg of mouse weight 30–45 min prior to seizure induction by audio or HC stimulation. For *in vitro* experiments, CNO was dissolved into dimethyl sulfoxide (DMSO) at a concentration of 10 mM and diluted 10,000-fold into ACSF for experiments.

Immunohistochemistry

Mice were deeply anesthetized with phenobarbital and transcardially perfused with 10 ml ice-cold Dulbecco's PBS (DPBS) followed by 10 ml ice-cold 4% PFA. Brains were removed and immersed in 4% PFA overnight at 4°C. Brains were then washed with DPBS with 0.1% sodium azide at 4°C prior embedding in 2% agarose and making 40 μm transverse sections using a vibratome (Leica Microsystems, Wetzlar, Germany, VT1200). Tissues were mounted on slides using AquaMount (Polysciences, Warrington, PA, United States). Microscopy and imaging were done using the Neurolucida system (MBF Bioscience, Williston, VT, United States) with a Zeiss Axioskop microscope with computer-driven stage and Zeiss MRc camera.

Statistical analysis

All data points in **Figures 3I**, **4G, H**, **5B, E, F** denote biological replicates (i.e., no animal was used more than once for the same test). Data comparing spontaneous and electrically stimulated seizures (**Figure 1**) and data comparing in current clamp neuronal responses to CNO (**Figure 3**) are technical replicates and the animal numbers are reported in the figure legends. All average data values are expressed as mean ± SEM. Descriptive statistics and statistical tests were computed using GraphPad Prism version 9 (GraphPad Software, Inc., San Diego, CA, United States), except for MANOVAs, which were computed using SPSS (IBM, Armonk, NY, United States). Comparisons were considered statistically detectable when $P < 0.05$. All data was evaluated for normality of residuals by the Shapiro–Wilk test, and the appropriate non-parametric test was used when residuals were not normal.

Results

Comparison of spontaneous, audiogenic, and hippocampal stimulated seizures in D/+ mice

Spontaneous (**Figure 1A**), HC electrically stimulated (**Figure 1B**), and audiogenic (**Figure 1C**) seizures of D/+ mice all have very similar semiology (**Supplementary Video 1**), including brief wild running prior to a pronounced tonic phase coincident with apnea (Wengert et al., 2021b; Wenker et al., 2021, 2022). One difference stands out: all recorded spontaneous and HC stimulated seizures produced cortical ictal activity that preceded the tonic phase; however, cortical ictal activity was delayed until after initiation of the tonic phase of audiogenic seizures (**Figures 1C,D**), suggesting that cortical ictal activity is not necessary for production of the tonic phase or apnea. To further investigate this we crossed the D/+ mice with mice homozygous for *Emx1*-Cre and LSL-GiDREADD receptors, to produce mice with GiDREADD receptors expressed in forebrain excitatory neurons of D/+ mice to selectively inhibit forebrain excitatory neurons during both audiogenic and HC stimulated seizures.

Confirmation of forebrain excitatory neuronal inhibition by Clozapine-*N*-Oxide

To verify whether Cre-mediated recombination of *Emx1*-Cre mice is restricted to forebrain excitatory neurons, *Emx1*-Cre mice were crossed with Ai9 mice to produce Ai9$^{Emx1-Cre}$ mice (**Figure 2A**), resulting in TdTomato production in cells that express Cre. As previously reported (Iwasato et al., 2000; Gorski et al., 2002; Madisen et al., 2012), many neurons were fluorescently labeled in the forebrain of Ai9$^{Emx1-Cre}$ mice (**Figure 2B**) and no cell bodies were observed in the brainstem. In the brainstem, labeling was exclusively found in neuronal fibers and not in cell bodies (**Figures 2C,D**), with the exception of sparse labeling of somata in the cerebellum (**Figure 2C2**). As expected, neuronal fibers were mostly restricted to the pyramidal tract, a major output of excitatory neurons of the motor cortex. Fiber tracts with robust labeling were also found in the tractus solitarius and its terminal fields in the nucleus of the solitary tract, the primary nucleus for visceral sensory relays (Llewellyn-Smith and Verberne, 2011; **Figure 2D2**).

To confirm that CNO does inhibit cortical neuronal activity we recorded individual pyramidal neurons in the motor cortex *in vitro* and motor cortex ECoG *in vivo* in *Emx1*-Cre mice crossed with LSL-GiDREADD mice to produce GiDREADD$^{Emx1-Cre}$ (**Figure 3A**). We performed whole-cell current-clamp recordings from layer V pyramidal neurons that were identified by their anatomical location, shape, and firing properties (Wengert et al., 2019, 2021a). Depolarizing current injection steps resulted in generation of APs (**Figure 3B**) that were reversibly inhibited by application of 1 μM CNO (**Figures 3C,D**). CNO (1 μM) detectably reduced AP firing across a range of current pulse amplitudes [**Figure 3E**; $p = 0.0084$, $F_{(1,8)} = 12.08$, treatment factor of 2-way ANOVA] and detectably hyperpolarized resting membrane potential (RMP; **Figure 3F**; $p = 0.0393$, 2-tailed paired t-test). We also monitored ECoG activity (**Figure 3G**, top) and spectral power (**Figure 3G**, bottom) from the motor cortex in GiDREADD$^{Emx1-Cre}$ mice before and after i.p. injection with CNO or saline. Spectral power was decreased 30–50 min after injection of CNO across 0.5–20 Hz (**Figure 3H**, bottom), while saline injection had minimal impact (**Figure 3H**, top). The decrease in spectral power due to CNO injection was detectable at 1, 3, and 5 mg/kg doses [**Figure 3I**; $p = 0.0004$, 0.0039, and 0.0008, respectively; after significant treatment factor of 2-Way ANOVA, $p = 0.0071$, $F_{(1,4)} = 25.79$].

Inhibition of cortical excitatory neurons does not affect tonic phase apnea of audiogenic seizures

To test the hypothesis that excitatory neurons of the cortex do not contribute to tonic muscle contraction and apnea, we used chemogenetics to inhibit cortical neurons while inducing audiogenic seizures. We crossed D/+ mice with *Emx1*-Cre and LSL-GiDREADD mice to produce mice heterozygous for *Emx1*-Cre, LSL-GiDREADD, and the N1768D mutation, which we refer to as EGiD mice (**Figure 4A**). Seven mice were each tested at four doses of CNO: 0 (i.e., saline control), 1, 3, and 5 mg/kg of CNO (**Table 1**). Audiogenic seizures were tested ∼45 min after injection (**Figure 4B**). Mice were always given a minimum of 48 h to recover between trials and the order of CNO dosage administration was random. Even at a high dose of CNO (5 mg/kg), seizure threshold was unchanged (**Figure 4C**; $p = 0.6056$, Chi-square = 0.2667, Gehan–Breslow–Wilcoxon test). As described above, all audiogenic seizures have apnea coincident with the tonic phase and cortical ictal activity occurs after the tonic phase begins (**Figure 4D**). Administration of both high (5 mg/kg) and low (1 mg/kg) doses of CNO suppressed cortical ictal activity measured directly from the motor cortex region (**Figures 4E,F**, black traces). However, tonic muscle contraction and apnea were unchanged (**Figures 4E,F**, red and blue traces). To quantify this, we assessed ictal ECoG power by FFT (**Figure 4G**, top), apnea duration as the time between observed inspirations (**Figure 4G**, middle) and extent

TABLE 1 Mouse usage for audiogenic and HC stimulated seizures.

Mouse	Seizure type	Saline	1 mg/kg CNO	3 mg/kg CNO	5 mg/kg CNO
EGiD_44	Audiogenic	X	X	X	X
EGiD_117	Audiogenic	X	X	X	X
EGiD_132	Audiogenic	X	X	X	X
EGiD_134	Audiogenic	X	X	X	X
EGiD_143	Audiogenic	X	X	X	X
EGiD_162	Audiogenic	X	X	X	X
EGiD_163	Audiogenic	X	X	X	X
EGiD_178	HC stimulated	X	X	X	No seizure
EGiD_184	HC stimulated	X	X	X	No seizure
EGiD_217	HC stimulated	X	X	No seizure	No seizure
EGiD_216	HC stimulated	X	X	X	No seizure
EGiD_219	HC stimulated	X	Not tested	X	X

"X" indicates seizure was stimulated in mouse at this dose and data as included for analysis.

of the tonic phase by RMS amplitude (Figure 4G, bottom; time constant = 0.5 s). ECoG power, but not apnea duration, was detectably decreased by 1, 3, and 5 mg/kg doses of CNO compared to control (Figure 4H; p = 0.0046, 0.008, and 0.007, respectively for ECoG power; p = 0.844, 0.847, and 0.954, respectively for apnea duration; using Dunnett's multiple comparison after significant MANOVA, Pillai's Trace = 1.325, F = 11.773, $p > 0.001$). Similarly, while ECoG power was detectably decreased by 1, 3, and 5 mg/kg doses of CNO compared to control, tonic phase AUC was not affected (Figure 4I; p = 0.0046, 0.008, and 0.007, respectively for ECoG power; p = 0.910, 1.000, and 0.985, respectively for apnea duration; using Dunnett's multiple comparison after significant MANOVA, Pillai's Trace = 1.205, F = 7.583, $p > 0.001$).

Inhibition of cortical excitatory neurons decreases hippocampal stimulated seizure susceptibility, but does not affect tonic phase apnea

We also wanted to test the hypothesis that excitatory neurons of the cortex do not contribute to tonic muscle contraction nor apnea during seizures that originate in the forebrain. To this end, we used HC stimulated seizures in D/+ mice, which have identical seizure semiology to that observed with spontaneous seizures (Figure 1). We injected EGiD mice either with saline or CNO prior to HC stimulation of seizures ∼45 min later (Figure 5A). Mice were always given a minimum of 48 h to recover between trials and the order of CNO dosage administration was random. For every HC stimulated seizure, the ADT was determined by increasing the current pulse amplitude until a seizure occurred. Only mice with successful seizure stimulation with low ADT under saline conditions were included. Five mice met these criteria. Of the five mice, we were able to generate HC stimulated seizures in only one mouse at 5 mg/kg CNO, 3 mice at 3 mg/kg CNO, and 4 mice at 1 mg/kg CNO (Table 1). In the 4 mice tested for HC stimulated seizures in the presence of 1 mg/kg CNO, mean ADT intensities were unchanged [Figure 5B; $p > 0.9999$, Holm–Sidak's multiple comparison test after significant one-way ANOVA, p = 0.0468, $F_{(3,10)}$ = 3.810]. At the higher concentration of 3 mg/kg CNO, ADT intensities were detectably elevated [Figure 5B; p = 0.0201, Holm–Sidak's multiple comparison test after significant one-way ANOVA, p = 0.0468, $F_{(3,10)}$ = 3.810]. Although seizure threshold was increased by 3 mg/kg CNO, the semiology of resultant seizures was unaffected (Figures 5C,D). Routinely, we found that HC stimulated seizure had two periods of cortical ictal activity. The first was initiated during or immediately after the electrical stimulation (downward arrows in Figures 5C,D). The second period of ictal activity occurred during the tonic phase (upward arrows in Figure 5C). The ictal activity that occurred during the tonic phase was detectably inhibited by 3 mg/kg CNO administration (upward arrows in Figure 5D) but not by 1 mg/kg CNO administration [Figure 5E; $p > 0.9999$ and p = 0.0201, for 1 and 3 mg/kg CNO, respectively, Sidak's multiple comparison test after significant one-way ANOVA, p = 0.0468, $F_{(3,10)}$ = 3.810]. Although cortical ictal activity during the tonic phase was inhibited by 3 mg/kg CNO, apnea duration was unaffected (Figure 5F; MANOVA, Pillai's Trace = 1.634, F = 8.934, p = 0.005).

Discussion

The primary finding of the current study is that synchronous activity of excitatory cortical neurons is not essential for instigation of the tonic phase and concurrent apnea of a seizure. This was true for both seizures that were initiated in the temporal lobe and audiogenic seizures, which are believed to originate in subcortical structures (Faingold, 2012). Thus, the current data suggests that regardless of how a seizure is formed, generation of the tonic phase and seizure-induced apnea is produced by neural circuitry in the mid- and hindbrain.

Scn8a mutant mice as a model of seizure-induced apnea and sudden unexpected death in epilepsy

SCN8A epileptic encephalopathy is a severe genetic epilepsy with a high risk of SUDEP (Larsen et al., 2015; Gardella and Møller, 2019). In addition, non-fatal tonic seizures have been witnessed in numerous patients. As we and others have reported, these tonic seizures present with similar semiology to those of the *Scn8a* mutant mouse models: that is, with apnea and generalized breathing dysfunction, in addition to bradycardia (Trivisano et al., 2019; Zawadzka et al., 2020; Wenker et al., 2021). Although cases of SUDEP in *SCN8A* patients have been unwitnessed, tonic seizure and the resultant apnea appear as a likely mechanism of fatality. Indeed, in *Scn8a* mutant mice we have found that tonic seizure and apnea produce the spontaneous death observed in these models (Wengert et al., 2021b; Wenker et al., 2021). As discussed below, tonic seizures are a feature of other models of SUDEP; thus, the *Scn8a* mutant mouse model utilized in this study represents both an ideal clinical and broadly applicable model of SUDEP and seizure-induced apnea.

The tonic phase and seizure-induced apnea

In the D/+ mice, spontaneous seizures are quite similar in semiology to both audiogenic (Wengert et al., 2021b) and HC stimulated seizures (Wenker et al., 2022). These seizures are of the tonic variety: a brief wild running phase is followed by a prolonged tonic phase, with occasional clonic activity prior to

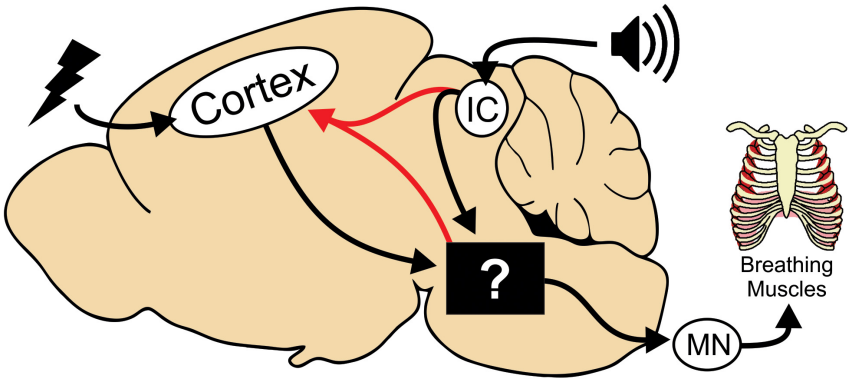

FIGURE 6
Model depicting neural circuitry involved in tonic phase apnea. In our mouse model, seizures can be initiated in the cortex or in the brainstem *via* the inferior colliculus (IC) for audiogenic seizures. The tonic phase and apnea are likely generated by neural circuitry in the brainstem, whose specific neuronal substrates are yet to be determined. This brainstem circuitry then recruits motor neurons (MN) and can also reactivate cortical ictal activity.

recovery (Wengert et al., 2021b). Tonic seizures appear common in other models of SUDEP (Faingold et al., 2017; Martin et al., 2020) and are associated with apnea in patients (Gastaut et al., 1963; Wyllie, 2015; Zawadzka et al., 2020; Wenker et al., 2021). Thus, the association between the tonic phase and seizure-induced apnea appears to be strong. In mice, there is also an association between the tonic phase and seizure-induced death–i.e., the experimental correlate of SUDEP (Martin et al., 2020; Wengert et al., 2021b; Wenker et al., 2021). Whether this tonic phase apnea occurs in instances of clinical SUDEP is unclear; however, some epilepsies that commonly present with frequent tonic seizures (e.g., tuberous sclerosis complex, Lennox-Gastaut syndrome, and *SCN8A* EE) do also experience higher mortality rates, often from SUDEP (Autry et al., 2009; Gardella and Møller, 2019; Parthasarathy et al., 2021). Thus, we propose the tonic phase and concomitant apnea as a possible mechanism of SUDEP, likely amongst others.

The role of forebrain excitatory neurons in seizure generation and semiology

We have previously shown that expression of a *Scn8a* gain-of-function mutation selectively in the forebrain is sufficient to produce spontaneous convulsive seizures and seizure-induced death (Bunton-Stasyshyn et al., 2019; Wenker et al., 2021). In the present study, we used mice with a *Scn8a* gain-of-function mutation expressed in the germline and expressed synthetic receptors that activate the Gi pathway (GiDREADD receptors) in excitatory forebrain neurons (Zhu et al., 2016) suppressing neuronal activity. We were able to demonstrate neuronal inhibition at the cellular level and *in vivo* during seizures. Perhaps unsurprisingly, we found that inhibition of excitatory forebrain neurons reduces susceptibility to HC stimulated seizures. However, once initiated, both HC stimulated and audiogenic seizures produced identical semiology: wild running followed by a prolonged tonic phase with apnea, and this was unaffected when cortical neurons were inhibited with CNO. Interestingly, we also observed fiber tracts projecting into the NTS that experienced recombination. It is likely that these peripheral neurons were also inhibited by CNO in our experiments, but this also had no impact on the tonic phase or seizure-induced apnea. Importantly, these results do not affect our conclusion that cortical neurons do not drive the tonic phase and apnea.

The neural circuitry of tonic phase apnea

Our interpretation is that although HC stimulated and audiogenic seizures are initiated in different regions of the brain, they impinge on the same circuitry to generate tonic phase apnea (**Figure 6**, black box). Interestingly, we observed cortical ictal activity during the tonic phase of both HC stimulated and audiogenic seizures. Since this cortical activity occurs with a slight delay to the start of the tonic phase, we propose that it is generated by seizure spread to the cortex from the original seizure focus (e.g., IC) or possibly from the neural circuitry that drives tonic phase apnea (**Figure 6**, red line). Previous work, while not causal, has implicated the broad region of the brainstem reticular formation in generation of the tonic phase of rodent seizures (Faingold and Randall, 1995; Faingold, 2012). In addition, numerous subcortical regions not depicted in our model could be critical for apnea generation. For instance, the amygdala is strongly implicated in apneas of pediatric epilepsies

(Dlouhy et al., 2015; Nobis et al., 2019; Rhone et al., 2020). While these apneas appear different than those we observe, most notably that they do not occur during the tonic phase, the amygdala and other regions could play an important role in seizure-induced apnea.

These ideas are not contradictory to our current study. In fact, our work could provide a framework for future studies using GiDREADD receptors to selectively inhibit different subcortical regions that may contribute to tonic phase apnea. For example, rhombomere-specific Cre mice have been produced that can target different regions of the mid- and hindbrain (Sun et al., 2017). Indeed, when excitatory Designer Receptors Exclusively Activated by Designer Drugs (DREADD) receptors were expressed under control of Cre in these mice, CNO administration produced apnea and death, similar to what seizure-induced death in our mouse models (Wengert et al., 2021b; Wenker et al., 2021, 2022).

Conclusion

Our findings suggest that while seizures may be generated in the forebrain, the detrimental behaviors (i.e., pathological motor activity and apnea) are generated in the mid- or hindbrain. Understanding the neural circuitry that generates these behaviors has translational value, as they could be targeted with the advancing genetic therapies, such as ASOs (Lenk et al., 2020; Hill and Meisler, 2021; Wengert et al., 2022). Future experiments examining brainstem circuitry that controls respiratory muscles could help us understand and treat potentially fatal seizures.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by University of Virginia Institutional Office of the Vice President for Research Animal Care and Use Committee.

Author contributions

IW and MP secured the funding, designed the experiments, and co-wrote the manuscript. AB, CL, AT, RM, JH, and PS the performed experiments and analyzed the results. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncir.2022.1002013/full#supplementary-material

SUPPLEMENTARY VIDEO 1
Side-by-side video of spontaneous, audiogenic, and hippocampal (HC) stimulated seizures in D/+ mice. Text indicates approximate timing of wild running, tonic phase, and recovery periods of the seizures.

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fMRI studies evaluating central respiratory control in humans

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A plethora of neural centers in the central nervous system control the fundamental respiratory pattern. This control is ensured by neurons that act as pacemakers, modulating activity through chemical control driven by changes in the O_2/CO_2 balance. Most of the respiratory neural centers are located in the brainstem, but difficult to localize on magnetic resonance imaging (MRI) due to their small size, lack of visually-detectable borders with neighboring areas, and significant physiological noise hampering detection of its activity with functional MRI (fMRI). Yet, several approaches make it possible to study the normal response to different abnormal stimuli or conditions such as CO_2 inhalation, induced hypercapnia, volitional apnea, induced hypoxia etc. This review provides a comprehensive overview of the majority of available studies on central respiratory control in humans.

KEYWORDS

central respiratory control, brainstem, fMRI, breathing, forebrain

Introduction

Regulating breathing is a response to alterations in blood levels of oxygen (O_2) and carbon dioxide (CO_2). Traditionally, this regulation process was attributed primarily on respiratory control centers located in the brainstem, particularly in the medulla and the pons. However, more recently, suprapontine structures such as the limbic areas, the diencephalon, the striatum and the cortex were ascribed to be essential in modulation of the respiratory drive of the brainstem (Horn and Waldrop, 1998; Pattinson et al., 2009b; Feldman et al., 2013). The basic pattern of respiration is generated in the medulla, and primarily regulated by pontine centers. These areas constantly regulate respiration, so that oxygen, carbon dioxide and acid levels are kept within normal limits. It is possible for someone to deliberately breathe faster or slower or to hold their breath, and it is also possible to not breathe at all for a period of time. This active control is regulated by the cerebral cortex, the amygdala and the hypothalamus, which participate in normal or exaggerated respiratory control, such as in stressful conditions (Horn and Waldrop, 1998). There are many brain regions sensitive to hypoxia and hypercapnia, and so the overriding of the will not to breathe comes from many regions (Guz, 1997). Both voluntary and automatic respiratory control systems are primarily integrated within the brainstem. However, in animal models, electrical stimulation of the cortex produces

respiratory responses directly through the dorsal cord and the respiratory motor neurons and indirectly *via* the corticobulbar pathways (Shea, 1996). Brainstem reflex respiratory response is inhibited if cortical inputs are altered, as shown in decorticated animals (Tenney and Ou, 1977), in patients with bilateral infarction (Heyman et al., 1958) or in opioid administration (Pattinson et al., 2009a).

Several acute and chronic neurological conditions are associated with altered breathing patterns. This is caused by changes that occur in central respiratory control centers located in the brainstem or in the forebrain (Nogues and Benarroch, 2008). In general, these changes are less severe in chronic diseases, like multiple sclerosis, compared to acute diseases like stroke (Nogues et al., 2002). They can also occur intermittently, such as during or after an epileptic seizure, where they might lead to sudden unexpected death in epilepsy (SUDEP).

Central nervous control

Medulla

Respiratory centers in the medulla are divided into the dorsal respiratory group (DRG) and the ventral respiratory column (VRC). The DRG represents the ventro-lateral portion of the nucleus tractus solitarius (NTS) and is mainly an inspiratory group (Alheid and Mccrimmon, 2008). It is described as a center of integration for afferents from peripheral chemoreceptors *via* the glossopharyngeal and vagus nerves (Alheid et al., 2011). The DRG sends constant bursts to respiratory motor neurons (Lalley, 1986). The VRC is a bilateral column formed by neurons in the lateral tegmentum and extending from the caudal part of the facial nucleus to the spino-medullary junction (Alheid and Mccrimmon, 2008; **Figure 1**). The caudal half of the VRC, termed the ventral respiratory group (VRG), contains bulbospinal respiratory premotoneurons that receive converging inputs from VRC rhythm generating neurons and from neurons outside the VRC, sculpting the activity pattern distributed to various pools of respiratory motoneurons (Alheid and Mccrimmon, 2008). The VRG is subdivided into the rostral (rVRG) and caudal (cVRG) group, based on the peak concentrations of inspiratory (rVRG) vs. expiratory (cVRG) bulbospinal neurons (Smith et al., 2013). The VRG is described as primarily expiratory, but it also contains inspiratory neurons. It consists of four groups of neurons, which generate a breathing rhythm through communication with each other: (1) the Bötzinger complex (BC); (2) caudal VRG jointly control the voluntary forced exhalation by sending input to intercostal and abdominal muscles. This is opposing: (3) the Prebötzinger complex (PBC); and (4) the rostral VRG that jointly acts to increase the force of inspiration (Ikeda et al., 2017). The PBC and BC are believed to be the central pattern generators of respiration (Smith et al., 2009).

Pons

The pons has two significant centers implicated in breathing regulation, both being part of the pontine respiratory group (PRG): (1) the pneumotaxic center; and (2) the apneustic center (**Figure 1**). The pneumotaxic center, located bilaterally in the dorsal rostral pons, is composed of the Kölliker-Fuse (KöF) and the parabrabrachial nuclei (PB) complex (Ikeda et al., 2017) and is involved in inspiratory off-switch. The KoF/PB complex is responsible for regulation of respiratory activity and respiratory phase transition (Ikeda et al., 2017). The apneustic center is located in the lower pons (Kahn and Wang, 1967). The PRG exerts "fine-tuning" influences over the medullary respiratory centers to help produce normal smooth inspirations and expirations (Douglas et al., 2004; **Figure 1**). Both centers communicate in order to control the rate and depth of breathing. The apneustic part is a "stimulator" and promotes inspiration by sending inputs to neurons in the DRG and VRG controlling inspiration. Although neurons involved with respiration are aggregated in certain parts of the brainstem, neurons that are active during inspiration are intermingled with those active during expiration (Smith et al., 2013).

Chemical control

Of vital importance in the control of respiration are chemoreceptors. These receptors respond to the CO_2 level in circulating blood, but the gas acts indirectly. CO_2 is capable of diffusing through the capillary blood-brain barrier. In the blood, dissolved CO_2 is neutralized by the bicarbonate-carbon dioxide buffer system and carbonic acid is formed, leading to the production of hydrogen and bicarbonate ions, and allowing the body to maintain a physiological pH (Alheid and Mccrimmon, 2008). When CO_2 is elevated, the concentration of hydrogen ions in the blood increases, lowering pH and resulting in acidosis. The central chemoreceptors are stimulated and respond to this pH change. The rise in blood CO_2 level, known as hypercapnia, thus triggers ventilation. The chemoreceptors that regulate respiration are located centrally near the medullary respiratory centers and peripherally in the arteries. Central chemoreceptors in the brainstem are continuously regulating breathing through monitoring of pH, the partial pressure of carbon dioxide (PCO_2) and oxygen (pO_2) in the blood. This regulation is insured by afferents from peripheral chemoreceptors located in the carotid body that are primarily targeting the solitary tractus (Lahiri et al., 1978). The partial pressure of oxygen (PO_2) will stimulate respiration when it reaches severe hypoxemic levels (Javaheri and Kazemi, 1987). The medullar structures playing the role of central chemosensors responding to changes in pH and $PaCO_2$

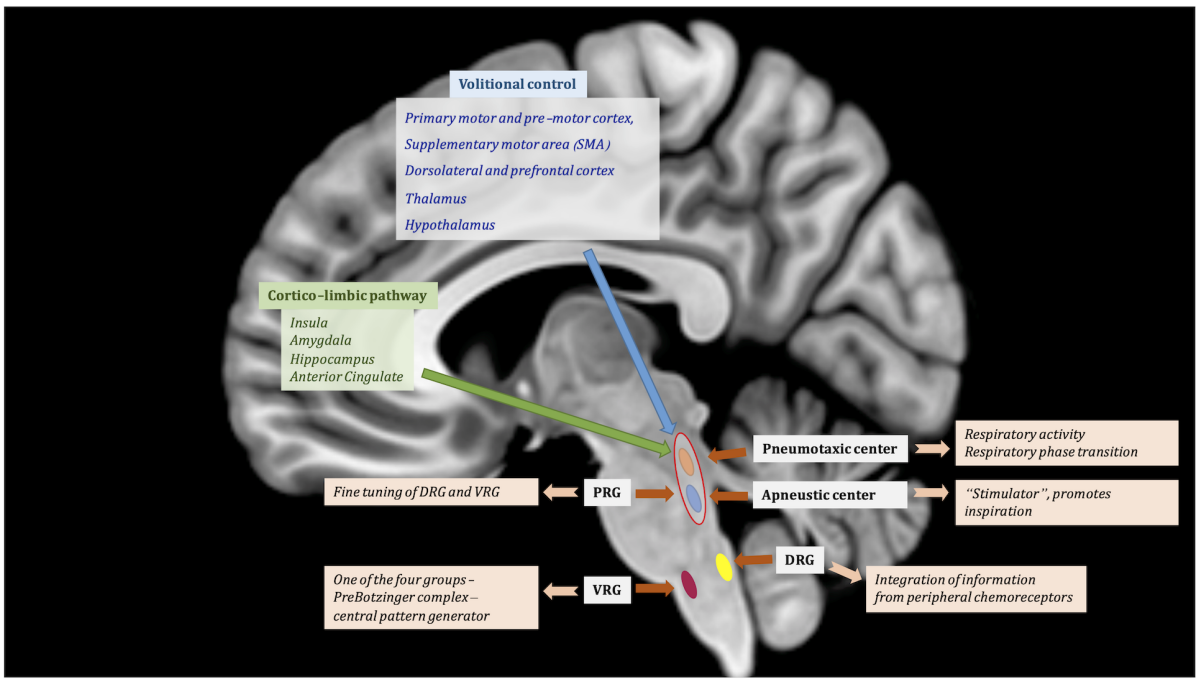

FIGURE 1
Central regulation of respiratory function. Suprapontine modulation of breathing through volitional control (blue) and corticolimbic pathway (green). Automatic regulation is assured by the brainstem centers contributing to the respiratory drive: (1) Pontine respiratory group (PRG) is represented by the pneumotaxic center and the apneustic center; and (2) Medulla—respiratory centers comprised of the dorsal respiratory group (DRG) and ventral respiratory group (VRG), the latter is part of the ventral respiratory column. The VRG is composed of four groups of neurons: (1) the Bötzinger complex; (2) caudal VRG; (3) the Prebötzinger complex; and (4) the rostral VRG. MNI T1 in sagittal projection was used for illustration.

in arterial blood or cerebral spinal fluid (CSF) are: (1) the raphe nuclei; (2) the arcuate nucleus; (3) the retrotrapezoid nucleus (RTN); and (4) the parafacial respiratory group (pFRG; Smith et al., 2013). The raphe nuclei are partly formed of serotoninergic neurons that participate in cardiorespiratory regulation (Morris et al., 2010). The arcuate nucleus is a group of neurons involved in the breathing rate control and is located bilaterally on both sides of the midline in the medulla (Mikhail and Ahmed, 1975). The pFRG and RTN, the most rostral structures of the ventral medulla, are respiratory-modulators and are also regulated by hypercapnia (Smith et al., 2013).

Peripheral chemoreceptors that regulate breathing are found in structures known as the carotid and aortic bodies. These bodies contain sensory neurons which react primarily to a reduction in oxygen supply. They are generally not involved in regulating respiration, since they do not act until O_2 drops to a very low level (Memmler et al., 1992). Recent studies have shown that astrocytes are also involved in chemosensing regulation in the brainstem. Low PO_2 stimulates astrocytes in the brainstem, and they promote a general respiratory response to hypoxia to deliver adequate oxygen to arterial blood (Angelova et al., 2015; Sheikhbahaei et al., 2018). Since there is usually a sufficient supply of O_2 in the blood, CO_2 has the most immediate effect on regulating breathing in the central chemoreceptors. As the level of CO_2 increases, increased respiration is required to remove excess gas. Chemoreceptors play an important role in developing the uncomfortable sensation of dyspnea, mostly due to direct connection to forebrain regions particularly in the limbic system, and they stimulate the respiratory system due to hypoxia/hypercapnia (Buchanan and Richerson, 2009).

Cerebral and limbic system control

While it is universally accepted that the brainstem drives the autonomic respiratory pattern, cortical modulation of central respiratory rhythm and the conscious perception of breathing are still poorly understood (Evans, 2010). Through the cerebral cortex, it is possible to consciously or unconsciously increase or decrease the rate and depth of the respiratory movements. The extra-pontine and extra-bulbar respiratory centers are involved in *volitional control, autonomic control, and cortico-limbic control* (**Figure 1**).

The *cortico-limbic pathway* involved in modulation of respiratory control is very similar to the one active during strong emotional or affective states (Ledoux, 2000; Nagai et al., 2010; Feinstein et al., 2022). It is mostly represented by the insula

and associated operculum (its anterior agranular part mostly), the head of the hippocampus, the amygdala, and the anterior cingulate cortex (ACC; Evans, 2010). Imaging studies have showed involvement of the amygdala in experimentally induced anxiety (Feinstein et al., 2022), of limbic structures in sleep disorders (Harper et al., 2014), and of the insula in obstructive sleep apnea (OSA; Li et al., 2015).

The *autonomic regulation* is mainly controlled by the brainstem, and it was mainly covered above, but inputs and outputs are generated by central structures acting in the control of cardio-respiratory functions, including the insula, temporal lobe, central operculum, and periaqueductal gray matter (Benarroch, 1993; Linnman et al., 2012). The fastigial nucleus of the cerebellum also contains CO_2/H+ intrinsic chemoreceptors and plays an essential role in compensating for extreme changes in blood pressure and modulating hypercapnia-induced respiratory response *via* monosynaptic projections to the medullary gigantocellular nuclei (Martino et al., 2007). The cerebellum is also thought to participate mainly in the expiration phase of breathing (Prasad et al., 2021).

The *volitional respiratory control* is mediated by the primary motor and pre-motor cortex, the supplementary motor area (SMA), the dorsolateral and prefrontal cortex for decision making and motor planning, and by thalamic nuclei (ventro-posterolateral, ventrolateral, anterior, pulvinar), as well as the hypothalamus. Various studies highlight the essential role of the dorsomedial hypothalamus in respiratory regulation as a result of different types of stressful situations (for review see Dampney et al., 2008). The caudal hypothalamus is also responsible for integration of respiratory output in relation to changes in homeostasis (Horn and Waldrop, 1998).

Afferents from pontine and medullary respiratory centers, cortical, limbic and other suprapontine structures descend along the anterolateral column of the spinal cord to the phrenic, intercostal and abdominal muscle motor neurons and generate respiratory movements.

Functional MRI of the brainstem in relation to respiration

The original fMRI studies of respiration were designed to detect central respiratory control in humans, but suffered from very limited sample size, lack of statistical power, and difficult interpretation of clusters observed around the midline (Gozal et al., 1995; Harper et al., 1998; Evans et al., 1999; Šmejkal et al., 2000). Furthermore, there was no correction for the various artifacts (motion artifacts caused by breathing, cardiac movement, cerebral spinal fluid pulsation) that impact BOLD signals generated in the brainstem or suprapontine structures (Dagli et al., 1999).

In recent years, the quality of MR imaging of the midline structures has improved significantly through the development of high field and ultra-high field MRI. However, imaging of the human brainstem remains challenging. Very few structures or nuclei can be reliably identified on a structural MRI, even on a high-field MRI scanner, let alone on a 1.5 or 3T scan. This becomes even more challenging for fMRI studies, since the BOLD signal is far more difficult to detect in the brainstem than in cortical areas due to a much lower signal-to-noise ratio (Beissner et al., 2014). However, there are solutions to tackle these issues, such as reducing the size of the acquisition field of view, which will consequently increase the spatial resolution and lead to a smaller voxel size, or correcting images for physiological movements.

Spontaneous fluctuations of BOLD signal during fMRI acquisitions are influenced by a multitude of physiological variables, including cardiac rhythm (Piche et al., 2009), respiratory movements (Hu et al., 1995; Kruger and Glover, 2001; Birn et al., 2006), head movements and changes in CO_2 (Iacovella and Hasson, 2011). The presence of major arteries and CSF in the vicinity of the brainstem adds to this physiological noise and further decreases the signal-to-noise ratio in fMRI studies of respiratory centers (Beissner et al., 2014; Beissner, 2015).

It is customary for fMRI studies dedicated to central respiratory control research to record all above-mentioned physiological signals to correct for their impact on BOLD signal. This requires using a pneumatic belt, end-tidal gas monitoring and head positioning foam pads during fMRI acquisition (Chang and Glover, 2009). De-noising of data from physiologically occurring signals can then be applied (Glover et al., 2000; Birn et al., 2006, 2008b; Chang and Glover, 2009). However, one needs to keep in mind that the removal of these signals, and in particular those related to respiratory movements, might partly hide the specific activation of respiratory centers elicited by the fMRI experiment (Iacovella and Hasson, 2011). Physiological noise can also be removed by computing both low frequency [physiological oscillations (∼0.01–0.15 Hz)] and high-frequency (driven by cardiac rhythm and normal breathing) physiological regressors (Windischberger et al., 2002; Birn et al., 2008a,b; Chang and Glover, 2009; Chang et al., 2009; Yuan et al., 2013; Cordes et al., 2014). Removal of these components from the fMRI series can induce significant changes and allow for a more reliable interpretation of the BOLD signal (Chang et al., 2009; Tong et al., 2019). This is especially valid for the reduction of susceptibility artifacts occurring along the vertebrobasilar arterial system and neighboring brainstem (Dagli et al., 1999). There are also other susceptibility artifacts that can induce changes in the magnetic field, such as the oscillatory chest movement, diaphragm shifting, and changes in the inhaled/exhaled gas. These artifacts will generate respiration-induced B_0 fluctuations and produce shifts in the phase of the MR image (Van De Moortele et al., 2002). A potential solution is parallel imaging, which increases the contrast to noise of echo-planar image (EPI) data by acquiring multiecho EPI (Poser

et al., 2006). Multiecho EPI samples the data at multiple repeated short echo times (TE). This type of acquisition with optimized echo weighting can reduce susceptibility-induced distortion and dropout artifacts in EPI images, while improving BOLD contrast sensitivity (Poser et al., 2006). The technique, in combination with Independent Component Analysis (ICA), has been shown to be effective in significantly reducing susceptibility artifacts in the brainstem (Kundu et al., 2012; Beissner and Baudrexel, 2014).

While total removal of these artifacts is improbable, their reduction is feasible either during the acquisition or in the pre-processing stage, by applying different denoising algorithms (Caballero-Gaudes and Reynolds, 2017). Yet, the intrinsic temporal relationship between the activation of the brainstem respiratory centers and respiration related movements makes such denoising a risk of masking the BOLD signal of interest. In addition, one needs to consider the degrees of freedom lost in this process. One of the most commonly used method in the field is RETROICOR, which is based on the Fourier transformation of cardiac and breathing rhythmic activities to correct for their related movement artifacts (Glover et al., 2000). A modified RETROICOR method has been developed to avoid overfitting the noise from physiological signals (Harvey et al., 2008; Jones et al., 2008; Wallace et al., 2017). Other approaches use ICA, CompCor toolbox from the CONN toolbox, or masked ICA (mICA) with a brainstem mask, to perform a signal decomposition directly from the acquired images into various components, including physiological artifacts such as breathing or heartbeat (Beissner et al., 2014; Moher Alsady et al., 2016; Jarrahi, 2021). Overall, modified RETROICOR and mICA are thought to enable removal of respiration-induced movements without masking the activity of brainstem respiratory centers (Beissner et al., 2014).

Reducing physiological noise in the brainstem is necessary for studies investigating its functions, such as control of breathing (Dunckley et al., 2005; Harvey et al., 2008). Denoising is particularly important in distinguishing between the true response of respiratory centers and the spill-over effect from neighboring vessels (Khalili-Mahani et al., 2013). Only few structures within the brainstem can be reliably identified on the traditional MRI (Matt et al., 2019). Parcellation techniques, widely used for segmenting MRIs, usually have one single label for the brainstem, or in some atlases, the brainstem is segmented into midbrain, pons, and medulla. Comparison of different preprocessing techniques for the brainstem described five methods that were sensitive to brainstem activation, including use of various parameters for normalization and smoothing in several commonly used software packages (Beissner et al., 2011). The small size of brainstem respiratory centers represents a significant issue in fMRI studies of respiratory control. Indeed, classical gradient echo images have a low spatial resolution, 3 mm^3 or lower (Weibull et al., 2008). This issue can be addressed by reducing the acquisition field of view by focusing on the brainstem (Pattinson et al., 2009b), or by using ultra-high field MR scanners which have provided promising results in the identification of brainstem nuclei. In addition, smoothing enables improving the high signal-to-noise ratio (SNR) of brainstem fMRI (Worsley and Friston, 1995; Beissner, 2015). Also, recent studies indicate that due to the particularly small size of nuclei involved in autonomic control, a small smoothing kernel of 6 mm for smoothing the data during preprocessing is advisable (Mckay et al., 2003, 2008; Evans et al., 2009; Hess et al., 2013). Yet, this results in blurring the distinction of closely spaced brainstem nuclei.

Correction models for respiratory rate and cardiac rhythm, along with direct measurements of CO_2 have also been applied successfully to quantify cerebrovascular reactivity (CVR) in the cerebrum (Golestani et al., 2015; Moreton et al., 2016; Prokopiou et al., 2019; Golestani and Chen, 2020). CVR is defined as the percentage signal change in CBF per mmHg change in arterial partial pressure of CO_2 ($PaCO_2$; Poublanc et al., 2015). It is often referred to as the ability of cerebral blood vessels to undergo diastolic contraction under the influence of hypercapnic challenges (Liu et al., 2019). CVR is also accompanied by mild hypoxia (Tancredi and Hoge, 2013; Chan et al., 2020). CVR is a marker of vascular reserve, and is complementary to basal cerebral hemodynamic measurements such as cerebral blood flow (CBF) and cerebral blood volume (CBV; Liu et al., 2019). Hypercapnia induced by CO_2 gas blend administration in patients with various neurological diseases effectively triggers CVR, enabling to evaluate their perfusion reserve (Spano et al., 2013). Cerebral regions that exhibit less or little BOLD CVR are usually affected in conditions such as gliomas, trauma, amyloid deposition, occlusion of major arteries etc. (Vernieri et al., 1999; Pindzola et al., 2001; Hsu et al., 2004; Ziyeh et al., 2005; Mandell et al., 2008). The CVR response to breathing challenges is different from the one induced by CO_2 inhalation, suggesting that CVR measurements in pathologies affecting the respiratory system may be inaccurate (Ogoh et al., 2019). One important issue in fMRI studies of respiratory centers is to distinguish BOLD signal changes due to CVR from those reflecting activation of brain respiratory response. In task fMRI the increase in the cerebral metabolic rate of O_2 and CBV is associated with increased local neuronal activity, which results in local decrease in the concentration of deoxyhemoglobin, which decreases the tissue-blood susceptibility differential. As a result there is a decrease in spin dephasing, and consequential BOLD signal increase (Bandettini et al., 1992). This process makes it feasible to measure the task related response from the brain areas involved in the processing of the task. For the CVR measurement, use of vasodilatory stimulus such as breath holding (BH) or exogenous CO_2 gas will induce only slight changes to cerebral metabolic rate of O_2 but a very robust global increased BOLD signal, primarily reflecting the augmentation of CBF (due to vasodilatation effect of the CO_2). The distinction between these two is the local and global

response to the task and the ways to measure the signal. The CVR BOLD quantifies the overall signal variance, the ratio between changes in the BOLD signal and end-tidal CO_2 change, whereas the BOLD response of the respiratory centers measures the local changes in BOLD intensity, which has a typical temporal profile known as the hemodynamic response function (HRF).

Removal of global signal changes is also fairly common (Macey P. M. et al., 2004). For the correction of CO_2 fluctuations on the fMRI signal, the HRF CO_2 can be convolved with end-tidal CO_2 data, and the output regressed out of the BOLD signal (Prokopiou et al., 2019). Use of end-tidal CO_2 measurements can also be useful when the user requires creation of the estimated CO_2 arrival time at each brain region and quantification of the hemodynamic response following elevation of CO_2 (Yao et al., 2021).

Using fMRI, there are several ways to obtain a response from the respiratory centers of the brain. This can be done either by: (i) inducing hypercapnia (through inhaling high concentration of CO_2 or through imposing prolonged breath holding); (ii) inducing hypoxia (through reducing the concentration of inhaled O_2); or (iii) through voluntary modulations of breathing (i.e., hyperpnea, breath-holding, slowing of respiratory pace). In particular, compliance with the breath-holding task is critical, given the role of prolonged volitional control in triggering an effective BOLD response from the respiratory centers. Breath-holding experiments can be either timed, using a fixed BH duration, or maintained for as long as participants can feel comfortable (Thomason et al., 2005; Mckay et al., 2008). Other methods to activate brainstem respiratory centers include hypercapnia and hypoxia challenges. Hypercapnia can be induced by administering a mixed gas with high concentration of CO_2 (5%) and 95% O_2, delivered for 2 min (Harper et al., 2005). For the hypoxia challenges, several paradigms have been used, including breathing a mixed concentration of 15% O_2 and 85% N_2 for 2 min (Macey et al., 2005), or undergoing five hypoxic episodes of breathing a mixed gas with 10% O_2 and 90% N_2 for 180 s followed by 90 s of normoxia (Gerlach et al., 2021). Because task-related changes in arterial gases, and notably pCO_2, can be affected by manipulation of breathing, some respiratory imaging studies have used mechanical ventilation as a passive condition and volitional control of respiration as an active condition, which allowed observing BOLD signal activations, unaffected by manipulation of pCO_2 (Ramsay et al., 1993; Evans et al., 1999).

Some of these studies are reviewed below.

Hypercapnia

Increased levels of pCO_2 and reduced pH trigger a response from the raphe pallidus, pFRG/RTN, and the NTS (Okada et al., 2008). Stimulation of these medullary chemoreceptors through an increase in pCO_2 leads to an excitatory response from the respiratory neuronal network, and to a hypercapnic ventilatory response (*in vivo* and *in vitro* study; Gourine et al., 2005; Fukushi et al., 2021). Usually, elevation of pCO_2 induces dyspnea or breathlessness in healthy subjects (Chonan et al., 1987; Burki and Lee, 2010) and this is used as a stimulus in investigations directed toward studying central respiratory control in dyspnea or air hunger (behavioral and pharmacological studies). The regional fMRI signal responses to hypercapnia, using 5% CO_2-95% O_2 for 120 s mixture, showed a pronounced increased response in regions not classically associated with breathing control, but traditionally related to affect, autonomic regulation, or motor coordination (Harper et al., 2005; i.e., the thalamus, dorsal striatum, insula, hippocampus, cingulate cortex, amygdala and hypothalamus; Brannan et al., 2001; Liotti et al., 2001; Von Leupoldt and Dahme, 2005). Limbic structures are also involved in breathing regulation such as air hunger (Banzett et al., 2000; Brannan et al., 2001; Liotti et al., 2001), urge-to-cough (Mazzone et al., 2007), or respiratory challenges such as induced hypercapnia by inhaling 5% CO_2 (Harper et al., 2005) or inspiratory breath holding (Macefield et al., 2006) and forced expiratory loading (Macey K. E. et al., 2004). Limbic activations are more notable in highly anxious subjects compared to low anxious participants when subjected to hypercapnia (Chan et al., 2019). The cerebellar and more rostral (midbrain, pons) involvement in mediating hypercapnia was also reported (Gozal et al., 1994; Harper et al., 1998, 2005; Kastrup et al., 1999b; Brannan et al., 2001; Parsons et al., 2001).

Hypercapnia-based studies are typically confronted by questions on how to obtain the response—either by volitional manipulation—end-expiratory or end-inspiratory BH, hyperventilation or by CO_2 gas mixture (which requires special equipment for the gas delivery, calibration, MR-safety measurements of all equipment etc). The advantage of such delivery is the control of inhaled and exhaled gas (Moreton et al., 2016), whereas the main constraint is the requirement of special equipment. Voluntary manipulations of breathing rely fully on subjects' compliance, which also can be viewed as a constraint. The advantage is that it can be easily implemented by means of finding a suitable stimulus onset/offset software and deciding upon which type of breathing manipulation to use in the experimental setting. Studies cited above have studied CVR (Okada et al., 2008) or central response to hypercapnia (Harper et al., 2005), voluntary expiration (Macey K. E. et al., 2004), inspiratory occlusion (Chan et al., 2019), Valsava maneuver (Harper et al., 1998), end-expiratory BH (Kastrup et al., 1999b). Few studies were conducted using CO_2 gas mixture (Gozal et al., 1994; Harper et al., 1998, 2005; Brannan et al., 2001; Liotti et al., 2001; Parsons et al., 2001). Some reference articles were using a PET technique (Brannan et al., 2001; Liotti et al., 2001; Parsons et al., 2001). Regarding the preprocessing of the data, some authors used data motion correction and global signal change removal (Macey K. E. et al., 2004; Harper et al., 2005), while some opted for no correction (Harper et al., 1998; Kastrup et al.,

1999b; Chan et al., 2019), or only the motion correction (Gozal et al., 1994).

Hypoxia

Hypoxia, reduction in pO_2, is detected by the carotid chemoreceptors, which trigger a response from the brainstem respiratory centers that leads to hyperventilation, for review please see Gourine and Funk (2017). Another review points to peripheral chemoreceptors, that also trigger forebrain response, descending through the hypothalamus to the DRG and VRG (Fukushi et al., 2021). Indeed, the hypothalamus is one of the key regions driving the central respiratory response to hypoxia (Horn and Waldrop, 1998). Hypoxia is an effective way to experimentally trigger dyspnea, and can be easily reversed with inhalation of O_2 (Fukushi et al., 2021). In an fMRI study, hypoxia was induced by repeated inhalation of 10% oxygen and 90% nitrogen, and contrasted with normoxia (Gerlach et al., 2021). Five distinct hypoxia-responsive regions were detected around the NTS (the nucleus ambiguous, intermediate reticular nucleus, dorsal motor nucleus of the vagal nerve, spinal trigeminal nucleus, and the inferior olivary nucleus; Gerlach et al., 2021), as well as three hypothalamic regions (the arcuate nucleus, anterior hypothalamic area/lateral hypothalamic area, and paraventricular nucleus; Gerlach et al., 2021). However, they did not monitor the end-tidal pCO_2 during their experiment, nor did they apply a physiological correction to the BOLD series, raising the possibility that the central chemoreflex response could have been merged with the activations detected during their experiment. They also employed mICA focusing on the lower brainstem and the hypothalamus. As for the hypercapnia induced by CO_2 inhalation, the advantage of inducing the hypoxia with a mixture of 10% O_2 and 90% N_2 is that it allows full control over the inhaled gas. The issue is that this method of gas delivery requires special MR safe equipment.

Voluntary modulation

Forebrain regions, such as the primary motor cortex, premotor area and supplementary motor area, are activated by volitional breathing (Brannan et al., 2001; Liotti et al., 2001; Von Leupoldt and Dahme, 2005). Voluntary modulation of breathing in 20 healthy volunteers who breathed at a slower pace than usual (i.e., 5.5 breaths per minute, similar to yoga practice, compared to 10 breaths per minute) resulted in increased BOLD activation pattern within the brainstem, across the dorsal length of the pons, in hypothalamic and thalamic regions, within cerebellar vermis and lateral cortices and in the striatum, the hippocampus and the motor, supplementary motor and parietal cortices (Critchley et al., 2015). These areas are part of an executive homeostatic network (Zaccaro et al., 2018). The observed activations hint to the link between the control of breathing and the baroreflex sensitivity. Hypoxic challenge (breathing 13% of O_2) in the same group of subjects led to activation within the dorsal pons, bilateral amygdala, thalamus and cerebellar cortices, along with activation of occipital, medial and dorsolateral prefrontal regions, an activation pattern typically observed in stressful conditions (Critchley et al., 2015). In the modeling of fMRI data, they added end-tidal CO_2, arterial oxygen saturation SaO_2, respiratory rate, tidal and minute volume (ventilation), heart rate and standard deviation of inter-beat interval. Another modulation of breathing, which consists of 6 s breath (3 s breathe in and 3 s breathe out) or 12 s breath (6 s breathe in and 6 s breathe out), provided comparable end-tidal CO_2 values and patterns of BOLD response compared to those resulting from inhalation of CO_2 (Liu et al., 2020). Authors accounted for variation of end-tidal CO_2 in their data preprocessing. An early fMRI study performed in five healthy men assessing volitional inspiratory control (voluntary hyperpnea ensured by a ventilator with creation of large tidal inspiratory volumes compared to passive expirations) showed activation within the superior motor cortex, premotor cortex and supplementary motor area (Evans et al., 1999). They used a 15 mm smoothing kernel, and no correction for any susceptibility artifacts was applied, but also no activation in the brainstem was reported. In another study where healthy participants executed voluntary hyperpnea (about three times faster as the normal breathing), the same cortical areas, along with medullar activation were detected (Mckay et al., 2003). They accounted for global signal changes in the volumes, and used a much smaller smoothing kernel—6 mm. Another paradigm consisted in contrasting unconscious to conscious breathing, by asking healthy volunteers to focus their attention (or not) on each inspiratory and expiratory movements. This resulted in modulation of the activation pattern in the premotor and parietal cortex (Šmejkal et al., 1999, 2000). Authors did not provide any information about data preprocessing.

The advantage of using voluntary respiration modulation relies on: (1) very little equipment required to perform the task; and (2) the facility to train subjects to perform the experiment. The drawback of this approach is: (1) little control over compliance; and (2) variability in subjects' ability to hold long breath holds.

Resting state fMRI

Resting state fMRI enables study of the synchronous spontaneous fluctuations between various cortical regions (Biswal et al., 1995). Several resting state networks have been described, however most studies focus on the default brain mode (DMN; Greicius et al., 2003). In the DMN, consistent regions of the brain are active at rest but reduce their activity when cognitive tasks are carried out. Abnormal resting state

connectivity has been observed in various diseases, including conditions where respiratory and cardiovascular regulation is impaired. Confounds, such as bulk motion, cardiac-related motion, white matter fluctuations, respiratory-related motion, and variations in end-tidal CO_2 account for 46% of signal variance in resting state fMRI data and must be considered when investigating low frequency variations of the BOLD signal (Harita and Stroman, 2017). Modulation of respiration also proved to influence resting state fMRI, with enhanced connections when pCO_2 is increased (Mcketton et al., 2021). Authors corrected for the end-tidal PCO_2 and for head motion, and used CompCor toolbox to extract physiologically related noise in the data. The same correction was used in a recent study, which reported that voluntary normal breathing through the mouth compared to nose breathing also resulted in increased functional connectivity (FC) throughout the DMN nodes (Jung et al., 2020). The authors linked this finding to the potential cognitive disturbances observed in subjects suffering from mouth breathing syndrome (Jung et al., 2020). Another study showed synchronized neural activity through a distributed network of limbic/paralimbic and brainstem regions during uninterrupted spontaneous respiration (Evans et al., 2009). Authors used a 6 mm kernel for smoothing, and used global regressors for correcting for variation of BOLD globally and the PCO_2 regressor.

Breath-holding fMRI

Breath holding (BH) is commonly used to simulate the effect of apnea and hypercapnia on the brain, resulting in autonomic down-regulation of heart rate, vasodilatation and simultaneous reduction in blood flow to the brain (Kastrup et al., 1998; Corfield et al., 2001). Breath-holding results not only in rapid increase in pCO_2 but also in reduction of PO_2 in the first 20–30 s (Dubois, 1952; Hong et al., 1971), and the cumulative amount of CO_2 for longer BHs (30–40 s) is lower than when the BHs last 20 s or less (Lindholm and Linnarsson, 2002). Hyperventilation before BHs will reduce the CO_2 and increase the reserve of O_2, and will diminish the urge to breath. While the PO_2 levels after very long BHs will descend to 20 mmHg, the levels of CO_2 will stay normal or slightly decreased (Lindholm and Lundgren, 2006). The breaking point of BH happens when the sum of lower PO_2 and higher CO_2 is sufficient to induce alveolar ventilation eight times higher the normal (Otis et al., 1948). The increase in PCO_2 will stimulate the central respiratory rhythm, which keeps its rhythmicity throughout the BH and cannot be controlled voluntarily (Parkes, 2006). The breathing patterns correlate with fluctuations in PO_2 and PCO_2 during BH, which are vasoactive triggers that modify global cerebral perfusion (Kastrup et al., 1999c). While the majority of fMRI studies cited in this review had reported PCO_2 or end-tidal CO_2 variations measured in their studies, only very few also focused on PO_2 variations (Gozal et al., 1995; Chan et al., 2020). That is probably due to the common concept that an increase in PCO_2 will imminently decrease the PO_2, as they work synergistically in the normoxic condition to stimulate peripheral chemoreceptors (Lahiri et al., 1978). Comparison of studies with and without measurement of PO_2, but with measurement of PCO_2 showed that variation of PCO_2 during BH and after BH are very similar (Otis et al., 1948; Gozal et al., 1995; Chan et al., 2020). Studies in which PO_2 was measured (Gozal et al., 1995; Chan et al., 2020) indicated that hypoxia and hypercapnia have synergistic effect, and reproduce results observed in animal studies (Honda et al., 1963; Lahiri et al., 1978; Chan et al., 2020).

Compared to inhalation of CO_2, where special equipment is needed, BH is safe, easy to use, suitable for various age groups, and offers fairly robust results (Godfrey and Campbell, 1968; Strohl and Altose, 1984; Li et al., 1999; Liu et al., 2002; Parkes, 2006; Mckay et al., 2008; Magon et al., 2009; Roberts et al., 2009; Bright and Murphy, 2013; Sutterlin et al., 2013; Tancredi and Hoge, 2013; Iranmahboob et al., 2016). Strong cortical, subcortical and medullary activations are usually observed during BH (data corrected for global intensity and smoothed with 6 mm kernel; Mckay et al., 2008) and the signal increase occurs with no apparent change in mean arterial pressure, but no information about data correction was reported (Kannurpatti et al., 2002). BH can be performed in different ways, either by: (1) end-expiratory BH (shortest due to lack of inhibitory lung stretch and a small lung volume reservoir to mix atmospheric air with arterial blood); (2) end-inspiratory BH (longer than expiratory BH); (3) end-inspiratory BH with previous hyperventilation (results in reduction of CO_2 due to hyperventilation, which allows this type of BH to last longer); and (4) end-inspiratory BH with previous hyperoxia [similar to (3)] (Skow et al., 2015). The most commonly used BHs in fMRI are standard end-expiratory and end-inspiratory BHs. Computer paced end-expiratory BHs were suggested to show a more intense BOLD signal compared to self-paced breathing, and data were corrected for the delay in onset of the BH (Scouten and Schwarzbauer, 2008). Breathing pace in inspiratory BH also influences the level of fMRI activation, with greater BOLD changes with faster breathing when data was corrected for head motion and delay in BH onset (Chen et al., 2021). A recent review recommended to practice expiratory BHs of 15 s with self-paced recovery period for optimal results (Pinto et al., 2021). BH fMRI studies can be applied in younger populations but suffer from significantly noisier and less activated voxels in children than in adults (Thomason et al., 2005).

A very early fMRI study (1993) of the effect of hypoxia following inspiratory BH reported a decrease in the intensity of BOLD, but no information about correction applied is provided in the article (Stehling et al., 1993). However, subsequent fMRI studies that used the inspiratory and expiratory BH maneuvers consistently observed an increase in activations, however no particular correction for the physiological noise

or else was used (Kastrup et al., 1998, 1999a; Li et al., 1999). Yet, end-expiratory BH was found to be associated with both increased BOLD response in the right insula, dorsal anterior cingulate, cerebellum, and fronto-parietal cortex, and decreased BOLD signal in the left insula, ventral anterior cingulate, precentral gyrus and hippocampus (Kimmerly et al., 2013; Sharman et al., 2014). Reported data was corrected for global signal intensity change (Kimmerly et al., 2013) and for respiration and heartbeat (Sharman et al., 2014). These regions are viewed as an autonomic cortical network controlling apnea-induced muscle sympathetic nerve activity (Kimmerly et al., 2013). Inspiratory BH was associated with fMRI activations in the midbrain, pons, cerebellum and lentiform nuclei, but no correction for the data was reported (Gozal et al., 1995). During resistive inspiratory load, higher BOLD activations were observed in ventrolateral and dorsal medulla (PBC) than in caudal ventro-lateral pons (pFRG; Hess et al., 2013). Data were corrected for respiratory volume per time, end-tidal CO_2, RR cardiac interval and saturation, and a small smoothing kernel was used, 6 mm.

Most of the studies examined here took into account the variation of end-tidal CO_2, likely because it is easy to measure and reflects the variation of O_2 in expired air. However, as discussed at the beginning of this section, CO_2 and O_2 fluctuations will also affect the BOLD signal and these confounds should be considered as part of the data model (Moreton et al., 2016). The first fMRI studies of central control of breathing have used little or no correction for susceptibility and movement artifacts, and also suffered from poor spatial resolution. Due to these limitations, findings from these pioneering studies were difficult to interpret, both in terms of the mechanisms underlying changes in BOLD signal and their precise anatomical location. Yet, most of these findings were confirmed in subsequent studies which have applied an appropriate methodology (Sharman et al., 2014; Chen et al., 2021).

fMRI of central respiratory control in different diseases

Sudden unexpected death in epilepsy (SUDEP)

Sudden unexpected death in epilepsy (SUDEP) is the most shattering outcome in patients with epilepsy, typically affecting adolescents and young adults between 20 and 40 years of age with drug resistant epilepsy. SUDEP may account for up to 1/3 of all causes of non-suicidal, non-accidental sudden death in this age range. Recent progresses have pointed to the primary role of post-ictal central respiratory distress (Patodia et al., 2021). Individuals who are at high risk of SUDEP, based on various clinical risk factors, exhibit regional brain structural and FC alterations compared with low-risk patients. In the former, FC was found reduced between pons and thalamus, between midbrain and thalamus (Tang et al., 2014), between the thalamus, brainstem, anterior cingulate, putamen and amygdala, and was elevated between medial/orbital frontal cortex, insula, hippocampus, amygdala, subcallosal cortex, brain stem, thalamus, caudate, and putamen (Allen et al., 2017, 2019). Structural alterations were also identified in patients who died from SUDEP, such as the presence of brainstem atrophy (Mueller et al., 2014) and reduced posterior thalamic gray matter volume, possibly linked to hypoxic challenges from apnea (Wandschneider et al., 2015). Finally, in a mixed population of SUDEP cases and individuals at high risk of SUDEP, fMRI showed impaired communication between several nodes involved in respiratory and cardiovascular regulation (La et al., 2019).

Congenital central hypoventilation syndrome

Congenital central hypoventilation syndrome (CCHS) is a genetic condition characterized specifically by a lack of sensitivity to CO_2 and is defined by an important alteration of the automatic control of breathing. Hypoxia, induced through inhalation of 10% oxygen and 90% nitrogen, elicited comparable BOLD responses in CCHS and control subjects in medullary and hypothalamic structures but significantly different patterns in cerebellar, dorsolateral pontine, thalamic, basal ganglia, limbic, and midbrain areas (Macey et al., 2005) and the prefrontal cortex (Zhang et al., 2011). The altered suprapontine control of respiratory function could also account for a dysfunction in the other cognitive processes supported by the same brain regions. In a single CCHS case study, spontaneous breathing showed an increased functional connectivity between the brainstem and the frontal cortex, whereas assisted breathing (mechanical ventilation) resulted in restoration of physiological DMN low frequency oscillations and improved patients' executive functions (Sharman et al., 2014). During Valsalva maneuvre, a forced exhale and hold task, the overall BOLD response in nine patients with CCHS was muted compared to healthy controls, leading authors to conclude that in CCHS the structures that mediate sympathetic and parasympathetic output are impaired (Ogren et al., 2010).

Obstructive sleep apnea

Obstructive sleep apnea (OSA) is characterized by episodes of complete or partial repetitive upper airway collapse during sleep. In 12 patients with OSA, the BH maneuver resulted in less CVR than in controls, while brain regions of decreased

CVR were larger in these patients than in controls (Buterbaugh et al., 2015). Expiratory loading in nine patients with OSA resulted in decreased global gray matter signal intensity, which was less pronounced than in healthy participants, and occurred twice as fast in OSA than in controls (20 s into the challenge compared to 40 s in controls; Macey et al., 2003). During this expiratory loading challenge, patients with OSA also showed increased activation in the ventral midbrain and the hippocampus, and decreased activation in the middle frontal gyrus and Broca's area, insula and anterior cingulate (Macey et al., 2003). Inspiratory loading in seven patients resulted in decreased BOLD signal in the dorsal and ventral striatum, frontal cortex, insula, hippocampus and midbrain, while there were significant increases in activation in the dorsal midbrain, medial cingulate, temporal and cerebellar cortex (Macey et al., 2006). fMRI study using the Valsava maneuver in 21 male subjects with OSA showed a decreased response compared to controls in the left inferior parietal cortex, anterior superior temporal gyrus, posterior insular cortex, cerebellar cortex, fastigial nucleus, and hippocampus (Henderson et al., 2003). Also, in patients with OSA, the DMN pattern is selectively altered (Li et al., 2016; Wu et al., 2020). The areas that show altered BOLD response are usually expressing regional structural changes in the white and gray matter and reduced cortical thickness (Macey et al., 2008, 2018; Canessa et al., 2011).

Conclusion

MR imaging in humans has demonstrated its ability to investigate the neural centers involved in the CNS control of respiration, using various types of experimental protocols. In particular, controlled hypercapnia, hypoxia, as well as breath holding maneuvers enable fMRI activation of brainstem respiratory centers and their hypothalamic, limbic and cortical controlling networks. Such investigations can now be used to explore medical conditions associated with known or suspected dysfunction of central respiratory control, in order to better understand their pathophysiology and to develop novel clinically relevant biomarkers.

Author contributions

CC collected books and articles for the review, conceived, designed, and wrote the review. SR and PR provided a methodological/clinical perspective and editing of the review, provided general advice on the review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Forebrain control of breathing: Anatomy and potential functions

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The forebrain plays important roles in many critical functions, including the control of breathing. We propose that the forebrain is important for ensuring that breathing matches current and anticipated behavioral, emotional, and physiological needs. This review will summarize anatomical and functional evidence implicating forebrain regions in the control of breathing. These regions include the cerebral cortex, extended amygdala, hippocampus, hypothalamus, and thalamus. We will also point out areas where additional research is needed to better understand the specific roles of forebrain regions in the control of breathing.

KEYWORDS

forebrain, control of breathing, limbic system, cortex, respiratory circuits

Introduction

Breathing is an essential function for humans during every waking and sleeping moment to provide movement of air in and out of the lungs (1–3). Our oxygen demands are dynamic and constantly changing depending on our activity level, emotional state, health status, and current behaviors. Because of this, the brain must constantly ensure that our breathing is appropriately matched to our physiological state and behavior. For example, our breathing rate and/or volume changes in anticipation of altered needs for gas exchange and tissue oxygenation during exercise or other physical activities (4). This feed-forward control is critical for maintaining homeostasis because there is no known mechanism for sensing gas exchange in the muscle or lungs. Breathing changes with emotional states as well, as the feelings of stress and fear can cause hyperventilation (5–7). Although the basic pattern of respiration (inspiration, post-inspiration, and expiration phases) is generated by neurons in the brainstem and transmitted to respiratory muscles via spinal circuits (1, 2, 8), these neurons are influenced by other brain regions in order to ensure that breathing is appropriate for the current situation.

Breathing, unlike other autonomic processes such as heart rate and blood pressure, can be modulated voluntarily in addition to autonomically (9–11). For example, singers and musicians that play wind instruments need precise control over their breathing to produce the correct notes and tones. Mindfulness exercises such as meditation and yoga utilize deliberate and precise breathing methods to elicit calming responses from the body, including lowering blood pressure and heart rate. Competitive weightlifters are among a variety of athletes that use methodic breathing techniques such as

hyperventilating before their lift to provide sympathetic activation to maximize strength during their lift. Swimmers pace their breathing to ensure that they do not inadvertently inhale water. Thus, intentionally pacing respiration or modulating breathing volume is a tool that humans and animals use to control their own physiology.

The forebrain is important for the planning and execution of movements, sensory processing, regulating sleep wake states and behavioral responses to emotions such as stress and fear (5, 7). Each of these functions can have an impact on breathing. For example, fear is linked with a variety of respiratory changes- you may gasp if you are startled, you might find yourself holding your breath when scared, or even hyperventilating as your body prepares its fight or flight response. Sleep/wake states strongly influence the control of breathing, with important consequences if this relationship is dysfunctional, such as sleep apnea, congenital central hypoventilation syndrome, sudden infant death syndrome, or sudden unexpected death in epilepsy. Thus, we propose that the forebrain may be important for ensuring that breathing matches current and anticipated emotional, behavioral, and physiological needs. However, the circuits and mechanisms by which the forebrain exerts control over breathing are only partly understood. This review will summarize anatomical and functional evidence implicating forebrain regions in the control of breathing. These regions include the cerebral cortex, extended amygdala, hippocampus, hypothalamus, and thalamus (Figure 1). We will also point out areas where additional research is needed to better understand the specific roles of forebrain regions in the control of breathing.

Brainstem and midbrain control of breathing

The brainstem is critical for the generation of respiratory rhythm, patterning of motor output, and adapting respiration to changes in blood gasses to ensure adequate ventilation at all times (1–3, 8, 12). Historically, the brainstem has been broadly divided into the ventral respiratory column, dorsal respiratory group, the parafacial respiratory group, and the pontine respiratory group. Here, we describe the main structures within these groups responsible for the control of breathing to provide a foundation for understanding how forebrain structures might modify breathing via their connections to these structures.

Ventral respiratory column and the triple oscillator model

The pre-Bötzinger complex (preBötC), post-inspiratory complex (PiCo), and lateral portion of the parafacial respiratory group (pF_L) are thought to be oscillatory rhythm generators responsible for driving the inspiratory, post-inspiratory, and expiratory phases of breathing, respectively (13). The preBötC is part of the ventral respiratory column that also includes the Bötzinger complex (BötC), rostral ventral respiratory group (rVRG), and caudal ventral respiratory group (cVRG) (1). The preBötC drives inspiration *via* connections to bulbospinal neurons in the rVRG (14). PiCo is an oscillatory neuronal population adjacent to the BötC and parafacial respiratory groups that is active in the post-inspiratory period (15). The pF_L drives expiration *via* connections to the bulbospinal neurons in the cVRG (1). Expiration is passive at rest and expiratory muscles are only recruited when metabolic/ventilatory demand is high, such as during exercise. The BötC contains inhibitory neurons that are active during post-inspiration and/or expiration and inhibit the preBötC and other brainstem regions (16). Inhibitory connections between the three oscillators are thought to maintain the three distinct phases of breathing (13). Forebrain regions with connections to the ventral respiratory column could potentially alter the inspiratory rhythm, coordination between the three oscillators/phases of breathing, or transmission of the respiratory rhythm to motor neurons.

Parafacial respiratory group

The parafacial respiratory group consists of a lateral part (pF_L) and a ventral part (pF_V), located adjacent to the facial motor nucleus. The pF_L is thought to generate the rhythm responsible for the expiratory phase of breathing, as discussed above. The pF_V (also referred to as the retrotrapezoid nucleus) is critical for central chemosensation- responding to changes in pCO2 and pH in the blood (12). These two groups can be distinguished by their location as well as expression of Phox2b only in the pF_V (17, 18). Thus, forebrain connections to the pFRG region could potentially alter expiratory rhythm, control passive vs. active expiration, or modulate chemosensory responses.

Dorsal respiratory group

The dorsal respiratory group consists of a population of respiratory neurons within the nucleus of the solitary tract (NTS) in the medulla. The NTS receives sensory input from the periphery and projects throughout the brainstem to modulate autonomic functions such as breathing and heart rate (19, 20). It is a major information processing and relay station, though only a portion of the NTS is involved in respiratory function. In the context of respiration, the NTS relays sensory afferent information from the vagal and glossopharyngeal nerves to the ventral respiratory column and receives information from the carotid bodies about circulating blood gas concentrations

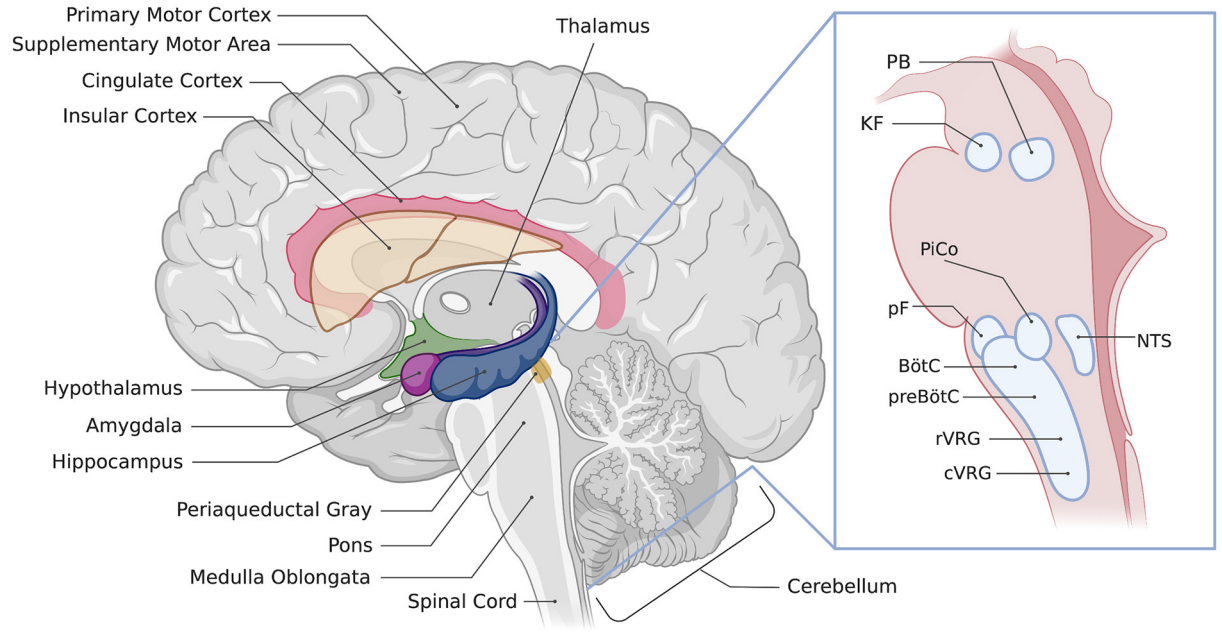

FIGURE 1
Location of regions involved in the control of breathing. Illustration of a sagittal section of the human brain showing the approximate location of respiratory-associated brain regions discussed in the text. The inset image shows brainstem respiratory regions, PAG, periaqueductal gray; PB, parabrachial nuclei; KF, Kölliker-Fuse nucleus; PiCo, post-inspiratory complex; pF, parafacial respiratory groups; NTS, nucleus of the solitary tract, BötC, Bötzinger complex; preBötC, preBötzinger complex; rVRG, rostral ventral respiratory group; cVRG, caudal ventral respiratory group. Created with BioRender.com.

(19, 20). Therefore, forebrain regions connected to the dorsal respiratory group are likely to modify respiratory responses to peripheral sensory information or alter sensory processing.

Pontine respiratory group

The pontine respiratory group is comprised of the Kölliker-Fuse nucleus and parabrachial nuclei. These are a collection of neurochemically diverse structures that are important for coordinating respiratory muscles during breathing and other orofacial behaviors such as vocalizing, coughing, swallowing, and emesis (21–23). The Kölliker-Fuse nucleus patterns respiratory muscle activity during the transition from the inspiratory to expiratory phases, mediates the inspiratory off-switch that triggers glottal closure, and controls upper airway patency (22, 24). The parabrachial nuclei pattern airway and expiratory muscle activity as well as receive chemosensory (i.e., hypoxia and hypercapnia) and mechanosensory information (i.e., negative airway pressure) important for arousal from hypercapnia (25, 26). These areas are likely critical for coordinating airway and respiratory muscles during diverse respiratory-related and orofacial behaviors driven by the forebrain.

Periaqueductal gray

Located in the midbrain, the periaqueductal gray (PAG) serves as an interface between the forebrain and the brainstem to produce integrated behavioral responses to internal or external stressors (e.g., pain or threats) (27). The PAG coordinates respiratory, cardiovascular, and pain responses, as well as plays a part in vocalization, cough, sneeze, swallow, crying, laughter, micturition, arousal, thermoregulation, and sexual behaviors (27–30). As part of the "emotional motor system," a major role of the PAG is likely to regulate breathing in response to emotional challenges and survival programs such as "fight or flight" or freezing responses (30).

The PAG receives inputs from the prefrontal cortex, amygdala, hypothalamus and nociceptive pathways and coordinates respiratory, cardiovascular, motor, and pain responses *via* efferents to the brainstem, forebrain and spinal cord (27, 30). Electrical or chemical stimulation of the PAG changes breathing patterns in rodents (31–33). Importantly, the pattern produced is dependent on which part of the PAG is stimulated. The effects of PAG stimulation on breathing include increasing respiratory frequency (e.g., tachypnea) and inspiratory effort (dorsal PAG, ventral part of lateral PAG), lengthening (e.g., apneusis) or shortening the inspiratory period (lateral PAG), or apnea (ventrolateral PAG) (32). The PAG is

part of a descending system that modulates airway sensory processing, critical for control of breathing and breathing related behaviors, *via* projections to the nucleus of the solitary tract (29). The PAG can also directly control breathing through projections to the preBötzinger complex that modify the activity of pre-inspiratory neurons (32, 34). In addition, the PAG projects to the nucleus retroambiguus, a medullary region important for airway control during breathing, vocalization, and other behaviors (30, 35), as well as the Raphe nuclei and adrenergic nuclei (29). Thus, the PAG relays information from the forebrain to the brainstem to ensure that breathing patterns suit current behavioral or emotional needs such as fleeing predators, freezing, talking, crying, coughing, etc.

Cerebellum

The cerebellum contains nearly half of the neurons in the central nervous system (36) and is a hub for processing information from many regions of the nervous system including the motor cortex, brainstem, and sensory afferents (37, 38). Although not principally a "respiratory region," respiration is among the many motor and non-motor aspects modulated by the cerebellum (39–43). The ventral respiratory group sends numerous projections to the cerebellum and cerebellar deep nuclei send projections back to the ventral respiratory group (44–46), providing evidence that the cerebellum is actively involved in respiratory regulation. The cerebellum also has numerous reciprocal connections to other brainstem and forebrain regions, including those associated with responses to hypercapnia and air hunger (42–44). Additional evidence also suggests that the cerebellum is involved in the response to chemical (hypoxia and hypercapnia), and mechanical (tracheal occlusion and positive pressure breathing) respiratory challenges (41, 43, 45, 47). Although the role of the cerebellum in control of breathing is not fully understood, it's connectivity to both forebrain and brainstem respiratory centers makes it a potential hub for the forebrain to exert control over breathing.

Forebrain control of breathing

The following sections will describe the anatomical and functional evidence supporting a role for each of the following structures in the control of breathing: cerebral cortex, amygdala, bed nucleus of the stria terminalis, hippocampus, hypothalamus, and thalamus (Figure 1).

Cerebral cortex

The cerebral cortex of the brain is responsible for a diverse array of functions including voluntary motor functions, sensory processing, emotional processing, executive functions, attention, perception, memory, language, and cognition (48). Respiration is a motor function that is modified by sensory processes and emotional state, indicating that the cerebral cortex likely plays multiple roles in the control of breathing (49). This section will discuss evidence that different regions of the cerebral cortex likely play different roles in the control of breathing.

Motor cortex

The motor cortex is responsible for selecting, planning, and executing movements. Early evidence for a role of the cortex in breathing came in the late 1950's when it was noted that stimulation of the motor cortex in cats activated the phrenic nerve with a short latency, indicating a possible direct cortico-motoneuronal connection from the motor cortex to the phrenic motor nucleus (50), which was corroborated by various other studies (9, 51, 52). Investigators have suggested that activation of the diaphragm *via* the motor cortex is congruent with similar experiments that activate limb muscles through corticospinal pathways (53). In fact, the diaphragm region of the motor cortex can control forelimb muscles following a phrenic nerve transfer to the forelimb (54). It has since been shown that direct cortico-motoneuronal as well as cortico-reticulospinal and cortico-propriospinal-motor neuron pathways can mediate cortical control of breathing (9, 51, 55) and that these pathways do not involve medullary respiratory regions (9, 52, 56). The primary motor area, premotor area, and the supplementary motor area likely cooperate to modulate breathing. Respiratory linked activity has been observed in all three brain regions through a combination of EEG recordings, transcranial stimulation experiments, and neuroimaging efforts (57–61). Vocalizations require the deliberate and precise modification of respiration, and much of these signals originate in premotor cortices (11, 59, 62–64). The supplementary motor area exhibits a tonic drive to phrenic motoneurons that is thought to play an important role in the wakefulness drive to breathe as well as modulating breathing for speech (59, 63). A better understanding of how the motor cortex exerts volitional and tonic drive to spinal respiratory circuits could lead to new therapies to improve breathing in cases of disease or injury in which respiratory drive is insufficient.

Somatosensory cortex

The somatosensory cortex is activated by respiratory loads as well as low tidal volume, presumably *via* lung and chest wall mechanoreceptors (49). Low tidal volume also activates association motor cortices. Hypercapnia, which is sensed by carotid body and medullary chemoreceptors, does not activate the somatosensory cortex. Intriguingly, human subjects are able to distinguish between different magnitudes of respiratory loads,

but not hypercapnia, consistent with the somatosensory cortex being important for discriminatory processes (49).

Insular and cingulate cortices

The insular and cingulate cortices have gained attention for their role in the sensation of dyspnea, the feeling of being unable to breathe (65–67). Therefore, these regions are particularly responsive to respiratory challenges such as hypercapnia, low tidal volume, and respiratory loads (49, 68–73). Due to their ties to the limbic system (65, 74) these regions also integrate emotional valence to sensory information (67, 71, 75) and likely play a role in generating behavioral responses to uncomfortable respiratory sensations (49).

The extended amygdala: Amygdala and bed nucleus of the stria terminalis

The amygdala is part of the limbic system and evaluates the emotional importance of sensory information to prompt an appropriate response. It is known for its roles in processing fearful or threatening stimuli, reward processing, and stimulating aggressive behavior (5, 7, 76).

The bed nucleus of the stria terminalis (BNST) is a limbic structure adjacent to the amygdala that is also involved in fear and aggressive behaviors (7, 77, 78). We refer to these structures together as the extended amygdala due to their close functional association. This section of the review will discuss what is currently known about the role of the amygdala and the bed nucleus of the stria terminalis in the control of breathing and suggest directions for future research.

The extended amygdala has connections to brainstem respiratory regions

Functional imaging and electroencephalogram (EEG) studies have shown that the amygdala, along with other cortical and limbic brain regions, exhibits a high degree of coordination with the respiratory cycle predominantly within the lateral amygdaloid region (10, 76, 79). However, ablation of the amygdala has no effect on eupnea in rodents (80). Anterograde and retrograde tracing studies demonstrated that there are reciprocal connections between the extended amygdala and the ventral respiratory group (VRG) (44), which could potentially mediate the coordination of activity. In fact, the central nucleus of the amygdala (the output center of the amygdala), has direct, monosynaptic projections to the preBötzinger Complex, as identified by viral tracing experiments (11, 81). Although the central nucleus is predominantly composed of inhibitory GABAergic neurons, it has connections to both excitatory and inhibitory neurons of the preBötzinger Complex (preBötC) and thus is poised to exert a variety of effects on the preBötC, possibly dependent upon emotional states (82–85). These reciprocal connectivity between the extended amygdala and VRG may serve as a substrate to regulate breathing in response to fear/anxiety, regulate fear-related behaviors in response to breathing rhythm, or both. Functional testing of this hypothesis in animal models awaits future studies.

The extended amygdala also has connections to other brain regions important for the control of breathing. Retrograde-tracing studies in rats and mice have shown that both the bed nucleus of the stria terminalis and central nucleus of the amygdala have afferent projections to the nucleus of the solitary tract, which is the viscerosensory tract that bears information from the vagus nerve among others (19, 86). However, since both the nucleus of the solitary tract and extended amygdala have broad functions in autonomic control, it is unclear whether this connection has a role in respiration. The midline apneic site, a medullary brain region related to the raphe nuclei and partially containing the raphe pallidus, receives projections from both the BNST and central nucleus of the amygdala (87). This connection could provide an explanation for the respiratory depression and central apnea that is elicited by stimulation of the central amygdala and bed nucleus of the stria terminalis (see below).

Amygdala stimulation can inhibit breathing

Direct proof that the amygdala can influence breathing comes from animal studies demonstrating that electrical stimulation of the lateral amygdala region can reduce phrenic nerve output and slow ventilation (82). Working with human epilepsy and non-epileptic patients, multiple studies have noted that electrical stimulation of the amygdala can cause apnea (83, 85, 88, 89). The location of this site, called the amygdala inhibition of respiration (AIR) site, has been mapped in pediatric patients (85). Intriguingly, stimulation of the amygdala in excess of 30 s evoked apnea; yet the subjects showed no signs of discomfort (i.e., dyspnea) or arousal (83, 85). For comparison, subjects were unable to voluntarily hold their breath for longer than 20 s without experiencing dyspnea. Interestingly, patients stimulated in the amygdala were still able to breathe by bringing their attention (6, 89). Another interesting finding is that apnea only occurs when the patient is breathing through their nose; apnea did not occur following amygdala stimulation when the patient was instructed to breathe through their mouth (89). This discovery is likely mediated through higher brain regions, as voluntary breathing is known to override amygdala stimulation-induced apnea and cortical structures have a known impact on volitional breathing. These findings may have important implications for sudden death in epilepsy (SUDEP) or sudden infant death syndrome (SIDS) as they suggest a mechanism whereby altered amygdala function could cause prolonged apneas while at the same time inhibiting dyspnea and arousal, leading to death.

The extended amygdala and sudden death in epilepsy

There is a growing body of evidence implicating the extended amygdala in seizure-induced respiratory dysfunction. In several mouse models of epilepsy, induction of seizures can result in central apnea and peri-ictal respiratory depression that can lead to death (80, 90–92). Studies in epileptic patients have shown that seizure spread to the amygdala is correlated with apneas (83, 85, 93). Moreover, electrolytic lesions of the amygdala reduce the occurrence of seizure-induced respiratory arrest in a mouse SUDEP model (80). Together with the studies described above showing that direct stimulation of the amygdala can cause apneas (see: *Amygdala stimulation can inhibit breathing*), these findings suggest that aberrant activity of the amygdala during or following seizures could lead to breathing dysfunction and SUDEP. Currently, it is not clear whether the amygdala directly alters breathing *via* connections to the VRG, NTS and other brainstem structures, and/or alternatively *via* other forebrain or midbrain regions. The bed nucleus of the stria terminalis has also been implicated in seizure-induced respiratory changes as these neurons are activated by seizures in a mouse model of Dravet syndrome (78). The investigators demonstrated that neurons projecting from the bed nucleus of the stria terminalis to the parabrachial nucleus (a pontine structure that regulates breathing) are hypoexcitable in Dravet syndrome mice, suggesting a potential circuit leading to breathing dysfunction and SUDEP (78). Additional studies are warranted to identify and further test which circuits are responsible for seizure-induced breathing abnormalities in different forms or models of epilepsy and how they might lead to SUDEP.

Hippocampus

The hippocampus is a structure located in the archicortex or allocortex and is considered an extension of the temporal lobe of the cerebral cortex although anatomically and functionally distinct (94). Main functions of the hippocampus include, but are not limited to, emotional processing, memory and learning, and spatial navigation. The role that the hippocampus plays in respiration is still not well understood but current evidence will be discussed below.

Lack of evidence for direct connectivity to brainstem respiratory centers

Viral tracing studies in rats demonstrate that the hippocampus receives connections from the nucleus of the solitary tract *via* polysynaptic pathways, suggesting the hippocampus may play a role in processing afferent information from the vagus nerve (95). However, whether the hippocampus receives information from lung afferents has not yet been determined. Diffusion imaging studies in humans provide evidence that there are connections between the hippocampus and the brainstem and cervical spinal cord (96). Electrophysiological studies in rodents have demonstrated sleep-state dependent functional connectivity between the hippocampus and brainstem (97). However, the imaging and electrophysiological studies could not assess the direction of signaling between the brainstem and hippocampus. Moreover, evidence of direct connections from the hippocampus to brainstem respiratory centers is lacking (44, 76, 81, 87, 98). Effects of the hippocampus on breathing are likely to be indirect (i.e., polysynaptic), mediated through another region such as the thalamus, cortex or amygdala, or the connections could be so sparse that they are difficult to label and trace.

Hippocampus stimulation alters breathing

Cells of the hippocampus have also been reported to discharge in phase with respiratory patterns in humans and rodents (10, 84, 99–102). Electrical stimulation of the hippocampus can result in the cessation of breathing (6, 88, 103). Like the amygdala, hippocampal-produced apnea can be overcome by volitional breathing or speech (6). Varying the levels of stimulation intensity or location can result in a spectrum of breathing differences (6). For example, stimulating the hippocampus during expiration can halt expiration or induce a phase switch to inspiration (100). The hippocampus may also play a role in triggering sighs, or "augmented breaths." Sighs are a normal component of breathing driven by the preBötzinger complex that are important for reinflating collapsed alveoli (1), but are also associated with the expression of mood and emotions. Poe et al. (101) noted that hippocampal activity in freely behaving cats increased prior to the initiation of a sigh or the end of an apnea. However, another study demonstrated that electrical stimulation of the ventral hippocampus inhibits sighs (99). Thus, it has been proposed that the hippocampus may play a role in controlling the timing of sighs (99). The ventral region of the hippocampus is a primary region dealing with fear and anxiety. Thus, it seems likely that the hippocampus is part of a limbic circuit that controls emotional aspects of sighing *via* indirect connections to the preBötzinger complex.

Hypothalamus

The hypothalamus is a structure located just above the brainstem with well-known roles in homeostatic regulation: from metabolism and endocrine function to sleep regulation and the circadian rhythm (48, 104). Since the hypothalamus is involved in many autonomic processes, researchers have explored its role in respiration. For a more focused discussion on the role of the hypothalamus in control of breathing, we

recommend Fukushi et al. (105). Here, we first discuss the main neuropeptide hormones produced by the hypothalamus that influence breathing (orexin and vasopressin). We then describe potential roles of the distinct hypothalamic regions in the control of breathing, including: the paraventricular nucleus, perifornical area, dorsomedial region of the hypothalamus, and lateral and posterior hypothalamus.

Neuropeptide hormone signaling by the hypothalamus

The hypothalamus is responsible for the production and release of numerous hormones that regulate a broad variety of autonomic and behavioral functions (106–108). Two in particular, vasopressin and orexin, appear to be involved in respiratory physiology and will be discussed below (109, 110).

Vasopressin, also known as antidiuretic hormone, is generated in the paraventricular and supraoptic nuclei of the hypothalamus by processing of the same pre-pro-hormone that generates neurophysin II and copeptin (109). It is well known for its role in maintaining the balance of water and electrolytes in the kidneys and circulatory system but can also influence other homeostatic functions such as glucose regulation, cardiovascular regulation, and breathing (109). Vasopressin can act as a hormone in the periphery or as a neuropeptide within the central nervous system by binding to one of three different G-protein coupled receptors (V1a, V1b, V2) (109). The receptor most significant for the control of breathing is likely the V1a receptor (V1aR), which is expressed in the lungs, carotid bodies, and circumventricular organs (subfornical organ, area postrema, and organum vasculosum laminae terminalis). Within the brainstem, V1aRs can be found in the rostral ventrolateral medulla, the rostral ventral respiratory column, and the preBötzinger complex, as well as the nucleus of the solitary tract, and the phrenic nuclei (111–113). A link between vasopressin and control of breathing was established by studies showing that the same stimuli that release vasopressin also result in changes to ventilation (114–118). The effects of circulating or central release of vasopressin on breathing can vary by target region, but it is generally inhibitory to breathing (109). Vasopressin is released during physical exercise, and it has been noted that increased levels of vasopressin accompany respiratory disorders such as COPD and pneumonia (119). Moreover, the expression of V1aRs has been shown to change in response to respiratory stresses. For example, hypoxia has been shown to increase the expression of V1aRs in the rostral ventrolateral medulla, the ventral respiratory column, and the phrenic nuclei (113). Additionally, hypercapnia has been shown to activate the vasopressinergic neurons of the paraventricular nucleus in the hypothalamus (120). Thus, vasopressin likely plays a homeostatic role in the control of breathing by modulating the function of multiple brain and spinal cord regions in response to activity or respiratory stress. Although it is not clear why a hormone that is generally inhibitory to breathing is released under conditions of respiratory stress, it has been proposed to play a protective role in preventing hyperventilation (109). Additional research is warranted to better understand the role of vasopressin in control of breathing.

Orexin (also known as hypocretin) is a neuropeptide expressed exclusively in the hypothalamus that acts on G-protein coupled receptors throughout the central nervous system (110, 121, 122). It has two forms (orexin-A and orexin-B) derived from the same precursor protein. The loss of orexinergic neurons leading to narcolepsy, demonstrating its critical role in promoting wakefulness (123, 124). Orexin has also been implicated in regulating aspects of metabolism, homeostasis, reward seeking behavior, and respiration (122, 125). Orexinergic neurons can be found in the perifornical area, dorsomedial hypothalamus, and lateral hypothalamus (110, 121, 126) and have known projections to respiratory regions, including: the preBötzinger complex, nucleus of the solitary tract, Kölliker-Fuse nucleus, parabrachial nuclei, and the phrenic nucleus of the spinal cord (122, 124, 127–132). Orexin neurons are sensitive to CO2, implicating them in chemosensory responses (133, 134). Orexin is likely important for sleep state-dependent regulation of breathing, as its expression is greatest during wakefulness. Further, mice lacking orexin show a 50% decrease in the respiratory response to CO2 during wakefulness, but not during sleep (135), which can be remedied by orexin supplementation (123). Consistent with this data, orexin deficiency can lead to sleep apneas in animal models (123) and decreased orexin levels are found in patients with obstructive sleep apneas (136). At least some of the stimulatory effects of orexin on breathing appear to be mediated through projections to the Kölliker-Fuse nucleus, as injection of orexin-B into this region increases the respiratory frequency in rodent brainstem preparation (137). A better understanding of the role of orexin in sleep state-dependent regulation of breathing and arousal could have important implications for sudden infant death syndrome (SIDS) or sudden death in epilepsy (SUDEP).

Paraventricular nucleus

The paraventricular nucleus (PVN) is predominantly known for its role in the regulation of various autonomic functions including stress responses, metabolism, and reproduction (138–140). The PVN has vast connections to brainstem regions important for respiratory control including the periaqueductal gray, parabrachial nucleus, retrotrapezoid nucleus, nucleus of the solitary tract, preBötzinger complex, and the phrenic nucleus in the spinal cord (105, 139, 141, 142). Experiments in rats and rabbits found that electrical or chemical (glutamate) stimulation of the paraventricular nucleus increases respiration (140, 143). The paraventricular nucleus receives afferent input from other parts of the hypothalamus, as well as the subfornical organ (a chemosensory organ), and the BNST (see: *The extended*

amygdala: amygdala and bed nucleus of the stria terminalis) (144). Inputs from the hippocampus, amygdala, and lateral septum can influence magnocellular neurosecretory cells in the PVN, likely *via* short projections from other parts of the hypothalamus and/or from the BNST. Thus, the PVN may serve as a relay station or integration center for other forebrain regions to influence the brainstem and/or spinal circuits controlling breathing.

Perifornical area

The perifornical area is commonly known for its role in the hypothalamic defense system, which is important for the assessment of threats, and predatory threats in particular (105, 126, 145, 146). The perifornical area is a widely interconnected region, showing projections to the nucleus of the solitary tract, Kölliker-Fuse nucleus, parabrachial nuclei, and periaqueductal gray (121, 123, 147–153). Chemical inhibition of the perifornical area abolishes the respiratory response to stressful auditory and visual stimuli in rats (145). Further, chemical disinhibition of the perifornical area of rats increases respiration (146). Thus, this area is likely to work with limbic structures to drive appropriate respiratory responses to stress and fear.

Dorsomedial hypothalamus

The dorsomedial hypothalamus plays a prominent role in response to stress and arousal (105, 154) and is crucial for the processing of respiratory and other autonomic changes in response to psychological stressors (145, 155). Congruent with its role in stress and arousal, the dorsomedial hypothalamus receives dense projections from the amygdala and bed nucleus of the stria terminalis (126, 156). The dorsomedial hypothalamus is known to send projections to the ventral respiratory column, as well as the periaqueductal gray, nucleus of the solitary tract, and Kölliker-Fuse nucleus/parabrachial nuclei (121, 123, 149, 152, 157–160). Disinhibition of the dorsomedial hypothalamus by injecting bicuculline in rats leads to increased respiratory drive and hyperventilation (161, 162). Working in concert with the perifornical area, these neighboring regions form the center of the hypothalamic defense area that is known to elicit a variety of sympathetic changes including increases in cardiovascular and respiratory activity in response to stress (126, 146, 163).

Lateral hypothalamus

The lateral hypothalamus is classically known as the "feeding center" because of its association with driving the motivation to eat and drink (164, 165) and also plays a role in controlling sleep/wake states (122, 123). Destruction of the lateral hypothalamus or inhibition by barbiturates has been shown to decrease the frequency and depth of ventilation (104). The lateral hypothalamus contains orexinergic neurons and has known projections to the rostral ventral respiratory group (44). The lateral hypothalamus also has a role in central chemosensation as neurons in this region respond to changes in the levels of carbon dioxide (133, 134). This region is also known to receive projections from the preBötzinger Complex (166). Chemosensory activation of the lateral hypothalamus is likely to regulate breathing at least in part *via* release of orexin, which is released maximally during wakefulness (134) (see: *Neuropeptide hormone signaling by the hypothalamus*). This region is thus likely important for sleep/wake state-dependent regulation of breathing.

Posterior hypothalamus

The posterior hypothalamus, also referred to as the caudal hypothalamus, is involved in a variety of behaviors and processes including: cardiovascular regulation, cardiorespiratory responses, locomotion, circadian rhythms, and defense responses (105, 106, 110, 167). This region has strong connections to the periaqueductal gray and medullary respiratory centers (168, 169). The posterior hypothalamus may play a role in the respiratory increase that accompanies movement (i.e., exercise hyperpnea) (105, 155, 170). Prior to and during exercise, both feedforward (central command) and feedback (chemosensory) signaling mechanisms ensure that respiration is able to provide enough oxygen for the increase in metabolic demand. The posterior hypothalamus contains chemosensitive neurons that respond to hypoxia and facilitate respiration as well as GABAergic neurons that modulate the respiratory response to hypercapnia (105, 171, 172). The posterior hypothalamus likely plays a role in the feedforward mechanism of exercise hyperpnea as electrical stimulation or chemical disinhibition are able to induce both increased respiration and spontaneous locomotion (170, 173, 174). These results indicate that the posterior hypothalamus uses multiple mechanisms to ensure that respiratory activity is matched to behavioral and metabolic demands.

Thalamus

The thalamus is a structure of the diencephalon important for relaying sensory information to the cortex and motor information from the cortex to other brain regions (175–178). There are several thalamic nuclei that are known to send direct, monosynaptic projections to the rVRG, namely the parafascicular, mediodorsal, and subparafascicular nuclei (44). The thalamus may also influence respiration indirectly *via* connections to the cerebral cortex, hippocampus, extended amygdala or other brain areas that control breathing.

As different parts of the thalamus play different roles in gating sensory and motor information, it is not surprising that different parts of the thalamus appear to have different effects on breathing. Electrical stimulation of the mediodorsal

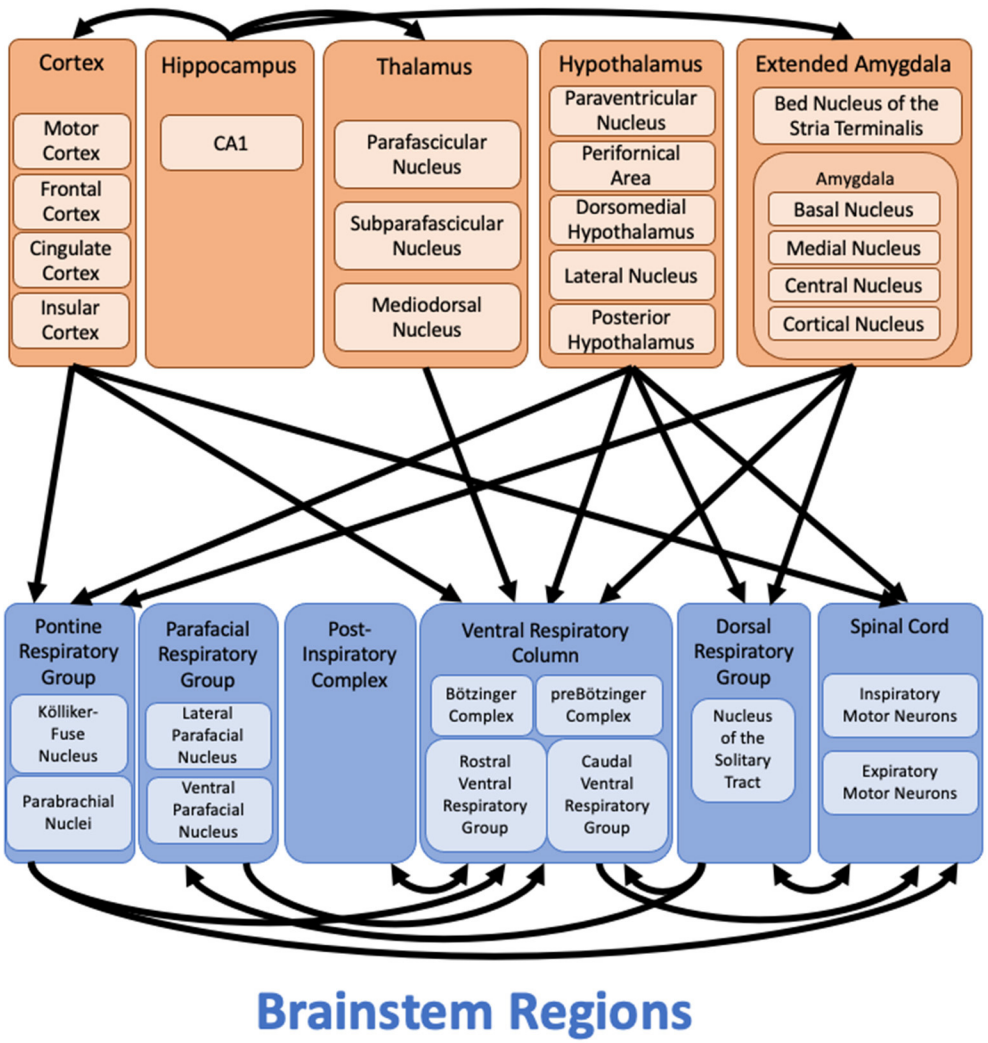

FIGURE 2
Forebrain projections to brainstem respiratory regions. Diagram of known projections from forebrain regions to brainstem respiratory regions as discussed in the text. Connections from the brainstem to forebrain regions are not shown. Connections from the hippocampus to other forebrain regions highlight potential pathways through which the hippocampus might influence respiration in lieu of known direct projections to brainstem respiratory regions. The extensive connections between other forebrain regions are not shown. Important connections within the brainstem highlight additional pathways through which the forebrain might influence breathing, even in the absence of direct connections to specific brainstem respiratory regions. Additional regions, such as the cerebellum and periaqueductal gray (not shown), may relay information from the forebrain to the brainstem.

nucleus of the thalamus in cats can increase respiratory rate (179). However, electrical stimulation of the parafascicular nucleus of the thalamus in fetal sheep reduces respiratory frequency (180). Consistent with this finding, lesions to the posteromedial thalamus, and particularly the parafascicular nuclei, abolished the normal response of the fetus to hypoxic conditions (181, 182). This region of the thalamus is involved with sleep regulation postnatally (181), but its involvement in sleep-disordered breathing is currently unclear. In a rat model for obstructive sleep apnea, rats exposed to chronic intermittent hypoxia revealed increased *c-fos* expression in the paraventricular thalamus (183). In this situation, the increase in neuronal activity indicated by the increase in *c-fos* expression suggests that the thalamus plays an important role in the stress response as it relays information to the prefrontal and insular cortices. Additional research is needed to better understand how sensory (or chemosensory) information is gated by the thalamus as well as how

respiratory motor output may be processed for the control of breathing.

Conclusions

Our brain uses a variety of feedback, feedforward, and homeostatic mechanisms to ensure that our breathing is appropriately matched to our physiological, emotional, and behavioral state. We propose that the forebrain regions reviewed here (the cerebral cortex, extended amygdala, hippocampus, hypothalamus, and thalamus) contribute to the regulation of breathing for this purpose. These forebrain regions have multiple connections to each other, as well as direct or indirect connections to brainstem regions known to be important for the control of breathing (Figure 2). For each region, there is functional evidence that they play a role in the control of breathing, at least during certain behaviors, conditions, or physiological states. Further research is necessary to elucidate the roles of these forebrain structures in the control of breathing under different conditions as well as the specific circuits and mechanisms involved.

Author contributions

KS and SC conceived, designed, and wrote the manuscript. KS designed and generated the figures. SC edited the figures and manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Perspectives on the basis of seizure-induced respiratory dysfunction

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Epilepsy is an umbrella term used to define a wide variety of seizure disorders and sudden unexpected death in epilepsy (SUDEP) is the leading cause of death in epilepsy. Although some SUDEP risk factors have been identified, it remains largely unpredictable, and underlying mechanisms remain poorly understood. Most seizures start in the cortex, but the high mortality rate associated with certain types of epilepsy indicates brainstem involvement. Therefore, to help understand SUDEP we discuss mechanisms by which seizure activity propagates to the brainstem. Specifically, we highlight clinical and pre-clinical evidence suggesting how seizure activation of: (i) descending inhibitory drive or (ii) spreading depolarization might contribute to brainstem dysfunction. Furthermore, since epilepsy is a highly heterogenous disorder, we also considered factors expected to favor or oppose mechanisms of seizure propagation. We also consider whether epilepsy-associated genetic variants directly impact brainstem function. Because respiratory failure is a leading cause of SUDEP, our discussion of brainstem dysfunction focuses on respiratory control.

KEYWORDS

cortical-brainstem connectivity, seizure propagation, spreading depolarization (SD), SUDEP (sudden unexpected death in epilepsy), apnea

Introduction

Epilepsy is a chronic disease associated with uncontrolled brain activity that results in recurrent seizures. Approximately 50 million people globally have epilepsy and people with this condition have a two-three-fold higher mortality rate than the general public. Sudden unexpected death in epilepsy (SUDEP)- defined as death in people with epilepsy that are not caused by injury, drowning, or other known reasons- is a leading cause of death in epilepsy patients (Pathak et al., 2022) and is second only to stroke in years of potential life lost to neurological disease, thus making SUDEP a significant public health problem (Thurman et al., 2014). Despite their potential lethality, most seizures are not fatal, and so a frequent question posed by family members of SUDEP victims

is "what was it about that [final] seizure that resulted in death"[1]? Considering seizures typically originate in the cortex and lethality involves disruption of autonomic (Thijs et al., 2021) and respiratory (Teran et al., 2022) function at the level of the brainstem, to address this question, we discuss two likely mechanisms by which cortical seizure activity propagates to the brainstem. We also consider whether the expression of epilepsy-associated genes in the brainstem contributes to epilepsy-associated cardiorespiratory dysfunction (**Figure 1**). It is our contention that SUDEP is a heterogenous process involving different mechanisms depending on the underlying cause of seizure activity. Highlighted here are what we consider the most likely mechanisms by which cortical seizure activity might propagate to the brainstem; however, it is also important to recognize that other regions and polysynaptic pathways may contribute to descending seizure propagation.

I. Descending seizure propagation through synaptic connectivity

Essential components of the respiratory circuit are located in the brainstem and include inspiratory rhythmogenic neurons in the pre-bötzinger complex (pre-bötC; Smith et al., 1991), neurons in the retrotrapezoid nucleus (RTN; Mulkey et al., 2004) and medullary raphe (Richerson, 2004; Ray et al., 2011) that regulate breathing in response to changes in CO_2/H^+ (i.e., function as respiratory chemoreceptors), parabrachial neurons that modulate inspiratory-expiratory phase transitions and integrate chemoreceptor, visceral and arousal information between the forebrain and brainstem (Kaur and Saper, 2019), and respiratory motor neurons that serve as the final common output for the respiratory system (Fogarty et al., 2018). These respiratory centers also receive input from suprapontine regions including the insular cortex (McKay et al., 2003), hippocampus (Harper et al., 1998), and amygdala (Feinstein et al., 2022); in humans, stimulation of these regions results in cessation of breathing (Ochoa-Urrea et al., 2020), presumably to allow for voluntary and emotional control of ventilation (Bondarenko et al., 2014; Ashhad et al., 2022). Evidence also suggests communication between brainstem respiratory centers and suprapontine structures is bidirectional; ascending respiratory activity entrains cortical and limbic network oscillations that are thought to be important for emotion and memory consolidation (Herrero et al., 2018; Karalis and Sirota, 2022). Furthermore, brainstem projections from the reticular formation to the thalamus and cortex *via* the reticular activating system modulate sleep-wake transitions and arousal (Kovalzon, 2016). In the context of epilepsy, cortical-brainstem connections provide an anatomical substrate for cortical seizure propagation to the brainstem, and as such, have long been implicated in seizure-induced cardiorespiratory dysfunction (Frysinger and Harper, 1990).

The amygdala stands out as a hub of the so-called brainstem-homeostatic forebrain connectome (Edlow et al., 2016). This region is located in the temporal lobe and sends extensive inhibitory projections to brainstem respiratory centers where it is thought to regulate fear-related respiratory responses (Nardi et al., 2009; Feinstein et al., 2022), particularly to external perceived threats but not necessarily interoceptive threats (Feinstein et al., 2013; for review see Feinstein et al., 2022). The amygdala is also highly susceptible to seizure activity (Aroniadou-Anderjaska et al., 2008), and animal models (Totola et al., 2019), as well as clinical work from pediatric (Rhone et al., 2020) and adult (Dlouhy et al., 2015; Lacuey et al., 2017; Nobis et al., 2018) epilepsy patients, showed that stimulation or seizure activation could elicit apnea. Consistent with its lack of involvement in interoceptive threats (Feinstein et al., 2022), amygdala-evoked apneas did not occur in conjunction with dyspnea (Dlouhy et al., 2015). Curiously, amygdala-evoked apneas are dependent on attention and nasal breathing (Nobis et al., 2018). This is interesting because cortical respiratory rhythms also depend on nasal breathing (Zelano et al., 2016), suggesting there is a hierarchical organization to cortical-brainstem communication where conscious effort through attention or elicited by mouth breathing can override coordinated activity between regions. This also suggests interventions that facilitate mouth breathing might limit seizure-induced apnea.

It should be noted that the amygdala is composed of multiple sub-nuclei and only a subset of which contribute to the respiratory activity. For example, stimulation of the basolateral, basomedial, and central regions consistently resulted in apnea, whereas stimulation of the more lateral amygdala failed to affect breathing (Rhone et al., 2020). Therefore, not all amygdala seizures result in apnea (Park et al., 2020). It is also worth mentioning that amygdala stimulation elicited apnea even during sleep (Nobis et al., 2018) when SUDEP occurs most frequently (Nobili et al., 2011). By contrast, another putative SUDEP mechanism, namely spreading depolarization (SD), is less likely to be favored during sleep (see next section below). In any case, these mechanisms are not mutually exclusive, but rather may occur simultaneously and in a synergistic manner. For example, SD will result in high extracellular potassium ($[K^+]o$) and this has been shown to facilitate excitatory more so than inhibitory synaptic

1 Partners Against Mortality in Epilepsy (PAME) hosts an annual meeting that brings together health care providers, basic scientists and families touched by epilepsy with the goal of improving our understanding of SUDEP. During previous PAME meetings we had the opportunity to talk with family members that lost a loved one to SUDEP. These interactions made a lasting impact on our perception of this disease and continue to motivate our work in this field.

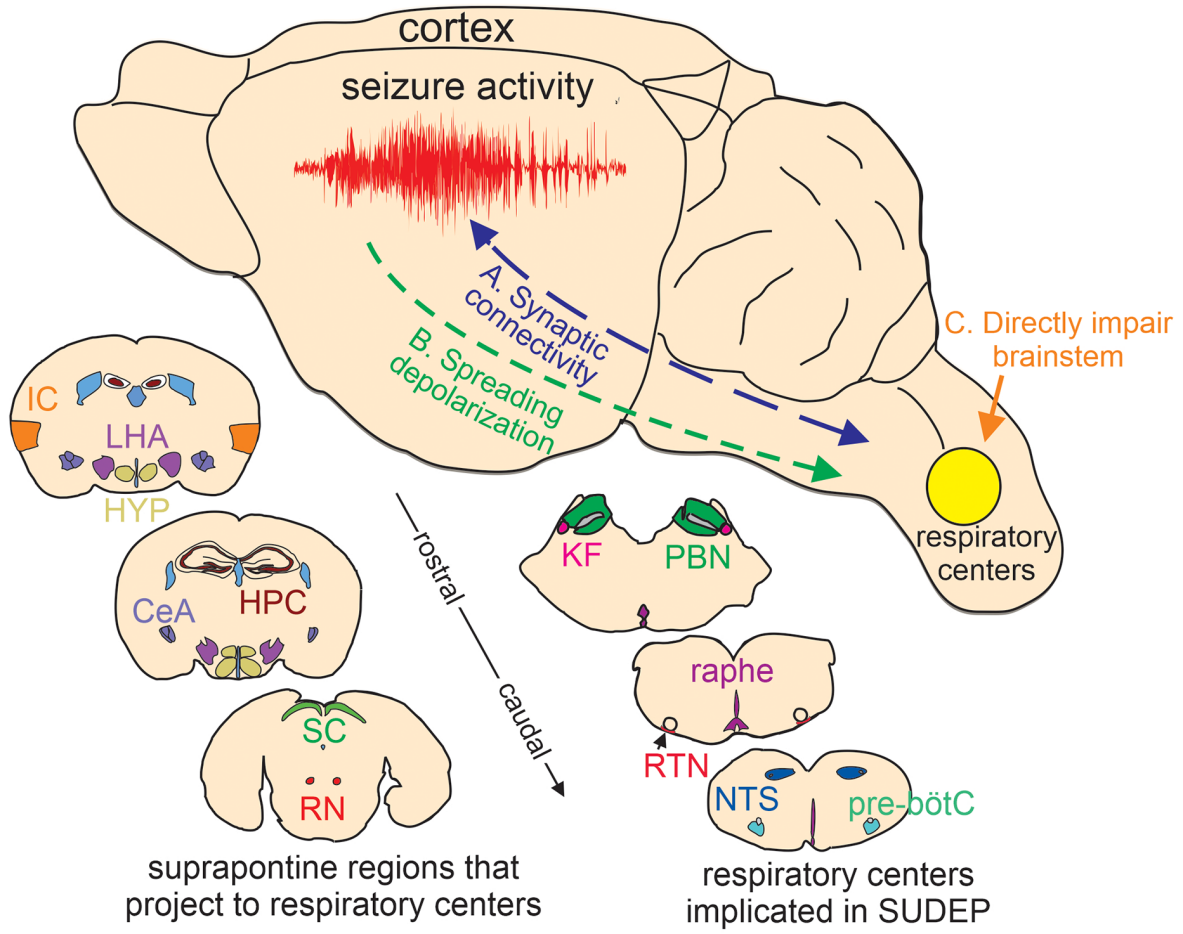

FIGURE 1
Putative basis of brainstem dysfunction in epilepsy. **(A)** Cortical-brainstem connectivity provides an anatomical basis for seizure propagation to the brainstem. Likewise, brainstem-cortical projections have been shown to synchronize cortical network activity and so may influence seizure propensity. **(B)** Spreading depolarization can propagate through contiguous tissue and if it reaches the brainstem will likely result in respiratory failure. **(C)** certain epilepsy-associated ion channel mutations are expressed in the brainstem where they directly impair respiratory function. Lower insets, cartoon coronal sections show the relative location of suprapontine regions that project to brainstem respiratory centers implicated in SUDEP (Harper et al., 1998; McKay et al., 2003; Rosin et al., 2006; Yang et al., 2020; Trevizan-Baú et al., 2021; Feinstein et al., 2022; Leitner et al., 2022). IC, insular cortex; LHA, lateral hypothalamus; HYP, hypothalamus; CeA, central amygdala nucleus; SC, superior colliculus; RN, red nucleus; KF, kölliker-fuse; PBN, parabrachial nuclei; raphe, medullary raphe nucleus; RTN, retrotrapezoid nucleus; NTS, nucleus of the solitary tract; pre-bötC, pre-bötzinger complex. Note, that the medullary raphe is part of the reticular activating system.

transmission (Rasmussen et al., 2020), thus promoting synaptic seizure propagation. Likewise, excitatory synaptic transmission has been shown to facilitate SD in a seizure-related mouse model of familial hemiplegic migraine type-1 (Tottene et al., 2009). Furthermore, SUDEP-prone mouse lines showed cortical epileptic activity that correlated with abnormal brainstem electroencephalographic (EEG) oscillations and suppression of brainstem activity (Gu et al., 2022), suggesting cortical-brainstem connectivity facilitated SD propagation to the brainstem.

In addition to individual ictal events causing cardiorespiratory arrest as described above, it is also possible that repeated cortical seizures cause maladaptive changes to the forebrain and brainstem respiratory circuity that render the respiratory system vulnerable to failure. In this case, epilepsy patients are expected to exhibit background cardiorespiratory abnormalities. Indeed, interictal cardiorespiratory problems are common in epilepsy (Barot and Nei, 2019). Typical background autonomic and respiratory symptoms exhibited by Dravet syndrome patients and partly recapitulated in animal models of this disease include diminished heart rate variability, bradycardia, and hypoventilation with increased apnea (Delogu et al., 2011; Kim et al., 2018). The possibility the brainstem is disrupted in epilepsy is also supported by anatomical evidence showing patients with focal epilepsy have diminished brainstem volume (Mueller et al., 2018) including loss of both neurons (Patodia et al., 2021) and astrocytes (Patodia et al., 2019) in respiratory control centers. Evidence

also suggests that seizure activity can alter synaptic connectivity between cortical and brainstem structures, and such circuit level changes may alter brainstem function and perpetuate excitotoxicity-related damage (Armada-Moreira et al., 2020). For example, amygdala neurons located ipsilateral to the temporal lobe seizure foci exhibited a stronger convergence of cardiorespiratory information compared to neurons on the contralateral side (Frysinger and Harper, 1990), thus indicating seizure activity enhanced excitatory coupling between cortical and brainstem respiratory areas. Nevertheless, the possibility that altered cortex-brainstem connectivity contributes to brainstem dysfunction or cell death remains unknown. From a bottom-up perspective, it is conceivable that enhanced respiratory-driven oscillations in cortical regions might increase neural network synchronization and seizure propensity.

It has also been speculated that seizure-induced amygdala-dependent apnea is independent of the underlying cause or type of epilepsy (Rhone et al., 2020). However, considering the central nucleus of the amygdala provides a primary output of this region to the brainstem (Feinstein et al., 2022) and since neurons in this region are mostly inhibitory and suppress breathing presumably by synaptic inhibition, it seems unlikely such a mechanism would be effective in forms of epilepsy associated with loss of inhibitory tone. Consistent with this, amygdala neurons that make monosynaptic projections to the parabrachial pneumotaxic center are hypoexcitable in a mouse model of Dravet syndrome (Yan et al., 2021). Also, inhibitory neurons in the amygdala appear prone to seizure-induced damage (Tuunanen et al., 1996), which conceivably will favor epileptogenesis in the amygdala and nearby hippocampus but is less likely to increase inhibitory bombardment of brainstem respiratory centers.

In sum, seizure activation of cortical to brainstem inhibitory projections can disrupt cardiorespiratory function and contribute to SUDEP (**Figure 2**). Repeated activation of cortical-brainstem circuits may also alter network connectivity in maladaptive ways that compromise cardiorespiratory control, favor seizure propagation to the brainstem or increase synchronized cortical activity to increase seizure propensity.

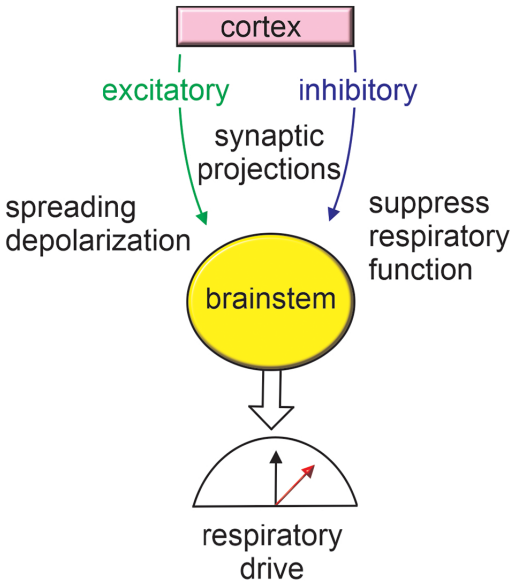

FIGURE 2
Cortex-brainstem connections provide a framework for seizure-induced respiratory failure. Seizure activation of inhibitory cortical projections to brainstem respiratory centers has been shown to result in respiratory arrest. Seizure activity may also stimulate excitatory brainstem projections thereby resulting in metabolic stress and favoring the propagation of spreading depolarization. For more information on anatomical regions refer to **Figure 1**.

II. Spreading depolarization

SD is a pathological event associated with migraine headache, ischemic, or traumatic brain injury and epilepsy (for reviews see Pietrobon and Moskowitz, 2014; Cozzolino et al., 2018). It is thought to be triggered by a severe depolarization that leads to large-scale loss of ion and transmitter homeostasis. In particular, a pronounced increase in $[K^+]o$ and glutamate can initiate a self-propagating wave of depolarization and cytotoxic edema (Hinzman et al., 2016; Hubel et al., 2017). The ability of such a wave to propagate into and through contiguous tissue is strongly influenced by ongoing neural activity and metabolic status (Aiba and Noebels, 2015). SD is followed shortly thereafter by a wave of neural inactivation (presumably caused by depolarizing block) that results in depression of EEG activity as frequently observed following generalized tonic-clonic seizures (GTCS; Surges et al., 2011). In the cortex, this so-called spreading depression may serve a protective role by limiting further seizure activity (Tamim et al., 2021). However, if such an event were to occur in the brainstem it is expected to have a negative impact on cardiorespiratory control. Consistent with this, GTCS are the most common type of seizure associated with SUDEP (Ryvlin et al., 2019), and mechanisms underlying GTCS are thought to involve dysregulation of the ascending reticular activating system and descending reticulospinal projections to result in characteristic features of GTCS including loss of consciousness and muscle convulsions (Sedigh-Sarvestani et al., 2015).

In epilepsy patients it is unclear whether post-ictal EEG suppression is an independent risk factor SUDEP (Ryvlin et al., 2019); however, pre-clinical experiments using monogenic SUDEP models showed that brainstem SD correlated with cardiorespiratory failure and mortality. For example, cortical-evoked seizures in two SUDEP mouse models (Kv1.1 null and *Scn1a*$^{R1407X/+}$ loss of function) resulted in brainstem SD and cardiorespiratory arrest (Aiba and Noebels, 2015). Similar results were also observed in mice expressing a $Ca_v2.1$ gain of function mutation associated with familial hemiplegic migraine type 1

(*Cacna1a*$^{S218L/+}$; Jansen et al., 2019) and mice expressing a ryanodine receptor-2 gain of function mutation associated with catecholaminergic polymorphic ventricular tachycardia (Aiba et al., 2016). Also, in *Cacna1a*$^{S218L/+}$ mice the superior colliculus, a midbrain structure that receives input from both the cortex and brainstem, was particularly effective at propagating seizure-induced SD to the brainstem (Cain et al., 2022), suggesting anatomical connectivity may facilitate SD.

Interestingly, expression of certain epilepsy-associated channel variants in the cortex but not the brainstem can cause cortical seizures and SD but in the absence of increased mortality. For example, Kcnq2 channels produce a subthreshold K^+ conductance (Abbott, 2020) and loss of *Kcnq2* function is associated with neonatal epileptic encephalopathy (Orhan et al., 2014; Kim H. J. et al., 2021). Conditional deletion of *Kcnq2* from forebrain excitatory neurons (*Emx1*$^{Cre/+}$::*Kcnq2*$^{f/f}$; *Kcnq2* cKO) resulted in cortical seizures and SD. Despite this, only a small subset of these animals died prematurely (Aiba and Noebels, 2021). However, a caveat to these experiments is that *Emx1*$^{cre/+}$ is not restricted to cortical excitatory neurons but rather is also expressed by peripheral autonomic ganglia that provide modulatory feedback to cardiorespiratory centers (Ning et al., 2022). Excluding potential confounding effects of peripheral nerves, these results suggest compromised brainstem function is required for cortical seizure- or SD-induced respiratory arrest and premature death (see Section "III. Direct effect of epilepsy associated mutations on brainstem function" below for more detail).

The initiation phase of SD is dependent on the concentration of ions and transmitters in the extracellular space which are themselves inversely related to extracellular volume (ECV). Also, ECV increases during sleep (Ding et al., 2016), therefore, we speculate the threshold for SD induction will be higher during sleep when SUDEP is thought to occur most frequently (Nobili et al., 2011). Consistent with this, spontaneous cortical SD in *Kcnq2* cKO mice (Aiba and Noebels, 2021), as well as seizure-induced death in Kv1.1 null mice (Moore et al., 2014) and *Scn1a*$^{R1407X/+}$ mice (Teran et al., 2019), occurred primarily during the dark/active state when ECV is expected to be minimal and the impact of $[K^+]o$ on neural activity is most favored (Ding et al., 2016). Therefore, these results are consistent with the involvement of high $[K^+]o$ as a key determinant of SD threshold and propagation. However, these results are not consistent with clinical evidence suggesting SUDEP occurs primarily during sleep (Buchanan et al., 2021). Note that once SD has been initiated the corresponding cellular edema is expected to negate this issue; thus, SD propagation is not expected to be sleep-wake state dependent.

Seizure events can deplete energy availability, thus limiting ion and transmitter buffer capacity and lowering SD induction threshold (Major et al., 2020). Consistent with this, Kv1.1 null and *Scn1a*$^{R1407X/+}$ models showed a low threshold for SD elicited by metabolic stress. Furthermore, repeated seizures may facilitate pathological remodeling that can lead to progressively more severe outcomes. For example, mice that express the *Scn1a* loss of function variant R1648H exhibit a mild/asymptomatic phenotype under resting conditions that can be transformed into a severe phenotype by subjecting the mice to heat- or chemoconvulsant-induced seizures (Dutton et al., 2017; Salgueiro-Pereira et al., 2019). This study also showed that wild type mice subjected to the same seizure induction protocol did not develop a severe seizure phenotype. These results suggest loss of *Scn1a* function lowered the seizure threshold, as expected, and is required for remodeling following repeated seizures that can lead to severe phenotypes and SUDEP. However, contrary to this, early work with chemoconvulsant models of epilepsy suggests frequent seizures can confer resistance to SD. For example, a pentylenetetrazol rat model of epilepsy showed that frequent seizures increased the SD threshold (Koroleva et al., 1993). Furthermore, patients with chronic epilepsy and pilocarpine-treated rats exhibited a similar high $[K^+]o$ threshold for SD (Maslarova et al., 2011). Based on this, it was speculated that chronic seizures promote a compensatory increase in $[K^+]o$ buffering capacity.

A critical function of astrocytes is to regulate extracellular ion and transmitter homeostasis and as such are important determinants of $[K^+]o$ buffering. The dynamics of $[K^+]o$ are complex and depend on several factors. Here, we focus on inward rectifying Kir4.1 channels because these are the main determinant of astrocyte resting membrane potential (by K^+ efflux) and can serve as a conduit for K^+ uptake when the reversal potential for K^+ is depolarized to resting membrane potential as can occur during increased neural activity. Glutamate uptake by astrocytes is also an electrogenic process favored at more negative membrane potentials (Grewer and Rauen, 2005). Astrocytes are also highly sensitive to seizure activity and in chronic epilepsy these cells are known to proliferate (gliosis) and transition into a pro-inflammatory state (so-called reactive astrocyte) characterized by the release of cytokines and growth factors that can increase seizure propensity or promote tissue repair (for review see Wetherington et al., 2008; Verhoog et al., 2020). Although there is some evidence suggesting Kir4.1 expression increased in a pilocarpine model of temporal lobe epilepsy (Nagao et al., 2013) and a mouse model of Dravet syndrome (Miljanovic et al., 2021), most studies suggest the opposite, that astrocyte Kir4.1 channel expression is diminished in epilepsy (Kinboshi et al., 2020; Ohno et al., 2021). Indeed, loss of function variations (missense and nonsense mutations) in *KCNJ10* (the gene encoding Kir4.1) causes an epileptic disorder known as EAST/SeSAME syndrome (Bockenhauer et al., 2009) characterized by early onset tonic-clonic seizures, sensorineural deafness, ataxia, intellectual disability, and electrolyte imbalance. Also, astrocyte Kir4.1 expression or function has been shown to be reduced in humans (Heuser et al., 2012; Steinhauser et al., 2012; Kitaura et al., 2018) and various animal models of epilepsy

(Harada et al., 2013) including DBA/2 model of audiogenic seizures (Inyushin et al., 2010). Note that Kir4.1 channels also contribute to K^+ buffering by oligodendrocytes, and loss of oligodendrocyte Kir4.1 channels also increases seizure activity (Larson et al., 2018). Also, loss of serotonergic signaling by raphe neurons contributed to seizure propensity and respiratory arrest in DBA/2 mice (Cervo et al., 2005). This is interesting because Kir4.1 channels can heteromerize with Kir5.1 to form a CO_2/H^+ sensitive conductance (Xu et al., 2000), and recent work showed that both Kir4.1 and Kir5.1 transcript are expressed by medullary serotonergic raphe neurons and so may contribute to CO_2/H^+ detection by these putative chemoreceptors (Puissant et al., 2017). Moreover, loss of Kir5.1 (*Kcnj16* gene) resulted in an audiogenic seizure phenotype with increased mortality in a rat model of salt-sensitive hypertension and chronic kidney disease (Manis et al., 2021), probably by a mechanism involving disruption of heteromeric Kir4.1/5.1 channels since Kir5.1 does not form functional homomeric channels (Pessia et al., 1996). Therefore, disruption of homo or heteromeric Kir4.1 channels may be a common substrate for breathing problems and seizure propensity.

As expected, astrocyte-specific deletion of *Kcnj10* also disrupted K^+ and glutamate uptake and resulted in increased seizure activity and premature death (Djukic et al., 2007). Kir4.1 channels also colocalize with aquaporin-4 (Aqp4) water channels (Nagelhus et al., 2004) and Aqp4 knockout mice exhibited slowed $[K^+]o$ clearance (Amiry-Moghaddam et al., 2003) and longer duration seizures following neural stimulation (Binder et al., 2006). Therefore, disruption of Kir4.1 may impact Aqp4 function and consequently regulation of cell size and ECV. This may be important because the ability of astrocytes to influence neural activity by paracrine signaling or regulation of extracellular ions and transmitters is proximity-dependent and inversely related to ECV (Murphy et al., 2017).

It is also possible that the loss of Kir4.1 containing channels will facilitate the release of various neuroactive signaling molecules from astrocytes. In particular, inhibition of Kir4.1 chanenls in cultured astrocytes increased expression of brain-derived neurotrophic factor (BDNF; Kinboshi et al., 2017), a growth factor that signals through TrkB receptors to regulate neural growth, differentiation, and synaptic plasticity (Cowley et al., 1994; Meakin et al., 1999; Huang and Reichardt, 2003). This is of interest because BDNF is a potent modulator of epileptogenesis; BDNF expression reportedly increased in the brains of epileptic patients and animal models of epilepsy (Jankowsky and Patterson, 2001), whereas disruption of BDNF/TrkB signaling suppressed seizure activity in epileptic mouse models (Kokaia et al., 1995; Hagihara et al., 2005; Liu et al., 2013). Although the link between the loss of Kir4.1 and increased BDNF expression remains murky, pharmacological evidence implicates activation of the MAPK/ERK pathway (Kinboshi et al., 2017), possibly in response to the depolarization-induced increase in intracellular Ca^{2+}. Together, these results suggest seizure-induced changes in astrocyte Kir4.1 expression is maladaptive and likely to contribute to epileptogenesis.

It is also important to recognize that increased Kir4.1 expression will not necessarily diminish seizure propensity. For example, *KCNJ10* gain of function variants that result in increased channel expression (p.R18Q), diminish proton-dependent inhibition (p.R348H) or increased channel conductance (p.V84M) are also associated with seizure-like behavior (Sicca et al., 2011, 2016). Mechanistically, it is hard to imagine how increased Kir4.1 channel function in astrocytes might promote seizures. One possibility is that increased Kir4.1 channel activity may increase $[K^+]o$ buffering kinetics, thereby limiting $[K^+]o$ build-up during increased activity. This mechanism may minimize depolarization-induced Na^+ channel inactivation and allow neurons to fire at higher frequencies for longer periods of time (Niday and Tzingounis, 2018). Note that increased Kir4.1 expression is not expected to substantially decrease $[K^+]o$ because a prerequisite for K^+ uptake by astrocytes is high $[K^+]o$ and a depolarized K^+ reversal potential relative to resting membrane potential. As such, decreasing $[K^+]o$ will favor K^+ efflux.

Another interesting mechanism by which increased Kir4.1 might favor seizure activity involves dysregulation of brain pH. Astrocytes express high levels of the electrogenic sodium bicarbonate cotransporter (NBC; Turovsky et al., 2016). The most common NBC isoform expressed by astrocytes has 1 Na^+: 2 HCO_3^- stoichiometry and a predicted reversal potential of around −100 mV (Mulkey and Wenker, 2011). This value is negative to astrocyte resting membrane potential, thus under normal conditions HCO_3^- flux through the NBC is directed inward (Mulkey and Wenker, 2011). If this is the case, then increased expression of Kir4.1 is expected to hyperpolarize astrocyte membrane potential and decrease electrogenic HCO_3^- transport, thereby resulting in extracellular alkalosis. This is significant because just 0.2 pH unit increase in extracellular pH can cause seizures (Schuchmann et al., 2006).

In sum, there is no doubt that SD, once initiated, can have profound effects on neural activity, and preclinical studies clearly implicate SD as a cause of seizure-induced mortality. However, the correlation between postictal generalized EEG suppression (PGES), which presumably also reflects SD, and SUDEP is a matter of debate in the literature. Some studies suggest there is a correlation between PGES and SUDEP (Lhatoo et al., 2010; Moseley and DeGiorgio, 2015), whereas other studies found PGES duration is not a risk factor for SUDEP (Surges et al., 2011; Lamberts et al., 2013). Also, sleep-wake changes in ECV are not expected to favor the initiation of SD during sleep when SUDEP usually occurs. Furthermore, although chronic seizure activity may result in compensatory cellular responses to limit SD, such adaptations are not likely to involve increased astrocyte Kir4.1 expression since most evidence indicates loss of this channel in epilepsy. For this same

reason, astrocyte Kir4.1 channels may have some therapeutic potential in treating epilepsy, possibly by limiting SD. Consistent with this, certain antiepileptic drugs have been shown to stimulate Kir4.1 expression (Mukai et al., 2018).

III. Direct effect of epilepsy associated mutations on brainstem function

In addition to promoting seizure activity in the cortex, epilepsy-associated genetic mutations may also be expressed in the brainstem (Kuo et al., 2019) where they increase SUDEP risk in seizure-dependent and -independent manners (**Figure 3**). For example, as noted above *Emx1*$^{cre/+}$::*Kcnq2*$^{f/f}$ mice (*Kcnq2* cKO) showed spontaneous cortical seizures with SD but they did not die prematurely (Aiba and Noebels, 2021), suggesting the brainstem was protected from SD infiltration. Another study used a similar approach (*Emx1*$^{Cre/+}$) to express a dominant negative *Kcnq2* variant (M547V) in forebrain pyramidal neurons (but with some off-target expression in astrocytes) of *Kcnq2* heterozygous knockout mice (C57BL/6 background); unlike *Kcnq2* cKO animals, these mice showed a severe phenotype including seizures and premature death (Kim E. C. et al., 2021). In this case, global *Kcnq2* haploinsufficiency appears sufficient to allow cortical seizures to disrupt brainstem function and result in mortality.

The possibility that brainstem expression of epilepsy-associated mutations increases the risk of mortality is supported by evidence that SUDEP can occur without overt seizure activity (Lhatoo et al., 2016). Pre-clinical animal experiments also support this possibility. For example, polymorphisms associated with DBA/2 mice (a common model of audiogenic seizures; De Sarro et al., 2017) appear to disrupt brainstem serotonergic signaling and contribute to seizure-induced cardiorespiratory failure. Specifically, DBA/2 mice express a single amino acid substitution in the gene encoding tryptophan hydroxylase-2 that results in limited serotonin production (Cervo et al., 2005), and this likely contributes to seizure-induced respiratory arrest since the systemic application of serotonin reuptake inhibitors improved seizure activity and related apneic events in DBA/2 mice (Faingold et al., 2014). DBA/2 mice also express a *Kcnj10* loss of function mutation that has been shown to disrupt Kir4.1-dependent maintenance of extracellular K^+ and glutamate (Ferraro et al., 2004; Inyushin et al., 2010) and thus lower seizure threshold (**Figure 2**). It is also worth noting that Kir4.1 channels together with Kir5.1 may contribute to CO_2/H^+ chemosensation by serotonergic neurons (Puissant et al., 2017), thus loss of Kir4.1 could further compromise raphe chemoreception and worsen seizure-induced respiratory problems.

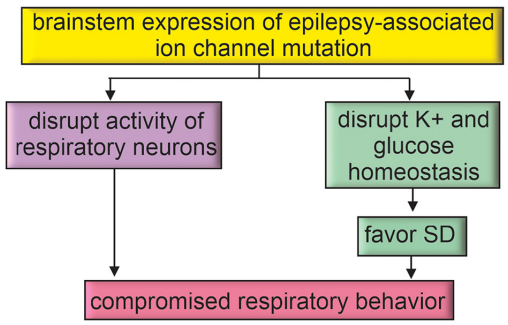

FIGURE 3
Epilepsy-associated genes may directly disrupt brainstem function. Brainstem expression of epilepsy-associated ion channel mutations may directly impact respiratory function or favor mechanisms for SD propagation to the brainstem.

The retrotrapezoid nucleus (RTN) is another important respiratory chemoreceptor region implicated in SUDEP (Patodia et al., 2018). For example, in the context of Dravet syndrome (caused by loss of function mutations in *SCN1A*), we showed that *Scn1a* transcript is expressed by inhibitory parafacial neurons in the region of the RTN (Kuo et al., 2019). We also showed that inhibitory somatostatin (SST)-expressing neurons in the region of the RTN are inhibited by CO_2/H^+ and contribute to RTN chemoreception by disinhibition of CO_2/H^+-activated glutamatergic neurons (i.e., RTN chemoreceptors; Cleary et al., 2021). Therefore, in addition to causing cortical seizure activity, Dravet syndrome-associated *Scn1a* mutations may disrupt the inhibitory modulation of RTN chemoreception. Consistent with this, inhibitory neurons in the region of the RTN in slices from mice that express a loss of function *Scn1a* mutation (A1783V) conditionally in inhibitory neurons under the vesicular GABA transporter promoter (*Slc32a*$^{1cre/+}$::*Scn1a*$^{A1783V\ fl/+}$) showed lower basal activity compared to control cells and fired fewer action potentials in response to depolarizing current steps (Kuo et al., 2019). Consistent with a disinhibitory mechanism, chemosensitive RTN neurons in slices from *Slc32a*$^{1cre/+}$::*Scn1a*$^{A1783V\ fl/+}$ mice showed increased baseline activity and enhanced output in response to increases in CO_2 (Kuo et al., 2019). However, at the whole animal level, Vgat$^{A1783V/+}$ mice showed reduced respiratory activity in room air and a blunted ventilatory response to CO_2 (Kuo et al., 2019). This outcome is not entirely unexpected because inhibitory signaling in the RTN (Cregg et al., 2017) and at other levels of the respiratory circuit (Baertsch et al., 2018) can facilitate respiratory output. Also, *Slc32a*$^{1cre/+}$::*Scn1a*$^{A1783V\ fl/+}$ mice have spontaneous seizures which, for reasons mentioned above, may propagate to the brainstem and disrupt respiratory control in a seizure-dependent manner. This later possibility is an important consideration since deletion of *Scn1a* only from forebrain inhibitory neurons also resulted in seizures and premature death (Cheah et al., 2012), suggesting in this mouse model that cortical seizure activity can cause brainstem dysfunction and SUDEP. This contrasts with evidence from

Kcnq2 cKO that as noted above exhibits cortical seizure activity that did not correlate with premature death (Aiba and Noebels, 2021). Both mouse models are maintained on a similar C56BL/6 background so the reason(s) for these divergent results remains unclear. That said, it is worth mentioning that disruption of *Scn1a* globally or conditionally only in forebrain inhibitory neurons caused sleep fragmentation with less non-rapid eye movement (NREM) sleep and more frequent waking episodes (Kalume et al., 2015). Although the relationship between sleep, sleep problems, and epilepsy have long been appreciated (Diaz-Negrillo, 2013; Wang et al., 2018), the basis for these associations is not clear. Based on evidence that regulation of the ECV is coupled to sleep-wake status (Ding et al., 2016) and decreased ECV positively correlates with neural activity (Walch et al., 2022), we speculate that disruption of sleep (as seen in Dravet syndrome; Kalume et al., 2015) will decrease ECV, lower seizure threshold and favor propagation of SD (**Figure 2**).

In sum, epilepsy-associated genes may be expressed by neurons or astrocytes in brainstem respiratory centers and so may contribute to background breathing problems that render the system vulnerable to failure. Altered neural activity or compromised astrocyte regulation of the extracellular milieu may also favor the propagation of SD into the brainstem.

Author contributions

DM drafted manuscript, drafted figures, and approved the final version. BM edited manuscript and figures, and approved the final version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Autonomic dysfunction in epilepsy mouse models with implications for SUDEP research

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Epilepsy has a high prevalence and can severely impair quality of life and increase the risk of premature death. Sudden unexpected death in epilepsy (SUDEP) is the leading cause of death in drug-resistant epilepsy and most often results from respiratory and cardiac impairments due to brainstem dysfunction. Epileptic activity can spread widely, influencing neuronal activity in regions outside the epileptic network. The brainstem controls cardiorespiratory activity and arousal and reciprocally connects to cortical, diencephalic, and spinal cord areas. Epileptic activity can propagate trans-synaptically or *via* spreading depression (SD) to alter brainstem functions and cause cardiorespiratory dysfunction. The mechanisms by which seizures propagate to or otherwise impair brainstem function and trigger the cascading effects that cause SUDEP are poorly understood. We review insights from mouse models combined with new techniques to understand the pathophysiology of epilepsy and SUDEP. These techniques include *in vivo*, *ex vivo*, invasive and non-invasive methods in anesthetized and awake mice. Optogenetics combined with electrophysiological and optical manipulation and recording methods offer unique opportunities to study neuronal mechanisms under normal conditions, during and after non-fatal seizures, and in SUDEP. These combined approaches can advance our understanding of brainstem pathophysiology associated with seizures and SUDEP and may suggest strategies to prevent SUDEP.

KEYWORDS

epilepsy, SUDEP, brainstem, mouse models, cardiorespiratory activity

Epilepsy and SUDEP

Epilepsy affects ~0.75% of all people (1), or ~50 million people worldwide, with an incidence of 4–10/1000 people/year. Epileptic seizures result from abnormal hypersynchronous neuronal activity (2, 3). Most seizures arise from both hemispheres simultaneously (generalized) or from restricted regions in one or both hemispheres but can propagate widely (focal) (4, 5). Anti-seizure medicines (ASMs) prevent seizures for ~67% of patients, but many well-controlled patients experience cognitive and behavioral comorbid disorders and ASMs side effects. One-third of patients have drug-resistant epilepsy and often take multiple and high doses of ASMs with greater comorbidities, adverse effects, impairments of quality of life, and higher mortality (6–8).

Sudden unexpected death in epilepsy (SUDEP) is a witnessed or unwitnessed, non-drowning, and non-traumatic death in a person with epilepsy which often but not always follows a convulsive seizure. SUDEP excludes status epilepticus and cases where post-mortem examination or toxicology reveals another cause of death (9). SUDEP is the leading cause of death in drug-resistant epilepsy (DRE), with an incidence rate of 1–5 cases per 1000 patients per year (10, 11). SUDEP is the second leading neurological cause of lost years of life after stroke (12). Case-control studies reveal the following risk factors: generalized tonic-clonic seizures (GTCS) (any in the last year and further increased risk with $\geq$ 3/year), lack of adequate medication, nocturnal seizures, and lack of nocturnal supervision (13, 14). Many SUDEP cases are undetected or misclassified, suggesting the incidence is higher than reported (13, 15).

The few SUDEPs recorded on video with electroencephalographic and electrocardiographic data are biased toward more severe focal epilepsy cases admitted for presurgical evaluation with rapid ASMs reduction (16). By contrast, SUDEP affects the full spectrum of people with epilepsy (17), and results from epilepsy monitoring units cannot be generalized. The underlying mechanisms of SUDEP remain poorly defined. Most occur during sleep and follow convulsive seizures, with reduced brain activity and respiratory impairments commonly observed, although cardiac dysfunction can contribute (18–20). Postictal disruption of brainstem regulation of arousal, respiratory and cardiovascular functions is considered the common final pathway of death in SUDEP (9, 20–23). In animal models, arousal and cardiorespiratory dysfunctions can result from fast direct synaptic circuit mechanisms (24–27) and slower phenomenons like spreading depressions (SD) (28–31). How cortical seizure activity impairs brainstem functions postictally is a critical research issue. Understanding this pathophysiology will inform preventative and therapeutic strategies. We review potential mechanisms of seizure propagation and spread that might contribute to SUDEP, examine models used to study the mechanisms, and highlight advances in investigating complex network interaction *in vivo* in mouse models.

Propagation of epileptic activity – the problem of a highly connected brain

This section reviews the following questions: how does epileptic activity spread to the brainstem? Is this a rare event, or common but usually compensated for (and if so, how)?

Rodent, primate, and human brains orchestrate multiple areas to optimally assess internal and external conditions and determine behavioral outputs. This requires high connectivity, precise coordination, and balance between interacting cortical-subcortical networks. During cortical seizures, affected areas are directly impacted by aberrant excitation and inhibition. In addition, areas beyond the epileptic network can be severely disturbed by ictal spread to resonating areas. The brainstem receives projections from cortical and subcortical brain areas (32–34). During and after seizures, these connections can alter brainstem activity and potentially impair arousal and cardiorespiratory functions and contribute to SUDEP (32, 34–36). Understanding why some cortical seizures propagate to other cortical and subcortical areas and how this disrupts brainstem activity is a major challenge in SUDEP research. The brain regions involved in epileptic circuits - cerebral cortex (37), hippocampus (38), amygdala (39, 40), and thalamus (41) - are directly and indirectly connected to the brainstem and exert powerful influences over it. The brainstem and more rostral cerebral regions share strong reciprocal connections, complicating our understanding. We review new techniques to study network interaction involved in SUDEP in epileptic mouse models.

General concepts of the spreading of pathological activity

Epileptic seizures can be provoked by disrupting neuronal E/I balance by altering intrinsic properties, or by altering synaptic transmission and network stability causing hypersynchronous activity (42). The mechanisms underlying seizure propagation and termination are less well characterized. Focal seizures influence other brain areas *via* rapid axonal connections or spreading depression (SD), a slow propagating depolarization wave that inactivates neurons (25, 31, 35, 36). This slow ictal wavefront propagation corresponds to the gradual evolution of seizure symptoms, as in the Jacksonian sensory symptom march (43). The ictal wavefront may evoke a feedback loop to the seizure focus which triggers the clinical symptoms. Failure of feedforward inhibition supports epileptiform activity and seizure spread *via* this slow route in addition to classic synaptic pathways (44, 45). While SD contributes to symptoms of migraine and epilepsy, the mechanisms may be conserved or divergent (29, 46). The propagation rate of SD in migraine and epilepsy are similar, but their onset, duration, impacted brain regions and EEG changes can differ (47–50). Different SDs might exert distinct influences on brainstem function and SUDEP risk (29, 51). Debate persists whether this risk is primarily an ictal or post-ictal phenomenon. While the ictal seizure spreading into the brainstem might cause direct autonomic dysfunctions (36, 52, 53), the disturbance in the post-ictal period might substantially outlast the seizures. The post-ictal EEG suppression is viewed as a potential contributor but only a weak SUDEP predictor (54–56).

Mouse models of familial hemiplegic migraine with mutations in *Cacna1a* (57, 58), *Atp1a2* (59) and *Scn1a* (60, 61) show increased mortality. In mice with *Cacn1a* variants, brainstem SD elicited by seizures can be fatal (31). Brainstem SD may directly impair cardiorespiratory function (21, 30, 31, 35, 40). In focal seizures, SD with seizure propagation may be restricted to cortical regions in most instances. SDs were directly triggered by high neuronal activity of focally induced seizures and prevented by applying tetrodotoxin (TTX; a potent sodium channel inhibitor) (62). The authors postulate that SD is an innate mammalian mechanism to prevent seizure propagation and generalization, and to induce seizure termination (62). However, if SDs reach brainstem autonomic centers, severe cardiorespiratory dysfunction may follow (31, 63).

Brain areas linked to autonomic control

Human studies used electrical stimulation or the time of seizure invasion to investigate cortical structures that alter breathing. These brain areas include the amygdala (32, 36, 64), the hippocampus head and body, anterior parahippocampal gyrus, and antero-mesial fusiform gyrus (65, 66). A pediatric study found apneas and seizure spread to the amygdala were strongly correlated (67), an adult study failed to replicate this (64). Electrical stimulation to the insula and left cingulate gyrus decreased cardiac output and induced cardiac asystole in epilepsy patients without effects on breathing (68, 69). However, electrical activation cannot precisely target specific neurons and circuits. Further, cortical and subcortical electrode coverage is limited. So the invasion of ictal activity to a region (e.g., amygdala) may be accompanied by spread to areas that were not sampled (e.g., hypothalamus, anterior cingulate, and orbitofrontal cortices). Also, correlating seizure invasion to apneas might reveal only some parts of the network involved in autonomic dysfunction. In animals and humans, physiological changes in subcortical areas (e.g., locus coeruleus) alter breathing (27, 70).

In addition to seizure invasion of cortical areas, altered connectivity between cortical areas and respiratory brainstem centers may be important (71). Functional magnetic resonance imaging (fMRI)-studies on epilepsy patients show reductions in resting-state functional connectivity and tissue loss in cortical, subcortical, and brainstem structures associated with impaired autonomic control and increased SUDEP risk (71–73). However, monitoring of patients who later died from SUDEP did not reveal a direct associated location or lateralization of the epileptogenic zone with their higher risk of death (16). Intracranial EEG recordings and stimulation studies implicate the amygdala, hippocampus, insular cortex, and seizure spread to the contralateral temporal lobe to correlate with ictal cardiorespiratory dysfunctions (32, 36, 53, 65, 67, 74). Thus, identifying the detailed and complex connectivity and the altered brain activity in regions controlling cardiorespiratory activity is crucial for SUDEP risk estimation.

Control of autonomic functions in the brainstem and SUDEP

Here, we review evidence of brainstem alterations (e.g., genetic or physiological/structural resulting from chronic epilepsy) associated with SUDEP risk. We discuss crucial brainstem areas generating and modulating autonomic rhythms, such as breathing, and discuss their potential role in SUDEP. The respiratory network flexibly adapts to environmental and metabolic changes while maintaining stability to guarantee effective gas exchange (75). This network integrates brainstem rhythm-generating nuclei with other central and peripheral neural regions (76). The brainstem respiratory network includes the parafacial respiratory group (pFRG), Bötzinger complex (BötC), pre-Bötzinger complex (pre-BötC), rostral ventral respiratory group (rVRG), and caudal VRG (cVRG). Pontine nuclei modulate respiratory activity *via* projections to medullary respiratory nuclei (34, 76, 77). The post-inspiratory complex (PiCo) provides excitatory input to generate post-inspiration patterns (78). Seizure-related effects on respiratory and cardiac brainstem centers can impair these functions and contribute to SUDEP (20, 21, 79, 80) (Figure 1). Cardiorespiratory dysfunction in SUDEP could result from the effects of higher cortical and limbic areas on brainstem function, direct brainstem alterations, or both, including descending and ascending circuitries (27, 38, 81) (Figure 2). Chronic alterations of respiratory control, such as reduced ventilatory responses to increased CO2 levels, occur in epilepsy patients (82).

Epileptic seizures can directly alter heart rhythms and heart rate variability (HRV), which reflects balanced sympathetic and parasympathetic activity (83–86). High sympathetic tone and elevated levels of several neuropeptides can follow seizures (87, 88). Other seizure-induced acute changes include asystole, brady- and tachy-arrhythmias are most common with seizure foci in paralimbic and limbic cortices (69) and may contribute to SUDEP (23, 89, 90). Reduced HRV can result from voltage-gated sodium channel gene variants (91), and low-frequency HRV power is associated with SUDEP risk (92). Temporal lobe seizures may disturb arousal and vigilance networks (93).

A critical challenge is distinguishing indirect vs. direct effects on brainstem autonomic centers. For example, PreBötC dysfunction can result from mutations in ion channels (94–96) and mitochondrial genes (97), as well as transcription factors (98). In animal SUDEP models with *Kcna1* and *Scn1a* mutations, the threshold to trigger brainstem SD is reduced (21). However, respiratory networks are state-dependent; neuromodulators influencing respiratory activity include norepinephrine, serotonin, acetylcholine, substance P,

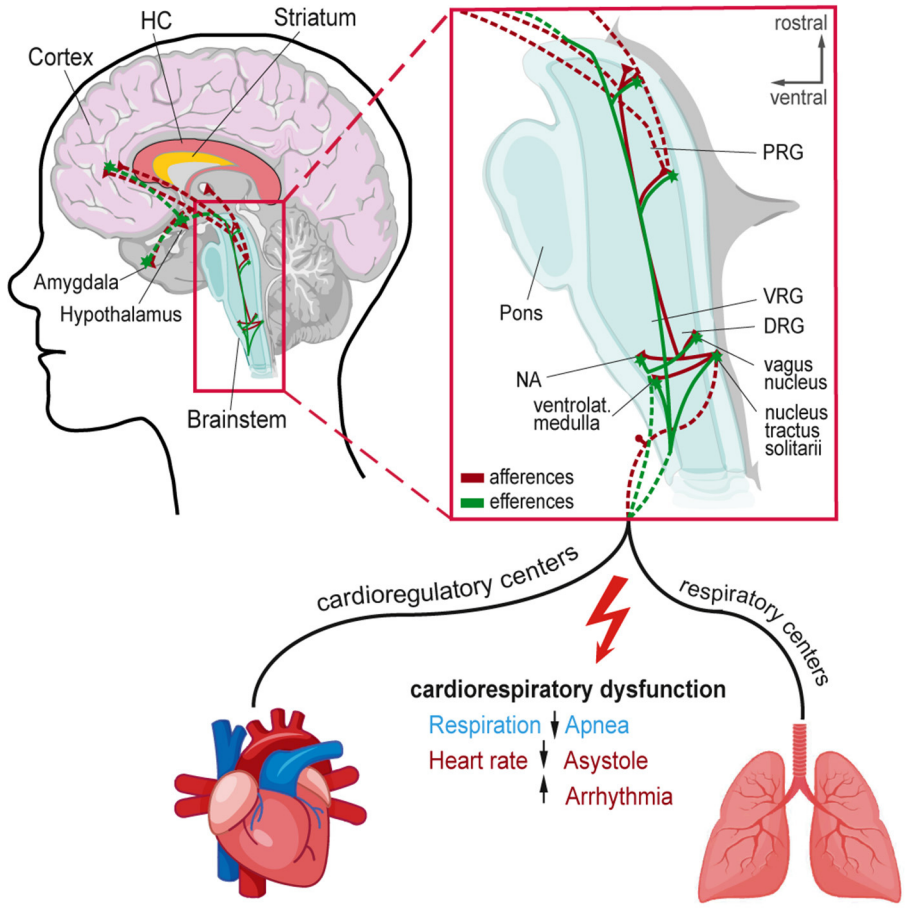

FIGURE 1
Afferent and efferent connections to central autonomic networks and proposed mechanisms involved in seizure-induced cardiorespiratory dysfunction leading to SUDEP. Cortical and subcortical regions involved in epileptic activity and cardiorespiratory modulation in the brainstem. Magnification shows connections and brainstem nuclei of cardioregulatory and respiratory centers that can be affected by seizure activity leading to cardiorespiratory dysfunction. DRG, Dorsal respiratory group; HC, Hippocampus; NA, Nucleus ambiguous; PRG, Pontine respiratory group; VRG, Ventral respiratory group.

ATP, somatostatin, dopamine, endorphins, and adenosine (99). Several have been shown to be elevated during and following seizures and potentially could contribute to SUDEP (88, 100–102).

The brainstem is crucial for controlling cardiorespiratory autonomic function impairments likely contribute to sudden infant death syndrome (SIDS), the sudden and unexpected death of a seemingly healthy baby under age 1 year (103–105). There are striking similarities between SIDS, sudden unexplained death in childhood (SUDC), and SUDEP (106–109) with the exclusion of other causes, nocturnal occurrence in the prone position, and an unwitnessed death (106). Arousal can be triggered by increased CO_2 (hypercapnia) and reduced oxygen levels (hypoxia), further preventing a build-up of end-tidal CO_2 and restoration of normal oxygen levels (103). This arousal response is linked to breathing and is normally initiated with a sigh (augmented breath) (110–112). Sighs are generated in the PreBötC by the same rhythm-generating network crucial for eupnea and gasping (95, 113). In addition, several other areas such as the dorsal raphe nucleus, the nucleus tractus solitarius, the parabrachial nucleus, and the retrotrapezoid nucleus are involved in arousal (106). Seizures in the amygdala [bed nucleus of the stria terminalis (BNST)] can activate projections to the brainstem, disturbing structures like the parabrachial nucleus involved in arousal and respiratory function (40). The BNST is highly interconnected to cortical regions, the hippocampus, the hypothalamus, the midbrain, and other brainstem nuclei and may serve as an integrator of autonomic and neuroendocrine responses (40, 114–117). As discussed above, massive release of neuromodulators (e.g., norepinephrine, serotonin, and acetylcholine) can disturb arousal. Since hypoxia and hypercapnia trigger arousal and gasping, they are a focus of SIDS research. However, another vulnerability phase is reoxygenation after a hypoxia/hypercapnia. This phase includes

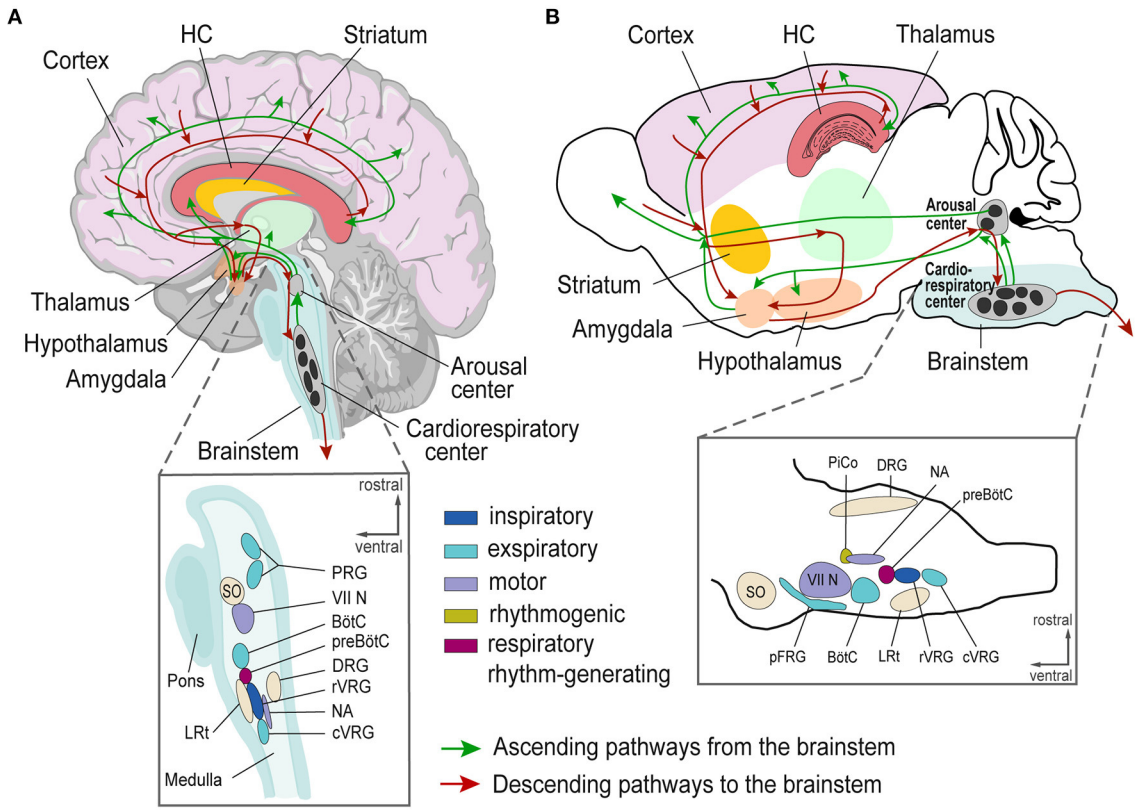

FIGURE 2
Schematic of brain regions involved in epileptic activity and cardiorespiratory regulation in humans **(A)** and mice **(B)**. **(A, B)** Top, Human and mouse brain with depicted Cortex (pink), Hippocampus (red), Striatum (yellow), Thalamus (green), Hypothalamus (orange), and brainstem (blue) with ascending (green lines) and descending (red lines) projections. Bottom, Magnification of brainstem nuclei. VII N, facial nucleus; BötC, Bötzinger Complex; cVRG, caudal ventral respiratory group; DRG, Dorsal respiratory group; HC, Hippocampus; LRt, lateral reticular nucleus; NA, Nucleus ambiguus; pFRG, para-facial respiratory group; PRG, Pontine respiratory group; PiCo, Post-inspiratory Complex; preBötC, preBötzinger Complex; rVRG, rostral ventral respiratory group; SO, superior olive; VRG, Ventral respiratory group.

post-hypoxic ventilatory depression (118, 119), which can occur after generalized tonic-clonic seizures and could be potentially prolonged in SUDEP. To dissect these mechanisms, modern experimental technology, including optogenetics and chemogenetics in animal models, as discussed below, is critical.

Future directions of SUDEP research seek to identify common molecular and cellular changes overlapping in several SUDEP animal models and potentially identify common changes in SIDS and SUDC models. While expression changes in RNA levels in brainstem areas of animals showing SD in cortical areas were detected (120), more investigations in epileptic animal models (genetic and induced) are needed to unravel molecular changes that participate in SUDEP.

ASM and brainstem function

Another potential SUDEP mechanism is direct ASM effects on brainstem function. ASM can reduce SUDEP risk by a reduction of seizure frequency and severity, thereby preventing seizure-induced impairment of brainstem autonomic centers. Under normal oxygen concentrations, mammals are eupneic, their robust respiratory network combines diverse synaptic and intrinsic signals in the respiratory network (99). During severe hypoxia, the respiratory network generates gasping (121, 122) through reduced mechanisms of rhythm generation (99, 113). During gasping, changes include reduced inhibition (123) and a switch to sodium-dependent intrinsic neuronal bursting securing rhythm generation (124–126). These altered rhythm-generating properties of the respiratory network alter the sensitivity to sodium channel-blocking ASMs and may interrupt the gasping response during seizure-induced postictal hypoxia (127). These direct brainstem effects may contribute to increased mortality associated with lamotrigine use observed in some studies (128). Moreover, during seizures, patients can experience repeated hypoxic episodes combined with increased norepinephrine and other neurotransmitter/modulator levels. This combination can destabilize PreBötC function (129)

and may parallel secondary changes induced by the hypoxic conditions during and after cortical seizures.

More investigations of the brainstem function and gene expression of the brainstem areas controlling respiratory and cardiac functions are needed in epileptic mice to get a better understanding of the mechanisms underlying SUDEP. New insights into chronic brainstem changes could stem from novel techniques of brainstem transcriptome using single-cell RNA-Seq or spatial transcriptomics (130).

Model systems for studying epilepsy and SUDEP

Model systems help to investigate the pathological interactions between brain regions that can result in a collapse of cardiorespiratory function and SUDEP. *In vitro* and *in vivo* models can study neuronal disease network mechanisms (131–133). *In vitro* models standardize experimental conditions, but oversimplify neuronal network function or whole organism interaction, which may be critical in SUDEP. *In vivo* models comprise diverse methods and model organisms (134–138). Brain areas involved in epileptic activity and cardiorespiratory regulation are similar in mice and humans (78, 139) (Figures 2A, B). Thus, mice can appropriately model human epilepsy. Modern techniques can target specific brain areas and predefined neuronal cell populations to decipher their role in epilepsy and SUDEP (140).

Epilepsy can result from diverse pathological processes, including trauma, stroke, tumors, infections, autoimmune disorders, and >150 genetic variants (141, 142). Epilepsy is often accompanied by comorbid disorders, including autism spectrum, cognitive, psychiatric, and hyperkinetic (143–148). The developmental and epileptic encephalopathies (DEEs) include a diverse spectrum of early-life epilepsies, often resulting from genetic disorders, and associated with developmental delays partly attributable to seizures and interictal epileptiform activity (149). Across these disorders, E/I imbalances occur in the amygdala, cortex, hippocampus, and other epileptogenic regions (150, 151). Complex and heterogeneous genetic mouse models recapitulate various human pathologies, offering insights into epilepsy and SUDEP and allowing controlled experiments on mechanisms by controlling for different confounds. Epilepsy mouse models are divided into induced and genetic models. In kindling models, stimulation (electrical, chemical, or acoustic) induces seizures, whereas in genetic models, gene mutations result in spontaneous seizures (136, 152). Mouse models can mimic focal and generalized epilepsies as well as post-traumatic epilepsy (153), temporal lobe epilepsy (TLE) (152), genetic variants (80, 154), and scores of rare genetic disorders (e.g., tuberous sclerosis complex, CDKL5, Rett Syndrome, Dravet Syndrome, FOXG1 syndrome, STXBP1 syndrome and many more) (Figure 3).

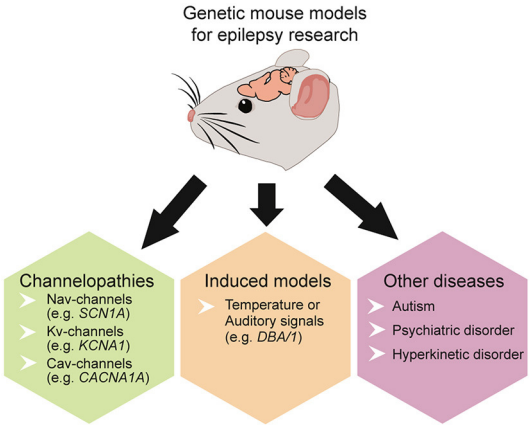

FIGURE 3
Overview of commonly used genetic mouse models to study epilepsy, SUDEP, and co-existing disorders.

Some genetically modified mouse lines model SUDEP with deadly seizure-induced cardiorespiratory abnormalities (39, 155). Many genetic models involve ion channels, including sodium voltage-gated channels (Na_V) (154, 156–158), potassium voltage-gated channels (K_V) (28, 159, 160), or calcium voltage-gated channels (Ca_V) (30, 161) (Table 1). Some display spontaneous epileptic seizures (e.g., *Scn1a*, *Scn1b*, *Kcna1*, *Kcnq1*, *Cacna1a*, *Shank3*) (31, 143, 159, 162, 163) while others are susceptible to heat or audiogenic-induced seizures (e.g. Scn1a, Scn8a, DBA/1) (80, 164) (Figure 3).

Commonly SUDEP mouse models carry mutations in the Na_V *(1.1, 1.6)* and K_V *(1.1, 7.1, 11.1)* genes (159, 162, 165). *Scn1a* mutations alter the $Na_V\alpha1$ subunit ($Na_V1.1$) and $Na_V1.1$ haploinsufficiency can cause Dravet Syndrome (DS). DS is a treatment-resistant early-onset epilepsy with 70-80% of cases due to *Scn1a* variants and high rates of SUDEP (9, 83, 166–169). $Na_V1.1$ is expressed in inhibitory neurons. A loss of function decreases their excitability, increasing network excitability, altering action potential (AP) dynamics (170–173) and impairs thalamic glutamatergic and GABAergic function, disrupting thalamocortical networks and facilitating seizure generation (174, 175). $Na_V1.1$ deficient mice recapitulate many aspects of human DS pathology including severe epilepsy, multiple neuropsychiatric comorbidities, and increased SUDEP risk (21, 22, 83, 173, 176–180). Other gene mutations (e.g., *Scn1b* and *Scn8a)* display similar symptoms (163, 164, 181, 182). Mice with mutations in genes encoding for K_V show cardiorespiratory failure including cardiac abnormalities and apnea observed in SUDEP (21, 183). $K_V1.1$-α1 subunits, encoded by the *Kcna1* gene, are crucial for neuronal excitability and are broadly expressed in the cortex, hippocampus, cerebellum, and brainstem (184, 185). *Kcna1* knockout mice display early-onset generalized tonic-clonic seizures, seizure-related death,

TABLE 1 Overview of common channelopathies in mouse models of epilepsy and SUDEP.

Gene	Channel	Expression	Disorder	Studies
SCN1A	Na^+ channel (α subunit of $Na_v1.1$)	Central nervous system and cardiac myocytes	Genetic epilepsy with febrile seizures plus (GEFS+), Dravet Syndrome	(22, 169, 170, 173, 174, 191)
SCN1B	Na^+ channel (β subunit of $Na_v1.1$)	Central and peripheral nervous system, skeletal, and cardiac muscles.	Genetic epilepsy with febrile seizures plus (GEFS+)	(163, 181)
SCN8A	Na^+ channel (α subunit of $Na_v1.6$)	Central nervous system	Epilepsy	(148, 154, 164, 182)
KCNA1	K^+ channel (α subunit of $K_v1.1$)	Central and peripheral nervous system	Epilepsy, Episodic ataxia	(159, 160, 183, 187)
KCNH2	K^+ channel ($K_v11.1$)	Brain and heart	Long QT syndrome	(165, 189)
KCNQ1	K^+ channel ($K_v7.1$)	Heart, intestinal cells	Long QT syndrome	(162)
CACNA1A	Ca^{2+} channel (α subunit of $Ca_v2.1$)	Brain	Epilepsy, Familial hemiplegic migraine, Episodic ataxia	(30, 31, 35, 161)

and cardiorespiratory dysfunction (159, 186–188). These mice exhibit apneas, increased respiratory variability, and precede cardiac failure as risk factors for SUDEP (183, 187). Further K_V-channelopathies (e.g., *Kcnh2* and *Kcnq1*) are susceptible to recurrent seizures and long QT syndrome (LQTS); i.e., arrhythmias and SUDEP (162, 189, 190).

Mutations in genes encoding for $Na_V1.1$, $K_V1.1$, and $Ca_V2.1$ are moreover linked to brainstem seizures, medullary SD, and cortical seizures propagating to the brainstem causing cardiorespiratory arrest (21, 30, 31, 35). Thereby, local brainstem SD can elicit EEG suppression, apnea, bradycardia, and asystole, mimicking the involvement of SD in epileptic activity propagation and its relevance as SUDEP models.

Thus, a number of model systems and especially mouse models, are nowadays available for epilepsy and SUDEP research. In the direct context of SUDEP, models with Na_V (*1.1, 1.6*) and K_V *(1.1, 7.1, 11.1)* mutations seem particularly promising. Of these, *Scn1a* models have been extensively studied and largely model the human SUDEP pathology and phenotypes well (152, 167, 191). Future studies need to extend to clinically and genetically characterized epilepsies to explore if common or distinct pathways of autonomic dysfunction mediate SUDEP.

Techniques to study network interaction involved in SUDEP

To understand SUDEP mechanisms, we need models and techniques to represent and measure cortical seizure generation and propagation as well as cardiorespiratory function. *In vivo* techniques allow direct epileptic activity measurements and manipulations (192, 193) of complex circuitries and brain connections. *Ex vivo* recordings from targeted brain regions allow cellular processes to be investigated at high resolution. *Ex vivo* measurements like histological reconstructions, stainings, and spatial transcriptomics (180, 194) can reveal anatomical brain changes associated with epilepsy, which may be the cause or effect of epilepsy or an epiphenomenon of the underlying pathology.

Next, we will discuss recent advancements in methods to investigate *in vivo* and *ex vivo* models, including optogenetics, electrophysiology, imaging, and other measurements (Figure 4).

Optogenetics is a technique to study specific cells and their relations to brain functions and disorders (195). Optogenetics utilizes the expression of light-sensitive proteins (opsins) in brain areas or specific cells. Depending on the opsin used, targeted neurons can be activated or inhibited (or even both) using light stimulation to precisely control neuronal activity. Optogenetics can trigger or prevention of epileptic activity (196) with millisecond temporal precision, enabling the assessment of how specific firing patterns affect brain cells and networks (197). Optogenetics can be applied invasively and non-invasively (198) and can be combined with electrophysiological recordings and imaging techniques. Chemogenetics can selectively modulate cellular pathways using restricted artificial chemogenetic receptors [e.g. DREADDs (Designer Receptors Exclusively Activated by Designer Drugs)] delivered to specific neuronal populations. Instead of light stimulation, chemogenetics systemically injected or microinfused can activate ligands that excite or inhibit targeted neurons (199). Optogenetics can be combined with chemogenetics to manipulate neuronal activity with a high temporal and spatial resolution (200). In epilepsy animal models, these combined methods can identify and manipulate specific neuron populations, brain regions, and neuronal circuitries involved in epileptic activity (201).

Seizures and interictal epileptic discharges (IEDs) can be restricted to certain brain regions and networks. Electroencephalographic (EEG) recordings can localize brain regions giving rise to seizures and examine epilepsy-related

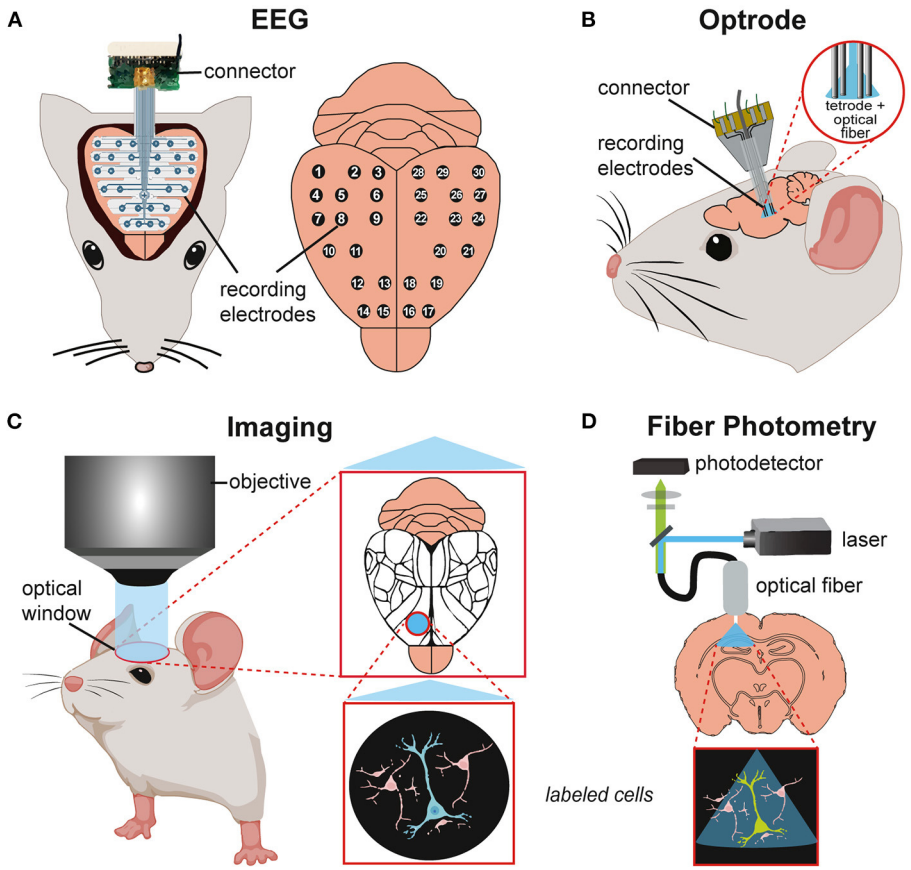

FIGURE 4
Schematics of *in vivo* techniques for studying neuronal activity in mouse models. **(A)** Electroencephalography (EEG) recording with implanted EEG probe and recording electrodes (30 channels) on the brain surface. **(B)** Implated optrode with integration of optical fiber and recording electrodes for simultaneous optical stimulation and electrophysiological recordings. **(C)** Imaging of the mouse brain through an optical window for visualizing brain areas (mapping, Allen Brain Atlas) or/and single-cell activity in the region of interest. **(D)** Fiber photometry of a target brain region with simultaneous optical stimulation (laser) and calcium imaging (photodetector) via an implanted fiber probe.

neuronal activity changes across brain regions (202). Scalp EEG records changes in electrical potentials caused by ion flow across neural membranes, mainly at the brain's surface. It can detect the origin and propagation of epileptic activity throughout different brain regions at a macro scale (203, 204) (Figure 4A). Invasive methods include intracranial EEG (iEEG) using depth or subdural EEG recordings (ECoG) to study seizure onset and spread as well as SD and seizure propagation in SUDEP models at a higher spatiotemporal resolution (22, 30). Stereotaxically inserted multi-channel electrodes can record local field potentials (LFPs) and single-cell activity (204, 205). Modulation of neuronal activity *via* electrical stimulation with these electrodes is possible but is much less precise than optogenetic manipulation. For example, SDs can be induced by electrical, and optogenetic techniques (206) whereas electrophysiological and optical recording methods can assess their propagation and effects on other structures (207, 208).

Combined optogenetics and electrophysiology *in vivo,* using optical microelectrodes are called *optrodes* (209, 210) enabling a direct readout of manipulated cell activity. Here, a single microelectrode probe with integrated optical fiber can simultaneously record and transmit light to genetically modified, opsin-expressing cells. Optrodes can study neuronal circuit dynamics in awake-behaving animals (211, 212) (Figure 4B).

Imaging allows the visualization/mapping of cortical activity with high spatial and temporal resolution (213, 214). Voltage-sensitive dyes (VSDs) or genetically encoded calcium indicators (GECIs) react to direct or indirect (Ca^{2+}) changes in neuronal activity. VSD imaging incorporates dyes into the cell membrane that signal membrane-potential differences as changes in fluorescence. VDS imaging can monitor synaptic transmission and propagation of cortical activity but has a low signal-to-noise ratio and lacks cellular specificity (215, 216). GECIs allow cell-specific targeting but have a slower temporal resolution. GECIs have been used to record population activity in wide-field calcium imaging experiments. Combined with two-photon imaging, GECIS can reveal activity dynamics of hundreds of

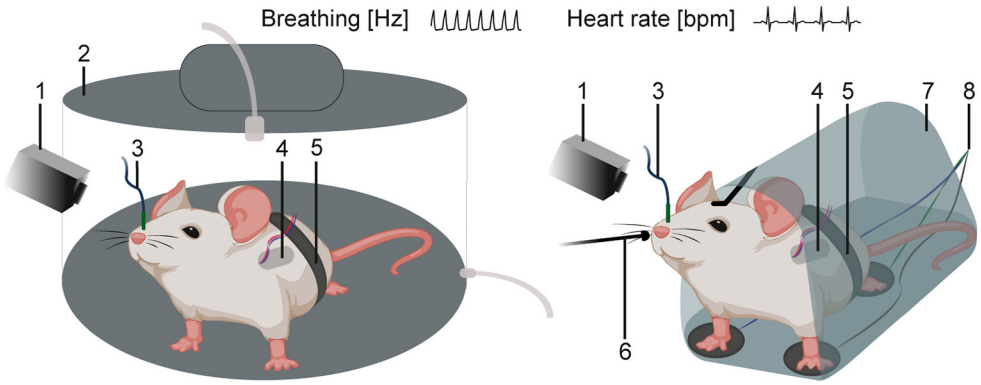

FIGURE 5
Schematics of *in vivo* techniques for the recording of cardiac and respiratory activity. Mouse in a whole-chamber plethysmograph **(left)** or in a restraining system **(right)** with additional recording methods. (1) video monitoring and infrared camera, (2) whole-chamber plethysmography, (3) intranasal cannula, (4) telemetry system, (5) movement sensor, (6) external temperature probe (thermocouple), (7) restraining system, and (8) 3-point ECG.

individual neurons (217). Since the activity-dependent changes in calcium-sensitive proteins can be visualized *in vivo* over months, large-scale longitudinal functional studies can assess activity before, during, and after seizures and in a single animal (Figure 4C). Imaging techniques can visualize seizures *in vivo* at high temporal and cellular resolution (172, 218).

Fiber photometry can combine imaging and optogenetics using an implanted fiber-optic cannula to deliver excitation pulses and monitor activity-dependent fluorescence changes (219). This technique is ideal for deep brain recordings and can study calcium signals in distinct epileptic brain regions in freely moving mice (220) (Figure 4D). Fiber photometry can be used simultaneously with electrophysiological recordings to combine cell-type-specific imaging with high temporal-resolution spike recordings in freely behaving mice (221).

These methods have provided new insights into the role of brain regions and cell populations in epilepsy. Optogenetics combined with optical manipulation, and electrophysiological recordings revealed the key role of inhibitory GABAergic interneuron signaling in seizure generation and ictal propagation in epileptic mice (212, 222). Other studies addressing brainstem excitatory neurons showed a direct correlation to reduced subcortical activity during seizures (223). Together, these techniques provide new research opportunities on epilepsy networks and seizure dynamics over the whole brain.

Investigating SUDEP and cardiorespiratory dysfunctions requires additional recording techniques for *in vivo* monitoring of autonomic functions including breathing and heart rate. Several methods are available in the mouse (155, 182, 183, 224). Cardiac activity is typically recorded *via* electrocardiography (ECG) (225, 226). Methods can monitor breathing (227) including invasive (telemetry systems and intranasal cannulas) and non-invasive methods (movement sensors, restraining systems, plethysmographs) (Figure 5). The whole-chamber plethysmography approach offers a non-invasive method in freely, non-restrained animals (95, 97, 228). This technique allows recordings of breathing under hypoxia/hypoxemia conditions (low blood oxygen levels and insufficient oxygen supply) linked to SUDEP (30, 52, 229).

Although *in vivo* methods provide insights into the network mechanisms, *ex vivo* studies offer more focused investigations of cellular changes. Histological reconstructions and stainings of brain regions can follow *in vivo* experiments to verify transgene expressions and precisely localize implanted electrodes or optical fibers (30, 180, 230). Brain slice preparations containing cortical, hippocampal, or brainstem microcircuits allow single-cell recordings or small network analysis to gain insights into pathophysiology (78, 95, 231, 232). Spatial transcriptomics can map the organization and connectivity of distinct genetically defined cell types (194, 233). In epilepsy research, this can provide a deeper exploration of disease mechanisms and pathogenic changes in the spatial organization and molecular signaling networks (234).

Thus, combining different techniques can provide a greater definition of the dysfunctions associated with epileptic activity and its interplay with autonomic functions on different levels to identify possible biomarkers for epilepsy, seizure onset, and SUDEP (202, 235).

Possible therapeutic approaches could be based on electrical or optical stimulation of specific brain areas to "rebalance" their E/I activity and maintain cardiorespiratory function during and after seizures (198, 236). Electrical stimulation in patients to map epileptic zones can inhibit or enhance respiration (237). Optogenetic neuronal activation has been shown to suppress seizure-induced respiratory arrest and exert an anticonvulsant

effect in a SUDEP mouse model (238). Further, *ex vivo* methods might provide opportunities for new molecular targets and drug screening (233, 234, 239). However, these invasive approaches will require far more refinement for their potential benefits to exceed their definite risks.

Conclusion and future perspectives

SUDEP is the leading epilepsy-related cause of death, affecting all age groups and epilepsy severities. SUDEP mechanisms are poorly understood but are critical for preventive and therapeutic strategies. Although ASMs can control seizures in most patients, they do not alter long-term prognosis or cure epilepsy (240). Further, their side effects can be severe (241, 242). 30% of the patients with ASM-resistant epilepsy suffer ongoing seizures and experience an increased SUDEP risk. Medications/treatments that prevent seizures in those that are currently uncontrolled with minimal side effects are desperately needed. Understanding SUDEP mechanisms in more detail is a desperate need.

Epilepsy mouse models with ion channel mutations mimic human epilepsies (176) and are critical in translational neuroscience research (243). They offer possibilities to investigate the link between genetic alterations and their underlying neurobiological mechanisms in much greater detail compared to humans. Translation of basic animal research to human epilepsy is exemplified by *SCN1A*-mice whose response to ASM has enabled the development of FDA-approved medications and gene therapy trials (191). Translational research with new molecular targets for anti-epileptogenic and anti-seizure research can empower novel drug discoveries and identify potential biomarkers for early diagnoses and more effective treatments (235, 243, 244).

Cardiorespiratory inhibition following epileptic seizures may be the common final mechanism of SUDEP. Cardiorespiratory dysfunctions from cortical or subcortical epileptic activity propagating to brainstem regions could cause SUDEP (21, 30, 31). SD might be directly involved in SUDEP-related seizure spread to the brainstem (29). Mouse models combining technological advances allow precise investigations of the brain networks implicated in SUDEP (235). These brain areas may provide new targets for interventions to prevent SUDEP.

In mice, invasive methods such as optical or electrical stimulations can manipulate neuronal networks (198, 245) whereas neurostimulation-based techniques can also be applied to epilepsy patients. Acute and chronic deep brain stimulation (DBS), as well as vagus nerve stimulation (VNS), are epilepsy therapies (236, 246–248). Combining neurostimulation and ASM may be more effective in controlling seizures than either alone (249).

There remains a critical need to better understand the mechanisms of epilepsy and SUDEP. Mouse models combined with precise methods are an important tools to assess these mechanisms and translate this knowledge into preventive and therapeutic strategies.

Author contributions

JB, OD, MR, and HK reviewed the literature and wrote this review article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Minocycline prevents hypoxia-induced seizures

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Severe hypoxia induces seizures, which reduces ventilation and worsens the ictal state. It is a health threat to patients, particularly those with underlying hypoxic respiratory pathologies, which may be conducive to a sudden unexpected death in epilepsy (SUDEP). Recent studies provide evidence that brain microglia are involved with both respiratory and ictal processes. Here, we investigated the hypothesis that microglia could interact with hypoxia-induced seizures. To this end, we recorded electroencephalogram (EEG) and acute ventilatory responses to hypoxia (5% O_2 in N_2) in conscious, spontaneously breathing adult mice. We compared control vehicle pre-treated animals with those pre-treated with minocycline, an inhibitory modulator of microglial activation. First, we histologically confirmed that hypoxia activates microglia and that pre-treatment with minocycline blocks hypoxia-induced microglial activation. Then, we analyzed the effects of minocycline pre-treatment on ventilatory responses to hypoxia by plethysmography. Minocycline alone failed to affect respiratory variables in room air or the initial respiratory augmentation in hypoxia. The comparative results showed that hypoxia caused seizures, which were accompanied by the late phase ventilatory suppression in all but one minocycline pre-treated mouse. Compared to the vehicle pre-treated, the minocycline pre-treated mice showed a delayed occurrence of seizures. Further, minocycline pre-treated mice tended to resist post-ictal respiratory arrest. These results suggest that microglia are conducive to seizure activity in severe hypoxia. Thus, inhibition of microglial activation may help suppress or prevent hypoxia-induced ictal episodes.

KEYWORDS

microglia, hypoxia, seizure, SUDEP, minocycline

Introduction

A seizure is a paroxysmal alteration of neurologic function caused by excessive hypersynchronous discharge of neurons in the brain, which causes temporary abnormalities in muscle tone or movements, behaviors, sensations, or states of awareness (Stafstrom and Carmant, 2015). Severe hypoxia induces seizures (Miyake et al., 2007; Gillam-Krakauer and Carter, 2012), which reduce ventilation and worsens the ictal state, occasionally resulting in sudden unexpected death in epilepsy (SUDEP) (So, 2008; Sowers et al., 2013; Fukushi et al., 2020). This negative spiral is a health risk to patients with epilepsy and comorbid hypoxic

respiratory pathologies, e.g., severe asthmatic attack or sleep apnea (Gullach et al., 2015; Harnod et al., 2017). However, pathophysiological mechanisms of hypoxia-induced seizures and post-ictal ventilatory depression remain unclear. Recent progress in glial physiology suggests that microglia are involved with brain neural processes (Wake et al., 2009; Baalman et al., 2015), neurotransmission (Hoshiko et al., 2012), and synaptic plasticity (Rogers et al., 2011). Microglia are also relevant to both epileptic and respiratory functions (Eyo et al., 2017; Camacho-Hernández et al., 2022). Here, we hypothesize that microglia are involved with the occurrence of hypoxia-induced seizures and hypoxic ventilatory responsiveness. We tested the hypothesis by comparing electroencephalogram (EEG) and ventilatory responses to acute severe hypoxia using minocycline, an inhibitory modulator of microglial activation (Tikka et al., 2001; Garrido-Mesa et al., 2013).

Materials and methods

Animal welfare

Experiments were approved by the Animal Experiment Ethics Committee of Murayama Medical Center and complied with guidelines for the Care and Use of Laboratory Animals of the National Research Council of the National Academies (8th edition, revised 2011) and Guiding Principles for the Care and Use of Animals of the Physiological Society of Japan.

Immunohistochemistry

We first performed immunohistochemical investigation to evaluate whether hypoxia activates microglia and minocycline blocks hypoxia-induced microglial activation in the piriform cortex, which is the crucial epileptogenic site in humans and rodents (Piredda and Gale, 1985; Vismer et al., 2015; Chee et al., 2022). We used eight adult male C57BL/6 mice aged 7.0 ± 0.0 weeks (mean ± SD). There were four groups of mice: with and without minocycline pre-treatment, without and with hypoxic loading (n = 2 each). Minocycline (Fuji Pharma, Tokyo, Japan) was diluted in saline, neutralized with sodium hydroxide solution to pH 7.4 (final concentration 11.8 mg/ml), and administered for three consecutive days before the experiment. Mice received 50 mg/kg minocycline twice daily for the first 2 days, and once for the next day (Zheng et al., 2015). Mice without minocycline group received saline as a vehicle in like manner. All injections were intraperitoneal. Mice without hypoxia loading were acclimated to a whole-body plethysmography chamber in room air for 60 min and then left in room air for 30 min. Mice subjected to hypoxia loading were also acclimated to the chamber environment for 60 min in room air and exposed to 7% O_2 hypoxia (N_2 balanced) for 30 min, followed by 30 min survival in room air. Then, under deep isoflurane anesthesia, mice were transcardially perfused with 4% paraformaldehyde (PFA)/phosphate buffered solution (PBS), pH 7.4, and brains were extracted. Immunohistochemistry was performed as described previously (Ikeda et al., 2007). To evaluate the cellular morphology of microglia, we immunostained ionized calcium-binding adaptor protein-1 (Iba-1), a constitutively expressed protein in almost all microglia, which is widely used as a microglia marker (Ito et al., 1998). The staining was performed using 1:2,000 anti-Iba-1 antibody (Wako, Neuss, Germany) as the first antibody, 1:1,000 fluorescent conjugated anti-rabbit antibody (Thermo Fisher Scientific, MA, USA) as the second antibody, and 1:2,000 alkaline phosphatase (AP)-conjugated anti-fluorescent antibody as the third antibody. The shape of stained resting microglia is ramified, and that of activated microglia is de-ramified round or ovoidal (Kettenmann et al., 2011; Fernández-Arjona et al., 2019). Signals were detected with nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Roche Diagnostics, Basel, Switzerland) as a chromogen. The piriform cortex was photographed with a high-resolution digital camera (DP70, Olympus, Tokyo, Japan) and the morphology of Iba-1-immunoreactive cells was examined.

Functional evaluation

Twenty adult male C57BL/6 mice aged 7.0 ± 0.0 weeks were used. The mice were divided into vehicle and minocycline groups of 10 each and were pre-treated with saline and minocycline, respectively, in the way as described above in the immunohistochemistry section.

EEG recordings

EEG electrodes were implanted as described previously (Fukushi et al., 2016, 2020, 2021; Terada et al., 2016). Briefly, the skull surface was surgically exposed under anesthesia with inhaled isoflurane (3.0%) and intraperitoneally injected pentobarbital (45–50 mg/kg). Then, two miniature screws (diameter 1.2 mm, length 2.0 mm) were inserted as recording electrodes into the skull over the frontal lobes, 2.5 mm posterior to the bregma, the third screw (diameter 1.2 mm, length 2.0 mm) along the midline, 4.5 mm anterior to the bregma, as a ground electrode. Dental resin and adhesive were used to fix the implanted electrodes, along with an additional screw for mounting the head. Screws for EEG electrodes were connected with a single amplifier by vinyl-coated flexible copper wires (O.D. 1.1 mm). The mice appeared to fully recover after 7 days, but the recovery period was extended by additional 4 days. The recording was conducted in the conscious, spontaneously breathing condition. EEG signals were amplified (JB-101J and AB-651J, Nihon Kohden, Tokyo, Japan) and bandpass filtered at 0.08–10,000 Hz. The EEG time signal was transferred to the frequency domain *via* a fast Fourier transform to calculate the power of theta (4–8 Hz) and gamma (35–45 Hz) bands. Each theta and gamma power was averaged across time in room air and during initial augmentation of ventilation in hypoxia before seizure appearance.

Ventilation recordings

A whole-body plethysmograph (PLY 310, EMMS, Bordon, UK), consisting of a recording and reference chambers placed inside a transparent acrylic box (size 20 × 20 × 20 cm), was used to non-invasively measure the respiratory flow, as described previously (Pokorski et al., 2014; Fukushi et al., 2016, 2020, 2021).

Briefly, the mouse was placed in a pre-calibrated recording chamber (volume 530 ml), which was maintained at 25°C throughout the experiment. The air in the chamber was suctioned at a rate of 250 ml/min using a constant flow generator (MV-6005VP, E.M.P-Japan, Tokyo, Japan). The pressure difference between the recording and reference chambers was measured using a differential pressure transducer (TPF100, EMMS), which was amplified with an amplifier (AIU060, Information and Display Systems, Bordon, UK) and bandpassed at 0.1–20 Hz. Since changes in the respiratory flow are proportional to those in the chamber pressure, the flow was calculated based on the pressure difference between the recording and reference chambers. Tidal volume was calculated for each breath (V_T; μl/g b.w.) by integrating the respiratory flow, and the counted respiratory rate (RR) was assessed as the number of breaths per min. Minute ventilation ($\dot{V}_E$; ml/g/min) was expressed as $V_T \times RR$. Respiratory variables were calculated in 1 min epochs. Controlled mixing of N_2 and air in the acrylic box was done to adjust the chamber O_2 concentration, which was monitored with an oxygen paramagnetic sensor (OxyStar-100, CWE, PA). An A/D converter (PowerLab4/26, ADInstruments, Colorado Springs, CO) was used to digitize pressure, EEG signals, and O_2 concentration data simultaneously at a sampling rate of 4,000 Hz. Ventilatory and EEG data were stored on a PC hard disk with LabChart7 software (ADInstruments). Signal processing was performed using MATLAB 2020a (MathWorks, Natick, MA).

The mouse was allowed to acclimate to the experimental setup in room air in the recording chamber for 60 min before the EEG and ventilation measurements began. After the normoxic recordings for 5 min, the gas was switched to 5% O_2 (N_2 balanced) for the continuous recording of ventilation for 20 min, followed by a switch back to room air. When respiratory arrest occurred during hypoxia, the gas in the chamber was immediately switched back to room air, and the recording was terminated. The moments of seizure appearance and cessation were determined by careful macroscopic observation of animals and counter-confirmed by EEG activity. The occurrence of respiratory arrest was defined as a point of flattening of the respiratory flow signal. Periods of sniffing, grooming, or licking that deviated from the mouse's normal breathing pattern were discarded. The periods of seizures, distorting the respiratory flow signals, were discarded as well. The variables recorded in the control group with saline were compared to those in the minocycline intervention group.

Statistical elaboration

The generalized Wilcoxon test was used to assess differences in the incidence of respiratory arrest between the two groups. The Mann-Whitney U test was used for differences in the time of seizure appearance during hypoxia between the vehicle and minocycline pre-treated groups. The mean values of $\dot{V}_E$, V_T, RR, theta power, and gamma power were respectively submitted to a two-factor within-subject ANOVA; with two pharmacological conditions (vehicle and minocycline), and two O_2 conditions (baseline room air and 5% O_2). When significant interactions were obtained, the *post-hoc* analysis was conducted using Welch's *t*-test between pharmacological conditions and paired *t*-test to determine the significant differences between the oxygen conditions. The criterion for significance was set at $p < 0.05$. All statistical tests were performed using SPSS 29.0 (IBM, Armonk, NY).

Results

The immunohistological investigation showed that the cortical Iba-1 positive cells were ramified in shape in mice breathing room air irrespective of minocycline pre-treatment or its lack (**Figures 1A,B**). In the hypoxic conditions, Iba-1 positive cells in the vehicle pre-treated mice were ovoidal (**Figure 1C**). However, those in the minocycline pre-treated mice were mostly ramified but intermingled with a small number of ovoidal cells (**Figure 1D**). These findings were consistent in the two sets of histological specimens investigated.

The functional investigation showed that severe hypoxia transiently augmented ventilation followed by the ventilatory fall-off in all mice. **Figure 2** shows raw recordings of respiratory flow and EEG. **Table 1** presents $\dot{V}_E$, V_T, and RR in room air and the initial hypoxic augmentation without and with minocycline. Respiration increased in response to hypoxia in both conditions; an increase remained unaffected by minocycline pre-treatment. Statistically, the ANOVA revealed the significant main effect on $\dot{V}_E$ of oxygen condition ($F(1, 18) = 94.482$, $p < 0.001$), no effect of pharmacological condition ($F(1, 18) = 0.281$, $p = 0.646$), and no interaction between pharmacological and oxygen conditions ($F(1, 18) = 1.243$, $p = 0.280$). Changes in V_T and RR were in harmony with those in $\dot{V}_E$. Likewise, there were main effects on both V_T and RR of oxygen conditions ($F(1, 18) = 90.816$, $p < 0.001$ and $F(1, 18) = 33.763$, $p < 0.001$, respectively), but no significant effects of pharmacological conditions on either variable. The time courses of $\dot{V}_E$, V_T, and RR in individual mice in the conditions without and with minocycline are shown in **Figure 3**. There appeared essentially no difference in ventilation between the two conditions in room air and during the early hypoxic phase with respiratory augmentation. However, as compared to the vehicle-pre-treated mice, the minocycline-pre-treated mice tended to resist respiratory depression and respiratory arrest during the late hypoxic phase.

Hypoxia also induced seizures in all but one minocycline-pre-treated mouse. **Figure 4A** shows representative raw traces of respiratory flow and EEG. At the onset of seizures, characteristic high amplitude aberrant waves in EEG were observed. In the mice without administration of minocycline, theta power, and gamma power were 44.84 ± 31.12 and 0.97 ± 0.75 $\mu V^2/Hz$ in room air, 27.19 ± 13.12 and 1.14 ± 1.10 $\mu V^2/Hz$ during the time period from hypoxic exposure to seizure appearance, respectively. In the mice pre-treated with minocycline, theta power, and gamma power were 15.59 ± 12.85 and 0.41 ± 0.45 $\mu V^2/Hz$ in room air, 8.45 ± 7.37 and 0.43 ± 0.41 $\mu V^2/Hz$ during the time period from hypoxic exposure to seizure appearance, respectively. The ANOVA revealed the significant main effect on theta power of pharmacological condition ($F(1, 17) = 9.472$, $p < 0.01$), no effect of oxygen condition ($F(1, 17) = 1.986$, $p = 0.177$), and the interaction between pharmacological and oxygen conditions ($F(1, 17) = 11.091$, $p < 0.01$). The *post-hoc* Welch's *t*-tests showed a significant reduction in theta power

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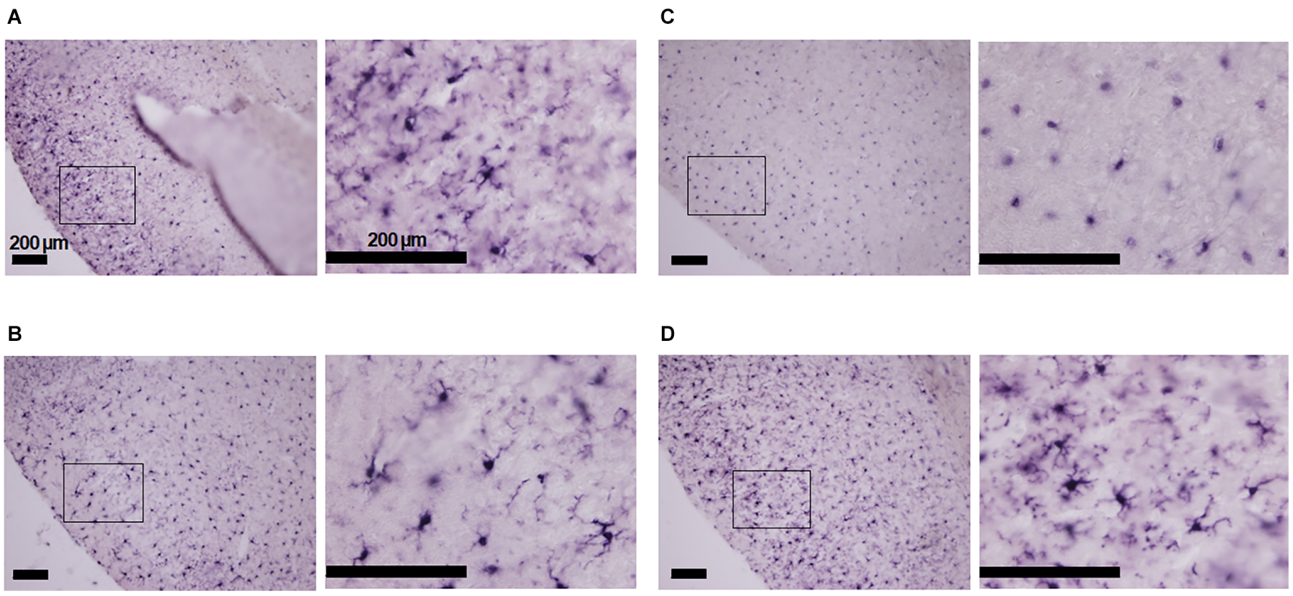

FIGURE 1
Morphology of Iba-1 positive cells in the piriform cortex of mice in four conditions: with and without minocycline, without and with hypoxic (7% O_2) loading. **(A)** Room air without minocycline: ramified non-active cells. **(B)** Room air with minocycline: ramified cells. **(C)** Hypoxia without minocycline: activated ovoid cells. **(D)** Hypoxia with minocycline: mostly ramified Iba-1 positive cells intermingled with some ovoid cells. Minocycline suppressed hypoxia-induced microglial activation to some extent in the epileptogenic piriform cortex. The panels' left-sided rectangles were blown-up in the corresponding right panels. The scale bars are all 200 µm.

from the vehicle to minocycline administration both in room air ($p < 0.05$) and in hypoxia ($p < 0.005$). The *post-hoc* paired *t*-tests showed a significant reduction in theta power from room air to hypoxia in conditions both without pre-administration of minocycline ($p < 0.05$) and with minocycline ($p < 0.01$). Changes in gamma power were not in harmony with those in theta power. There were no main effects on the gamma power of oxygen conditions ($F(1, 17) = 0.529$, $p = 0.477$), pharmacological conditions ($F(1, 17) = 3.733$, $p = 0.070$), and no interaction between the two ($F(1, 17) = 0.949$, $p = 0.344$). Time to seizure occurrence was significantly longer in the minocycline than in vehicle pre-treated mice ($p < 0.05$; Figure 4B). Following the seizures, mice frequently exhibited respiratory arrests. Figure 5 shows that the minocycline pre-treated mice tended to have fewer respiratory arrests compared to the vehicle pre-treated mice ($p = 0.12$).

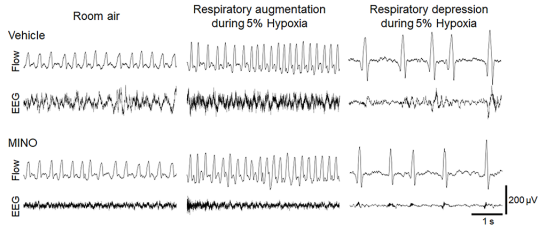

FIGURE 2
Representative raw recordings of respiratory flow (inspiration upward) and EEG signal in mice without and with minocycline in room air and 5% hypoxia showing the initial ventilatory augmentation and late ventilatory fall-off. Minocycline failed to influence the hypoxic ventilatory response.

Discussion

Here, we investigated the role of microglia in the appearance of severe hypoxia-induced seizures and post-ictal respiratory arrest. This comparative study used a pharmacologic tool consisting of minocycline to unravel the potential role of microglia in the ventilatory responses to hypoxia and the appearance of seizures. Our immunohistological investigation showed that Iba-1 positive cells converted from the resting ramified to activated

TABLE 1 Respiratory variables in room air and during the respiratory augmenting phase in 5% hypoxia in the two conditions, without and with minocycline pre-treatment.

	$\dot{V}_E$ [ml/g/min]		V_T [µl/g]		RR [breath/min]	
	Vehicle	Minocycline	Vehicle	Minocycline	Vehicle	Minocycline
Room air	0.82 ± 0.14	0.73 ± 0.12	5.00 ± 0.70	4.47 ± 0.73	163 ± 18	163 ± 14
Hypoxia	1.28 ± 0.26	1.26 ± 0.23	5.30 ± 0.81	6.09 ± 0.97	241 ± 22	207 ± 24

Values are means ± SD. $\dot{V}_E$, minute ventilation. V_T, tidal volume. RR, respiratory rate. All variables increased during hypoxia compared to room air ($p < 0.001$). Minocycline pre-treatment did not significantly affect the variables.

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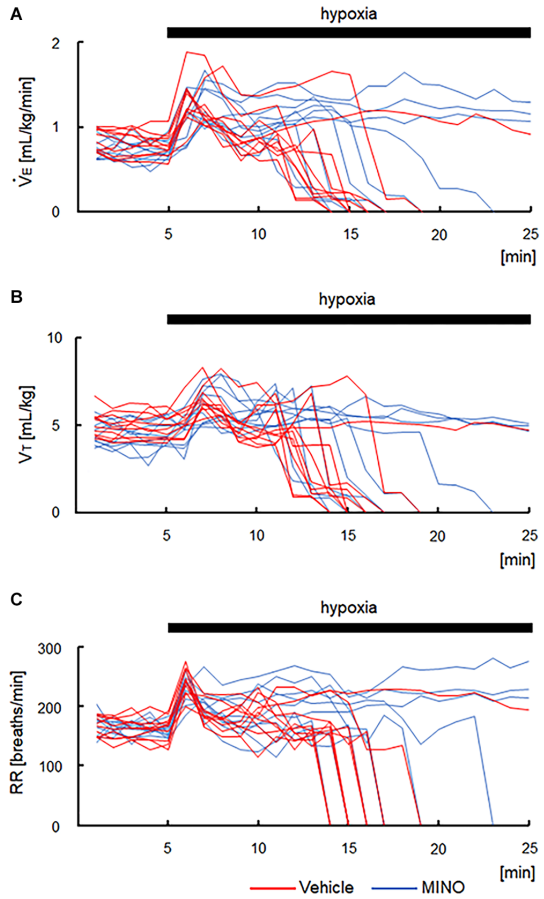

FIGURE 3
Time courses of respiratory variables in the vehicle- and minocycline pre-treated conditions before and during 5% hypoxia. **(A)** Minute ventilation ($\dot{V}_E$). **(B)** Tidal volume (V_T) in each mouse. **(C)** Respiratory rate (RR). As compared to vehicle-pre-treated mice, minocycline-pre-treated mice tended to resist respiratory depression and respiratory arrest during the late hypoxic phase. MINO, minocycline.

de-ramified ovoidal form in hypoxia in the control vehicle pre-treated condition. However, pre-treatment with minocycline inhibited the hypoxia-driven conversion. These results suggest that hypoxia did activate microglia, which was suppressed by minocycline. Severe hypoxia, applied for 20 min, initially increased ventilation, but subsequently caused seizures followed by the post-ictal ventilatory fall-off in all but one mouse. Further, compared to the vehicle group, minocycline pre-treated mice showed a delayed appearance of seizures and tended to resist respiratory arrest.

In the present study, minocycline administration did not affect respiratory variables in room air or the initial hypoxic augmentation. These results are at variance with minocycline-induced reductions in hypoxia-induced increases in $\dot{V}_E$ and V_T in rats in the study reported by Silva et al. (2017). However, it should be considered that microglia are positioned upstream of astrocytes. When microglia are activated, they transmit ATP that binds to P2Y1R on astrocytes and activates neighboring astrocytes in a paracrine manner (Pascual et al., 2012). Astrocytes enhance respiratory output by releasing gliotransmitters (Okada et al., 2012; Rajani et al., 2018; Sheikhbahaei et al., 2018). Astrocytes can also act as hypoxia sensors thereby modifying the hypoxic ventilatory response (Tadmouri et al., 2014; Angelova et al., 2015; Fukushi et al., 2016, 2021; Uchiyama et al., 2020). The present results did not support a mediatory role of astrocytes *via* microglia in hypoxic ventilatory augmentation, since minocycline failed to affect respiratory variables. Alternatively, the protocol might not allow sufficient time for the transmission of microglial activation to astrocytes to augment ventilation.

In animal models, activated microglia decrease the seizure threshold by releasing proinflammatory chemokines such as IL-1β or TNF-α, which promotes glutamate release by astrocytes (Volterra and Meldolesi, 2005; Vezzani et al., 2011; Galic et al., 2012; Vezzani et al., 2013; Benson et al., 2015). These effects may be relayed over astrocytes. Pre-treatment with minocycline reduces IL-1β and TNF-α mRNA levels in the rostral ventrolateral medulla after acute hypoxia in rats (Silva et al., 2017). The present findings lend support to scarce animal data showing that brain microglia activated by hypoxia could exert proconvulsive action through the production of IL-1β and TNFα if the suppression of these cytokines by minocycline delayed the onset of seizures.

Microglial and astrocytic activation are well-described features of epilepsy. Dysregulation of astrocytic function contributes to hyperexcitation of neuronal networks leading to seizures (Devinsky et al., 2013; Coulter and Steinhäuser, 2015; Fukushi et al., 2020). Microglial activation occurs before reactive astrogliosis in brain diseases (Sofroniew and Vinters, 2010; Shinozaki et al., 2014). Acute hypoxia activates microglia that enhance astrocytic function through the release of ATP as discussed above. Sano et al. (2021) reported that the sequential activations of microglia and astrocytes are essential for epileptogenesis and susceptibility to seizures in a drug-induced epileptic model. In the present study, hypoxia-induced microglial activation conceivably caused astrocytic activation and induced seizures. The inhibition of microglial activation by minocycline delayed astrocytic excitation and consequently the onset of seizures.

Microglia are activated in human epilepsy, including mesial temporal sclerosis, focal cortical dysplasia, tuberous sclerosis complex, and Rasmussen's encephalitis (Beach et al., 1995; Boer et al., 2006; Devinsky et al., 2013). Consistent with human studies, microglial activation has been found in animal epileptic brain tissue (Taniwaki et al., 1996; Vezzani et al., 2000; Rosell et al., 2003; Dubé et al., 2010; Zolkowska et al., 2012). In the present study, we found microglial excitation in the piriform cortex of mice under severe hypoxia, which was inhibited by pre-treatment with minocycline (**Figure 1**). These results suggest that hypoxia-induced microglial excitation in the brain regions critical for epileptogenesis plays a role in the onset of seizures. Minocycline by inhibiting hypoxia-induced microglial excitation would counteract the lowering of the seizure threshold.

In the present study, minocycline administration suppressed theta power in room air and further suppressed it during severe hypoxia. It has been reported that epileptic mice, but not non-epileptic mice, exhibit a decrease in theta power before a seizure (Milikovsky et al., 2017; Mazziotti et al., 2020). Essentially the same finding has been reported in humans (Bettus et al., 2008; Milikovsky et al., 2017). Our results are consistent with these reports. However, the relationship between theta oscillation and seizures remains unclear. Further research should be undertaken

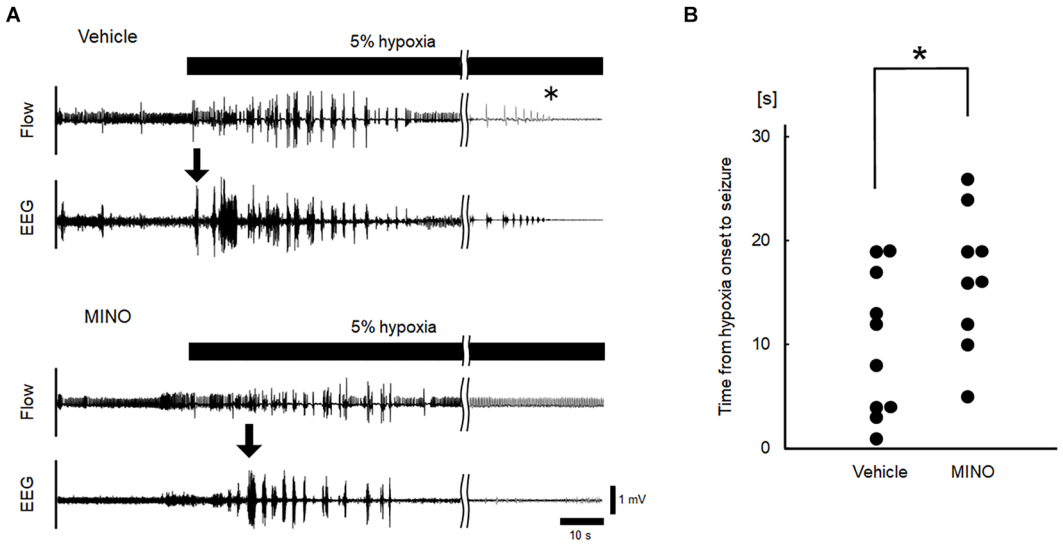

FIGURE 4
Inhibition of microglial activation delayed the occurrence of seizures. **(A)** Representative raw recordings of respiratory flow (inspiration upward) and EEG signals in hypoxia experiments without and with minocycline. Seizures were accompanied by high amplitude aberrant waves in EEG (the onset of seizures was indicated by downward arrows). The time of respiratory arrest was indicated by an asterisk. **(B)** Time from the onset of hypoxia to seizures in vehicle and minocycline pre-treated mice. Time to seizure was significantly longer in the latter group of mice (Mann-Whitney U test). MINO, minocycline. $^{*}p < 0.05$.

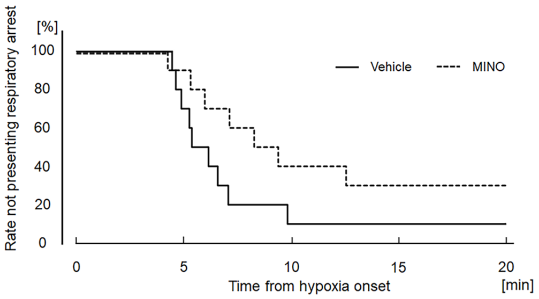

FIGURE 5
Kaplan–Meier curves showing the time from hypoxia onset to respiratory arrest in the vehicle (saline) and minocycline pre-treated mice; 10 mice each. There were no significant differences between the two groups. MINO, minocycline.

to investigate the relationship between EEG power in theta band and seizures. It has also been reported that gamma activity increases before seizures in rodents (Medvedev et al., 2000; Medvedev, 2002; Maheshwari et al., 2016). Our results did not agree with these reports, which may be due in part to the small sample size.

The definition of SUDEP is "sudden, unexpected, witnessed or unwitnessed, non-traumatic and non-drowning death in patients with epilepsy, with or without evidence of a seizure, and excluding documented status epilepticus, where post-mortem examination does not reveal a toxicological or anatomical cause of death" (Nashef, 1997). Dysfunction of the cardiorespiratory autonomic system and sudden death are frequently associated with seizures in chronic epilepsy (Kloster and Engelskjøn, 1999; Langan et al., 2000; So et al., 2000; Opherk et al., 2002; Seyal et al., 2010; Mulkey and Milla, 2022). When patients with epilepsy and respiratory disorders experience hypoxic episodes, microglia-activated neuroinflammatory changes may trigger seizures and cause post-ictal respiratory arrest and SUDEP.

In synopsis, the study suggests that microglia be conducive to seizure activity in severe hypoxia. The finding that minocycline pre-treated mice tended to resist post-ictal respiratory arrest suggests that microglia antagonism could help prevent seizures and SUDEP under severe hypoxia, which would be worthwhile to explore in clinical trials.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Animal Experiment Ethics Committee of Murayama Medical Center.

Author contributions

IF conceived and designed the study, performed the animal experiments, analyzed data, and drafted the manuscript. KI performed the immunohistochemical analyses, and drafted the manuscript. KT performed the statistical analysis and drafted the manuscript. YK, MY, and YH participated in the design of the study. MP edited and revised the manuscript. YO conceived and designed

the study, analyzed the data, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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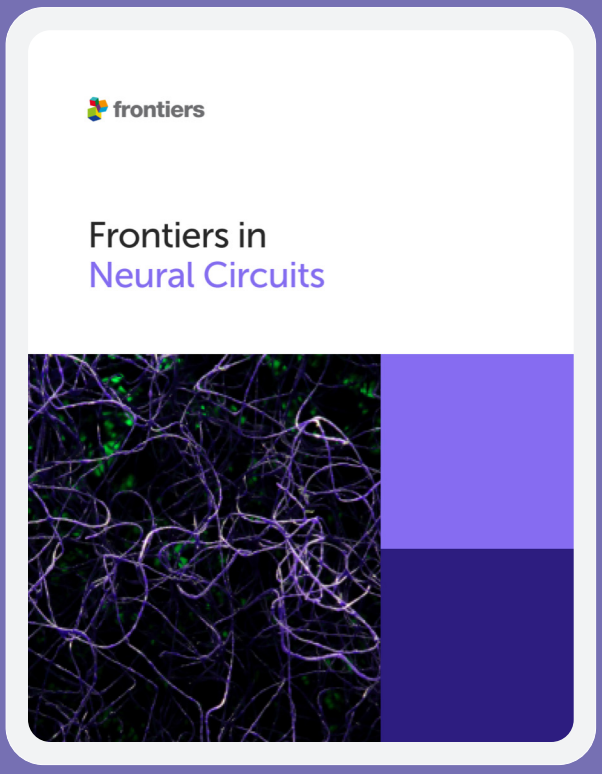

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