

FULL-LENGTH ORIGINAL RESEARCH

The effect of lamotrigine and other antiepileptic drugs on respiratory rhythm generation in the pre-Bötzinger complex

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Abstract

Objective: Lamotrigine and other sodium-channel blocking agents are among the most commonly used antiepileptic drugs (AEDs). Because other sodium channel blockers, such as riluzole, can severely alter respiratory rhythm generation during hypoxia, we wanted to investigate if AEDs can have similar effects. This is especially important in the context of sudden unexpected death in epilepsy (SUDEP), the major cause of death in patients suffering from therapy-resistant epilepsy. Although the mechanism of action is not entirely understood, respiratory dysfunction after generalized tonic-clonic seizures seems to play a major role. **Methods:** We used transverse brainstem slice preparations from neonatal and juvenile mice containing the pre-Bötzinger complex (PreBötC) and measured population as well as intracellular activity of the rhythm-generating network under normoxia and hypoxia in the presence or absence of AEDs.

Results: We found a substantial inhibition of the gasping response induced by the application of sodium channel blockers (lamotrigine and carbamazepine). In contrast, levetiracetam, an AED-modulating synaptic function, had a much smaller effect. The inhibition of gasping by lamotrigine was accompanied by a significant reduction of the persistent sodium current (INap) in PreBötC neurons. Surprisingly, the suppression of persistent sodium currents by lamotrigine did not affect the voltage-dependent bursting activity in PreBötC pacemaker neurons, but led to a hypoxia-dependent shift of the action potential rheobase in all measured PreBötC neurons.

Significance: Our results contribute to the understanding of the effects of AEDs on the vital respiratory functions of the central nervous system. Moreover, our study adds further insight into sodium-dependent changes occurring during hypoxia and the contribution of cellular properties to the respiratory rhythm generation in the pre-Bötzinger complex. It raises the question of whether sodium

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channel blocking AEDs could, in conditions of extreme hypoxia, contribute to SUDEP, an important issue that warrants further studies.

KEYWORDS

lamotrigine, pacemaker, persistent sodium current, pre-Bötzinger complex, SUDEP

1 | INTRODUCTION

Sudden unexpected death in epilepsy patients (SUDEP) is generally defined as death in epilepsy patients that is not triggered by a traumatic or structural cause.¹ The exact patho-mechanism of SUDEP is not entirely understood, but several clinical observations indicate that seizure-induced hypoventilation and cardiac arrhythmia play a major role.²⁻⁴ Data obtained from patients who died of SUDEP in a video-monitoring unit suggest that respiratory drive is crucial to restart the cardiorespiratory function after a generalized tonic-clonic seizure and to secure survival.³ This puts the study of respiratory activity in the focus of better understanding and preventing SUDEP.

The network-generating respiratory activity is located in the brainstem and contains the pre-Bötzinger complex⁵ (PreBötC), the site of rhythm generation of inspiratory activity for eupnea (normal breathing),^{6,7} sighs (augmented breaths),^{8,9} and gasping (the last respiratory efforts during severe hypoxia).^{10,11} The PreBötC is a core of interconnected neurons in the ventral respiratory group and has been identified and well characterized in mammals^{12,13}; it has also been recognized in humans.¹⁴ Evidence for the involvement of the brainstem in SUDEP also comes from recent animal studies, which have confirmed that spreading depolarization in the brainstem during and following seizures leads to cardiovascular arrest and sudden death in mice.^{15,16}

Apart from seizure activity, several studies have shown that the pharmacological modulation of sodium currents directly influences the activity of respiratory neurons^{17,18} and network function.¹⁹

Of interest, the seizure-blocking potential of two of the most commonly used antiepileptic drugs (AEDs), lamotrigine (LTG) and carbamazepine (CBZ), is also thought to partially stem from the inhibition of sodium channels.²⁰⁻²² Therefore, we wanted to know if sodium-channel modulating AEDs could influence respiratory circuits during normal conditions and under severe hypoxic events. For that purpose, we employed a murine *ex vivo* brainstem preparation containing the pre-Bötzinger complex (or PreBötC) and recorded the network and intracellular activity of the respiratory neurons. We believe that this could be of relevance not only to better understand mechanisms of action of widely used AEDs, but also in the context of

Key Points

- Antiepileptic drugs (AEDs) blocking sodium currents such as carbamazepine and lamotrigine did not affect respiratory rhythm during normoxia but disrupted the gasping response in transverse brain stem slices.
- Non-sodium current blocking AEDs (levetiracetam) had only a weak effect on gasping in transverse brainstem slices.
- Lamotrigine reduced the persistent sodium current but did not affect rhythmic bursting of pacemaker neurons in the pre-Bötzinger complex (PreBötC).
- Lamotrigine shifted the action potential rheobase in PreBötC neurons during hypoxia.

SUDEP, where respiratory depression appears to be a driving factor.

2 | METHODS

All animal experiments were performed using approved protocols. Mice were maintained with rodent diet and water available ad libitum in a vivarium with a 12 h light/dark cycle at 22°C. We used CD-1/NMRI F1 hybrids (male and female, P1-P15 for all experiments). All experiments were approved by the Regierungspräsidium Tübingen.

2.1 | The transverse slice preparation

Brainstem transverse slice preparations from mice of either sex (P1–15) were obtained as described previously.²³ Mice were deeply anesthetized with isoflurane (4%) before quick decapitation. Isolated brainstems were then placed in ice-cold artificial cerebrospinal fluid (aCSF) bubbled with carbogen (95% O₂/5% CO₂). The aCSF contained (in mmol): 118 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 24 NaHCO₃, 0.5 NaH₂PO₄, and 30 D-glucose, pH 7.4 with an osmolarity of 305–315 mOsm/L. Brainstems were glued rostral end up onto an agar block for mounting into a vibratome

(Microm, H650 V) and 150–250 μm thin slices were taken until the correct area of the brainstem was approached. As anatomical landmarks, the slices included the inferior olive (IO), nucleus of the solitary tract (NTS), hypoglossal nucleus (XII), and nucleus ambiguus (NA) (Figure S2A).²³ A single 550–650 μm thick slice was then prepared and used for extracellular experiments (including pacemaker blind-patch recordings) or two 300 μm thick slices were used for intracellular visual patch-clamp recordings. Slices for electrophysiology were transferred into a recording chamber, continuously superfused with oxygenated aCSF, and kept at a temperature of $30 \pm 1^\circ\text{C}$. To initiate and maintain fictive respiratory rhythmic activity, the potassium concentration of the aCSF was raised from 3 to 8 mM over a period of 30 min.

2.2 | Extracellular population recordings from the pre-Bötzing complex

In the transverse slice preparation, extracellular population recordings were obtained with suction electrodes positioned on the surface of the ventrolateral region containing the PreBötC. To obtain signal containing multi-unit action potential (AP) activity, extracellular signals were amplified 10 000-fold and filtered between 0.25 and 1.5 kHz using an AM Instruments (A-M Systems) extracellular amplifier or a NPI EXT 10 2F (NPI electronic GmbH). To facilitate the detection of bursts, this signal was rectified and integrated by using an electronic integrator with a time constant of 50–100 ms using an NPI integrator unit INR-011 (NPI electronic GmbH) or offline with a self-written Matlab (MathWorks) script. The bursting activity was categorized into (a) eupnea, (b) sighs (augmented biphasic bursts), and (c) gasping (bursting during hypoxia).^{10,19}

2.3 | Experimental design and statistical analysis

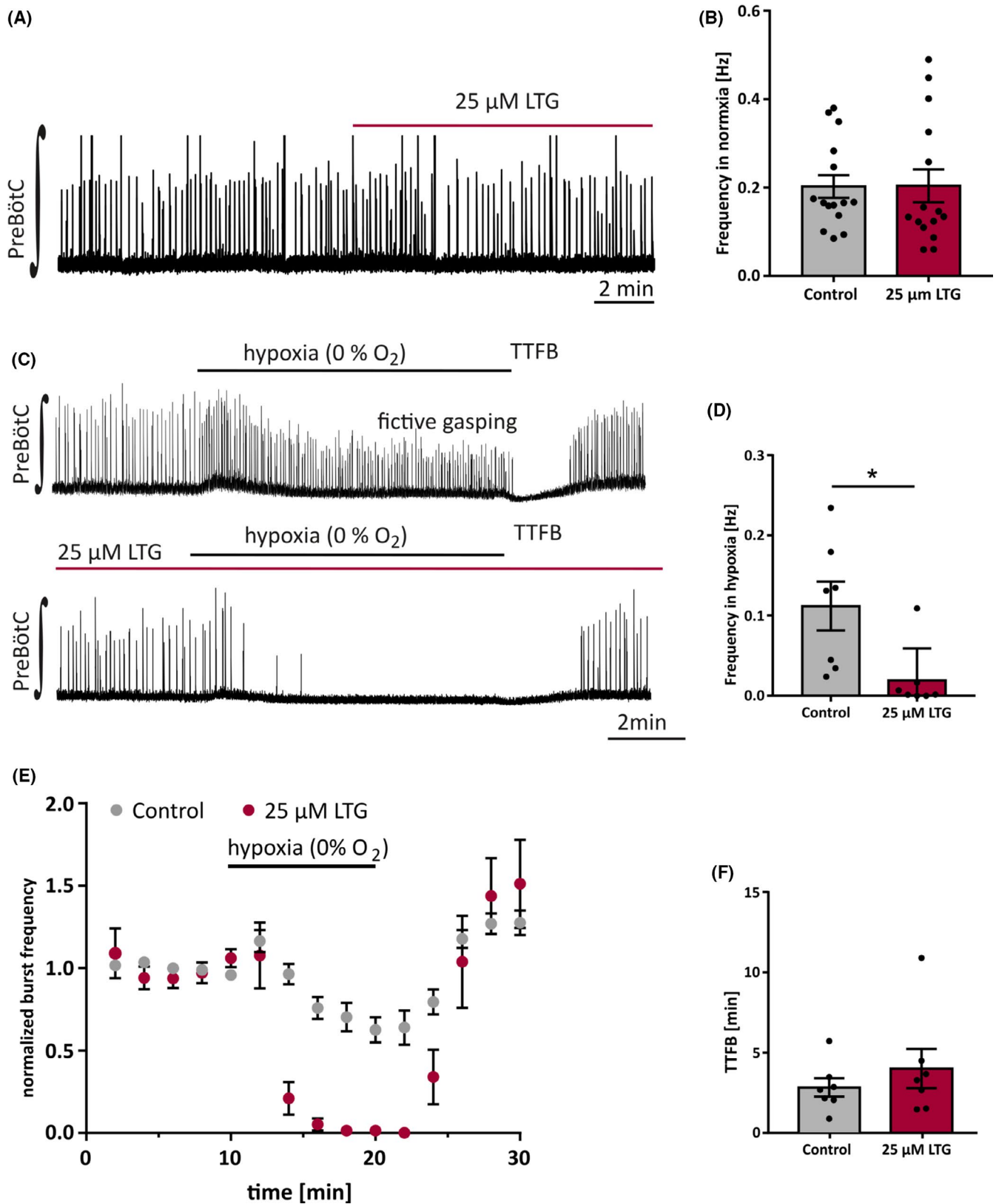
Normalization of values for frequency was performed to the 20-min baseline activity before the first hypoxic exposure. Two controls were used: The first hypoxia before application of the drug in each slice we called control 1. Control 2 was defined as the second hypoxia without application of a drug in a separate set of slices. To validate statistical significance between two groups of the same slice (with and without the drug) we used the Wilcoxon matched-pairs signed-rank test, for two independent groups i.e. control 2 vs a group with medication we used the unpaired Mann-Whitney test. Mean values \pm standard error of the mean (SEM) are presented in text and

figures, unless specified differently. Statistical testing was done with Prism 7.01 (GraphPad) and differences between groups were considered statistically significant when $p < .05$.

2.4 | Intracellular whole cell recordings from pre-Bötzing complex neurons

Whole-cell current-clamp recordings were obtained from PreBötC neurons using either the visual-patch or blind-patch technique and sampled at 20–100 kHz with a low-pass filter of 5–30 kHz. Recordings were performed with unpolished patch electrodes manufactured from borosilicate glass pipettes with filament (Science products). Patch-clamp experiments were performed with a patch-clamp amplifier (Axopatch 200B) or a NPI Bridge Amplifier (Model BA-01X), a digitizing interface (Digidata 1440A or 1550A Digidata), and pClamp 10 software (Molecular Devices). Current clamp recordings were performed after 1 h equilibrating in the patch setup using recording electrodes with a resistance of 3–5 M Ω and filled with an intracellular whole-cell patch-clamp pipette solution containing the following components (in mM): 140 K-gluconic acid, 1 CaCl₂ \times 6 H₂O, 10 EGTA, 2 MgCl₂ \times 6 H₂O, 4 Na₂ATP, and 10 HEPES, pH 7.2, 300 mOsm. Resting potential was measured directly after breaking the seal and APs evoked by a current ramp were measured 5 min after breaking the seal. The junction potential was calculated and subtracted offline to correct the membrane potential in current clamp mode. Pacemaker neurons were identified as spontaneously rhythmic discharging neurons in the presence of CNQX (20 μM) exhibiting voltage-dependent bursting properties as described before.¹⁹

For intracellular persistent sodium current measurements in voltage clamp, slices were maintained at $30 \pm 1^\circ\text{C}$ in a bath solution consisting of the following components (in mM): 100 CH₃SO₃Na, 40 TEA-Cl, 10 HEPES, 2 CaCl₂ \times 2 H₂O, 3 MgCl₂ \times 6 H₂O, 0.2 CdCl₂, 5 4-aminopyridine, 10 glucose, pH 7.4, 300 mOsm. Electrodes with a resistance of 2–5 M Ω were filled with an intracellular pipette solution containing the following components (in mM): 110 CsCl₂, 30 TEA-Cl, 1 CaCl₂ \times 2 H₂O, 10 EGTA, 2 MgCl₂, 4 Na₂ATP, 10 HEPES, pH 7.2, 310 mOsm. To record persistent sodium currents, neurons were held at -80 mV, and a voltage ramp from -80 to 10 mV was injected over a duration of 1.8 s. Cells with a persistent current <15 pA or a leak current >200 pA under control conditions were excluded from analysis. The intracellular whole-cell patch-clamp pipette solutions contained biocytin (5 mg/mL) in all experiments to allow for *post hoc* identification of the location and morphology of recorded neurons (Figure S2B,C).



To perform morphologic reconstruction of biocytin-filled cells, imaging was performed on a Leica TCS SP8 confocal laser-scanning microscope using a 40 \times /1.3NA oil-immersion objective. Z-stack tile scans of the region of interest were acquired. The Cy3 fluorescence was excited

at 548 nm using a tunable white light laser and emission was detected between 555 and 615 nm. For three-dimensional reconstruction of filled neurons, the slices were imaged at a voxel size of 0.2481 \times 0.2481 \times 0.7 μm . The tile scans were stitched using ImageJ-based plugin

FIGURE 1 The effect of lamotrigine (LTG) on the activity generated in the pre-Bötzinger complex (PreBötC). (A) Representative integrated and rectified trace of the activity recorded from the surface of the ventral respiratory group (VRG) under control conditions and under bath application of 25 μ M LTG during normoxia. (B) Averaged frequency of the last 2 min of the network activity under normoxic conditions with and without bath application of LTG. Generated rhythmic activity was not changed by 25 μ M LTG under normoxic conditions ($n = 15$, $p = .7615$). (C) Representative integrated and rectified trace of the activity recorded from the surface of the VRG under control conditions (upper trace) and under bath application of 25 μ M LTG (lower trace) during normoxia, hypoxia, and recovery. (D) Averaged values of the last 2 min of the control hypoxic bouts and the hypoxic bouts in the presence of LTG (25 μ M). Frequency during hypoxia was significantly reduced in the presence of 25 μ M LTG ($n = 7$, $p = .0156$). (E) Plot of the averaged and normalized frequency of the fictive respiratory activity before, during, and after a 10-min hypoxia period. Note the severe reduction of activity in the presence of LTG during hypoxia compared to control ($n[\text{control}] = 7$, $n[25 \mu\text{M LTG}] = 8$). (F) The time to first burst (TTFB) was determined from the moment hypoxia was ended to the re-emergence of bursting activity. In the presence of 25 μ M LTG, the time to first burst was not significantly longer than in control conditions ($n = 7$, $p = .0781$). Statistical analysis by Wilcoxon matched-pairs signed-rank test ($*p < .05$, $**p < .01$); data are presented as mean \pm standard error of the mean (SEM)

for grid-stitching developed by Preibisch et al. Filament reconstruction was performed using neuTube software (Version 1.0z, developed by Ting Zhao, Howard Hughes Medical Institute).

3 | RESULTS

To investigate the effects of commonly used AEDs on respiratory rhythm generation, we measured the effects of three different drugs (lamotrigine, carbamazepine and levetiracetam) on the activity generated by the PreBötC *in vitro*.

3.1 | The effect of the sodium channel blocker lamotrigine on the PreBötC during normoxia

Bath application of the sodium channel blocker lamotrigine (LTG) did not change the frequency or amplitude of fictive eupneic bursts generated in the PreBötC compared to control conditions of the same slices (Figure 1A,B). Increased (50 μ M) or decreased LTG concentrations (5 μ M) also did not change the frequency of fictive eupneic bursts (Figure S1B). Of interest, we found a significant increase in fictive sigh frequency in the presence of LTG at 25 and 50 μ M compared to control conditions (Figure S1E).

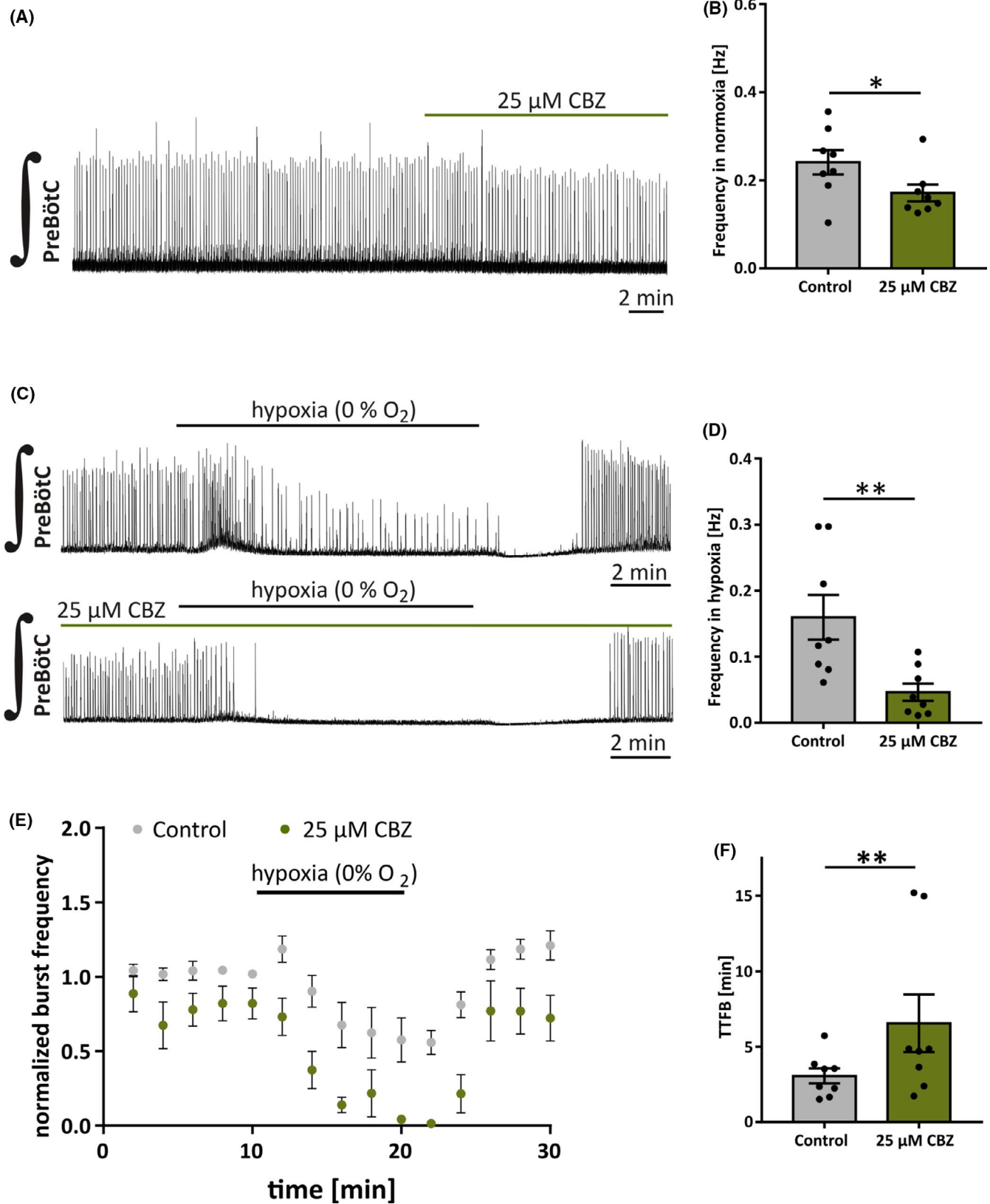
3.2 | The effect of the sodium channel blocker lamotrigine on the PreBötC during hypoxia

It has been postulated that postictal hypoperfusion can cause dysfunction in the brainstem and trigger a cascade of events eventually causing SUDEP.²⁴ Therefore,

we measured the effect of AEDs under hypoxia in our brainstem preparations. For that purpose, we applied a control hypoxia to the slice followed by a second hypoxic period with the drug after recovery (Figure 1C). First, we confirmed that respiratory activity during the second hypoxia is not significantly different from the first hypoxia (Figure S1A). In contrast, both the medium (25 μ M) and high concentrations (50 μ M) of LTG showed a significant reduction in the frequency of fictive gasping compared to their respective control hypoxia periods (Figure 1C-E and Figure S1C). Although the collapse of the inspiratory rhythm was reversible and recovered after removal of hypoxia to the same level as the control slices without LTG application (Figure 1C), the time to first burst (TTFB) after the hypoxic exposure was significantly longer in the presence of 50 μ M LTG compared to the respective control hypoxia periods (Figure S1D). Lower concentrations of LTG did not increase TTFB significantly (Figure 1F and Figure S1D).

3.3 | The effect of the sodium channel blocker carbamazepine on the PreBötC during normoxia and hypoxia

To investigate if the effect of LTG was likely mediated by a modulation of sodium currents, we also tested the effects of carbamazepine (CBZ), another clinically used sodium channel blocker. Similar to LTG, we found a significant reduction of bursting activity during hypoxia compared to control conditions (Figure 2C-E) as well as an increase in TTFB (Figure 2F) under application of 25 μ M CBZ. Of interest, in contrast to LTG, we additionally found a significant reduction in the bursting frequency during normoxia (Figure 2A,B) and a reduction (instead of an increase as caused by LTG) of the number of sighs (Figure S1F) in the presence of CBZ, suggesting additional effects by this drug.



3.4 | The effect of the AED levetiracetam on the PreBötC during normoxia and hypoxia

To investigate whether the effects on the activity generated in the PreBötC are due to a general depression of the

system caused by generic AED treatment, or alternatively whether they are specific to sodium channel blocker type AEDs, we tested the impact of levetiracetam (LEV) (a synaptic vesicle protein 2A ligand) on the PreBötC. LEV (100 μM) did not lead to any significant reduction of the

FIGURE 2 Effects of carbamazepine (CBZ) on pre-Bötzinger complex (PreBötC) under normoxia and hypoxia. (A) Representative integrated and rectified trace of the activity recorded from the surface of the ventral respiratory group (VRG) under control conditions and under bath application of 25 μ M CBZ. (B) Averaged frequency of the last 2 min of the network activity under normoxic conditions with and without bath application of CBZ. Generated rhythmic activity was mildly but significantly reduced by 25 μ M CBZ under normoxic conditions ($n = 8$, $p = .0156$). (C) Representative integrated and rectified trace of the activity recorded from the surface of the VRG under control conditions (upper trace) and under bath application of 25 μ M CBZ (lower trace) during normoxia, hypoxia, and recovery. (D) Averaged values of the last 2 min of the control hypoxic bouts and the hypoxic bouts in the presence of CBZ (25 μ M). Frequency during hypoxia was significantly reduced in the presence of 25 μ M CBZ ($n = 8$, $p = .0078$). (E) Plot of the averaged and normalized frequency of the fictive respiratory activity before, during, and after a 10-min hypoxia period. Note the severe reduction of activity in the presence of CBZ during hypoxia compared to control. (F) The time to first burst (TTFB) was determined from the moment hypoxia was ended to the re-emergence of bursting activity. In the presence of 25 μ M CBZ, the time to first burst was significantly longer than in control ($n = 8$, $p = .0078$). Statistical analysis by Wilcoxon matched-pairs signed-rank test (* $p < .05$, ** $p < .01$); data are presented as mean \pm standard error of the mean (SEM)

activity generated in the PreBötC under normoxic conditions (Figure 3A,B). However, we found a significant increase in the number of sighs (Figure S1G). In hypoxia, 100 μ M LEV slightly but significantly reduced the burst frequency (Figure 3C-E), albeit this was a lot less prominent than with LTG or CBZ. TTFB was not altered by LEV (Figure 3F). Because LEV (considered a non-sodium channel blocker type AED) only mildly mimicked the effects of LTG and CBZ, we hypothesized that LTG- and CBZ-mediated modulation of the respiratory network may be largely dependent on their sodium channel blocking properties.

3.5 | AEDs reduced the persistent sodium current of PreBötC neurons

Because persistent sodium currents play a major role in rhythm generation in the PreBötC,²⁵ we next investigated the effects of AEDs on persistent sodium currents in cells of the PreBötC. In whole-cell patch-clamp recordings under block of calcium (Cd^{2+}) and potassium channels (TEA, 4-AP), moderate (25 μ M) and high (100 μ M) concentrations of LTG—as expected—significantly reduced the persistent sodium current evoked by a voltage ramp (Figure 4A-C).²⁶ For both concentrations of LTG the persistent sodium current was reduced by $46 \pm 5\%$ (Figure 4B,C), whereas LEV (100 μ M) had a statistically significant but overall smaller impact on the amplitude of the persistent sodium current with a reduction of $27 \pm 3\%$ (Figure 4B,C). Meanwhile, hypoxia over 10 min did not change the persistent sodium current (Figure 4B,C).

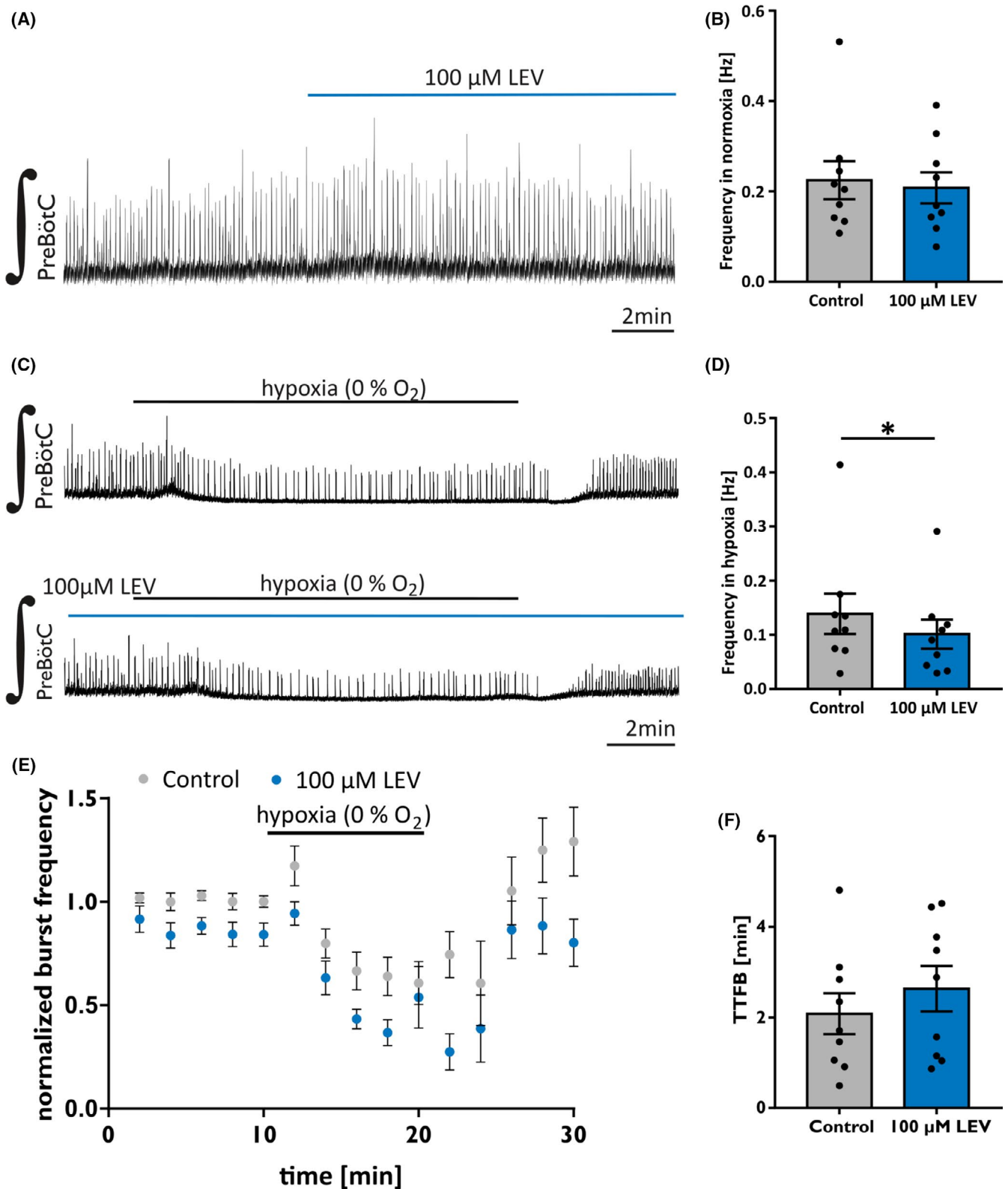
3.6 | LTG did not suppress voltage-dependent pacemaker properties

It is intriguing to speculate that the severely decreased fictive gasping by LTG demonstrated in Figure 1C,D is

mediated by the marked reduction of persistent sodium current (by LTG) in PreBötC neurons (Figure 4A-C) with subsequently impaired rhythm generation in pacemaker cells under hypoxic conditions. To test these hypotheses and to dissect a putative mechanism, we tested the effect of LTG on spontaneously firing cadmium-insensitive pacemakers in whole cell current clamp under normoxic conditions. PreBötC pacemakers were identified by the maintenance of spontaneous and voltage-dependent intrinsic bursting activity (Figure S4A) in the presence of 20 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Figure 5A). Of interest, 25 μ M and 50 μ M LTG did not affect bursting in pacemakers (Figure 5B, Figure S4B) under these conditions. Although this result seemed surprising at first, it is actually in line with our data that LTG does severely affect gasping, but does not interfere with network function under normoxia, indicating potentially state-dependent mechanisms requiring simultaneous hypoxia. In contrast, riluzole (20 μ M) (Figure 5B) abolished rhythmic firing activity in the tested pacemaker neurons as reported previously.¹⁸

3.7 | Lamotrigine decreased PreBötC network excitability under hypoxia due to an increase of neuronal rheobase

The finding that the reduction of the persistent sodium current by LTG had no effects on pacemakers under normoxic conditions led us to the idea that LTG may interfere with the excitability of PreBötC cells exclusively under hypoxia. Therefore, the PreBötC neuron firing threshold was analyzed via a repeating current injection ramp in current clamp mode (150 pA injection over 1 s, one sweep every 20 s) mimicking postsynaptic bursting inputs. In these experiments we did not differentiate between pacemaker cells or non-pacemaker cells, but solely measured cells located within the PreBötC. After 5 min, the cells were exposed to hypoxic conditions followed by wash-in of 25 μ M LTG 10 min later (Figure 6B). In hypoxia, neither firing rate



nor action potential (AP) rheobase changed compared to normoxia (Figure 6). After wash-in of 25 μM LTG under hypoxia, however, mean AP frequency in the range of 100–150 pA current injection decreased significantly (Figure 6A–C). AP rheobase was shifted significantly to higher currents as well (Figure 6A,B,D). This effect of combined hypoxia

and LTG on AP rheobase was reverted by changing back to normoxia (Figure 6D). These findings over the course of hypoxia and LTG wash-in and wash-out are comparable to the network recordings (compare Figure 1C and Figure 6B). Of interest, LTG or hypoxia did not affect AP generation using a standard step current injection protocol (Figure S3).

FIGURE 3 Effects of antiepileptic drug (AED) levetiracetam (LEV) on pre-Bötzing complex (PreBötC) under normoxia and hypoxia. (A) Representative integrated and rectified trace of the activity recorded from the surface of the ventral respiratory group (VRG) under control conditions and under bath application of 100 μM LEV during normoxia. (B) Averaged frequency of the last 2 min of the network activity under normoxic conditions with and without bath application of LEV. Generated rhythmic activity was not changed by 100 μM LEV under normoxic conditions ($n = 9, p = .4258$). (C) Representative integrated and rectified trace of the activity recorded from the surface of the VRG under control conditions (upper trace) and under bath application of 100 μM LEV (lower trace) during normoxia, hypoxia, and recovery. (D) Averaged values of the last 2 min of the control hypoxic bouts and the hypoxic bouts in the presence of LEV (100 μM). Frequency during hypoxia was slightly but significantly reduced in the presence of 100 μM LEV ($n = 9, p = .0195$). (E) Plot of the averaged and normalized frequency of the fictive respiratory activity before, during, and after a 10-min hypoxia period. Note the slight reduction of activity in the presence of LEV during hypoxia compared to control. (F) The time to first burst (TTFB) was determined from the moment hypoxia was ended to the re-emergence of bursting activity. In the presence of 100 μM LEV, the time to first burst was not significantly longer than in control conditions ($n = 9, p = .0977$). Statistical analysis by Wilcoxon matched-pairs signed-rank test ($*p < .05, **p < .01$); data are presented as mean \pm standard error of the mean (SEM)

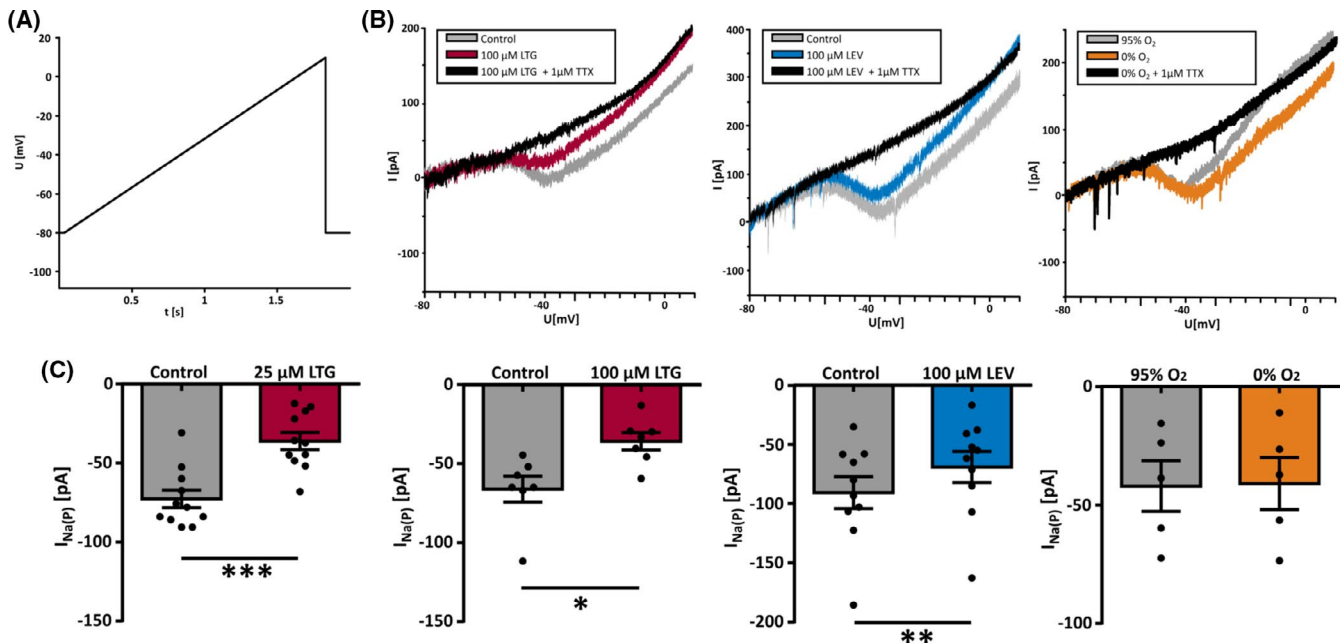


FIGURE 4 Effect of lamotrigine (LTG), levetiracetam (LEV), and hypoxia on the persistent sodium current of pre-Bötzing complex (PreBötC) neurons. (A) Illustration of the voltage ramp used to measure persistent sodium currents. (B) Representative traces of currents recorded before (gray traces) and after the exposure to LTG (red trace), LEV (blue trace), hypoxia (orange trace), and 1 μM Tetrodotoxin (TTX) (black traces). (C) Statistical analysis of recorded persistent sodium currents in pA. Moderate (25 μM ; $n = 11, p = .001$) and high (100 μM ; $n = 7, p = .0156$) concentrations of LTG significantly reduced the amplitude of the persistent sodium current. LEV 100 μM reduced the amplitude of the persistent sodium current slightly but significantly ($n = 10, p = .002$). Severe hypoxia did not change the amplitude of the persistent sodium current ($n = 5, p = .4375$). Statistical analysis by Wilcoxon matched-pairs signed-rank test ($*p < .05, **p < .01$); data are presented as mean \pm standard error of the mean (SEM)

4 | DISCUSSION

4.1 | Clinical relevance of the effects of sodium channel blockers on the activity in the pre-Bötzing complex

We conducted this study to further understand the mechanism of action of AEDs and their potential involvement in the fatal cascades leading to cardiorespiratory dysfunction and SUDEP following a seizure.³ In transverse brainstem preparations, we found that the sodium channel blocking AEDs lamotrigine (or LTG) and carbamazepine (or CBZ),

but not the SV2A modulator levetiracetam (or LEV), significantly impaired the generation of gasps under severe hypoxia. This might indicate that severe hypoxia, as experienced after generalized tonic-clonic seizures (GTCSs), could be aggravated in the presence of LTG and thus possibly increase the risk of SUDEP. Indeed, two initial studies reported increased risk for SUDEP in patients treated with LTG.^{27,28} Several subsequent studies also reported higher odds ratios for SUDEP on LTG in some patient populations; however, these effects were no longer present after correction for GTCSs.^{29–31} It is important to note that this confirms GTCSs and not AEDs as the most important

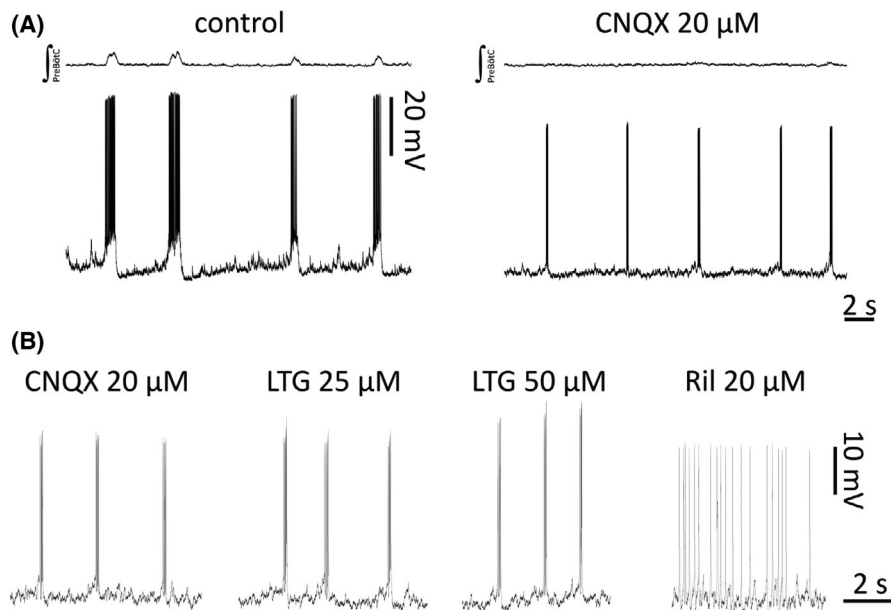


FIGURE 5 No alteration of pacemaker activity *in vitro* by bath application of lamotrigine (LTG). (A) Representative trace of pacemaker neurons. Pacemaker neurons were identified as neurons that fired in phase with the ventral respiratory group (VRG) population recording and continued to produce voltage-dependent bursting in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μ M). (B) Representative traces showing the effects of LTG (25 μ M, 50 μ M) and riluzole (Ril, 20 μ M) on pacemaker neurons. LTG did not affect intrinsic bursting properties at medium ($n = 6$) or higher concentrations, but intrinsic bursting properties were subsequently blocked by riluzole ($n = 3$)

risk factor for SUDEP^{1,4} and underscores the importance of well-balanced AED treatment to prevent GTCs. The exact interaction between AEDs and GTCs in the cascade leading to SUDEP is still poorly understood, and our data suggest it to be an important subject for further study.

The relevance of understanding the AED mechanism of action on the respiratory system is further emphasized by evidence that riluzole has been reported to drastically impair gasping in neonate mice.²⁵ Furthermore, our findings are in line with *in vivo* plethysmographic recordings of awake rats, whose breathing activity was recorded under normoxic, hypoxic (12% O₂), and hypercapnic (5% CO₂) conditions. In this model, intraperitoneal injection of 5 mg/kg LTG severely impaired the hypoxic response and even slowed breathing activity under normoxic conditions.³² It is interesting to note that this study also reported changes at very low concentrations of LTG for which we could not observe effects. Possibly, this could mean that living organisms are more susceptible to inhibitory effects of LTG than *in vitro* models. In addition, this study found an effect of LTG on peripheral chemoreceptors in suction-electrode recordings from an *in vitro* preparation. Like our recordings, 10 μ M LTG decreased chemoreceptor action potential frequency but not amplitude.

The concentrations of lamotrigine used to investigate respiratory network function in this study ranged from 1.280 μ g/mL (5 μ M) to 6.422 μ g/mL (25 μ M) to 12.844 μ g/mL (50 μ M). In patients undergoing surgery for intractable

epilepsy, CSF concentrations of LTG range from 2.15 μ g/mL to 5.99 μ g/mL.³³ These data underscore the clinical relevance of the LTG doses applied in our experiments and inducing hypoxic responses in our *in vitro* preparations.

4.2 | Persistent sodium current and the activity of the pre-Bötzing complex

In this study we investigated the direct effects of AEDs on the network activity generated in the PreBötC and the cellular mechanisms underlying these effects. The fact that both sodium channel blockers tested (LTG and CBZ) led to a significant reduction of the fictive gasping response measured in the transverse slice preparation, while only a mild reduction was found for the SV2A modulator LEV, suggested that the mode of action was dependent mainly on properties of sodium channel gating. This finding is in line with several previous studies that have investigated the role of sodium currents in pre-Bötzing complex neurons and the network response during normoxia and hypoxia.^{18,34–36} In line with our network measurements, we found a significant reduction of the persistent sodium current in single-cell voltage-clamp recordings, which was more pronounced in the presence of LTG compared to LEV. The observed smaller reduction caused by LEV is probably due to an off-target effect of this drug, as seen for many AEDs, and is unlikely to be mediated by its

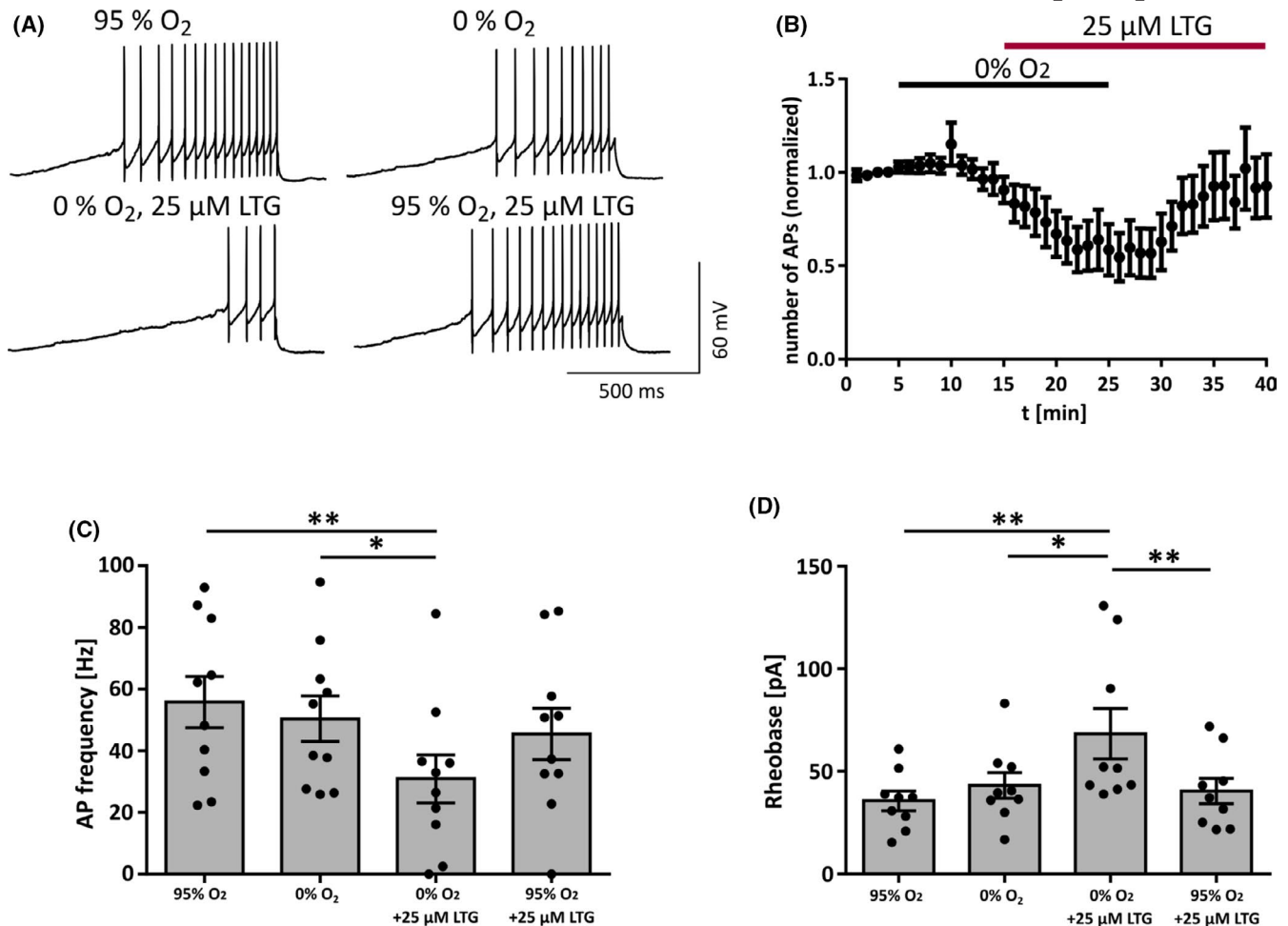


FIGURE 6 Effect of lamotrigine (LTG) and hypoxia on evoked firing in pre-Bötzing complex (PreBötC) neurons at action potential (AP) threshold level. (A) Representative response of a PreBötC neuron to a 150 pA current ramp over a duration of 1000 ms in the presence of normoxia, hypoxia, and 25 μM LTG. (B) Number of evoked APs (normalized to the normoxic control condition) by current ramp injection over the course of hypoxia and wash-in of 25 μM LTG. (C, D) Action potential (AP) frequency in the last 50 pA of injected current ramp injection (C) and rheobase (D) of patched PreBötC neurons under the four different conditions ($n = 6$). Statistical analysis was performed via one-way analysis of variance (ANOVA; Dunn's multiple comparisons test) with * indicating $p < .05$, ** $p < .01$. Data are presented as the mean values with standard error of the mean (SEM)

main mechanism of action, that is, interaction with the synaptic vesicle glycoprotein 2A. Similar to our findings for LTG, the drug riluzole has been shown to inhibit the persistent sodium current without reducing the frequency of the respiratory rhythm generated in the PreBötC under normoxic conditions.^{18,37} It is notable that these studies also established that riluzole at the same concentration abolishes intrinsic bursting of the sodium dependent pacemaker neurons,^{18,37} an effect that we did not find for LTG. Del Negro and colleagues argued that because riluzole at the tested concentrations abolished bursting in these pacemaker cells but no effect was observed on the network output, these cells were irrelevant for the generation of the respiratory rhythm. Peña and colleagues similarly reported that riluzole application did not decrease the frequency of the respiratory rhythm; however, they found that the drug completely abolishes gasping during

hypoxia, an effect that we also saw for LTG. Together with the fact that in contrast to the majority of other respiratory neurons the sodium-dependent (Cd-insensitive, CI) pacemaker neurons stay active during hypoxia, this led to the hypothesis that these “neurons become the essential drivers” of rhythm generation under hypoxic conditions.¹⁸ However, the results of our study seem to not entirely support this hypothesis, since similarly to riluzole LTG did show a reduction in persistent sodium current and a strong inhibition of the gasping activity, yet failed to reveal a clear effect on voltage-dependent bursting of the tested pacemaker neurons under normoxic conditions. In a set of these neurons ($n = 3$) we verified that this bursting behavior was suppressed by riluzole. Although we acknowledge that the sample size was rather small, we deem this to be a surprising finding that deserves further investigation. In addition, we showed that neurons

located in the PreBötC display a reduction of the firing frequency with an increased rheobase in the presence of LTG and hypoxia, which was reversible on the switch back to normoxia (Figure 6), suggesting that not only the intrinsic bursting properties of pacemaker cells but also the overall network excitability is an important factor for fictive gasping under hypoxic conditions.

4.3 | Limitations of this study and potential involvement of other regions in the hypoxic response

It has been shown that the response to hypoxia is multi-centered and involves many sites to finely tune the O₂ and CO₂ levels of the organism. Peripheral chemoreceptors like the ones in the carotid bodies are known to detect changes in blood oxygen levels. When oxygen tension in the peripheral blood decreases, peripheral chemoreceptors increase their rates of APs and send signals to the brainstem to trigger arousal and an increased breathing rate.^{32,38} In our study we used an *ex vivo* preparation of the PreBötC, meaning that we have no information on the activity of peripheral chemoreceptors. Nonetheless, the central nervous system (CNS) is also directly sensitive to changes in central levels of O₂ and CO₂.^{39,40} In the absence of carotid bodies, hypoxia still triggers a ventilatory response,⁴¹ and direct pharmacologically-induced hypoxia in the PreBötC in cats with sodium cyanide (NaCN) leads to excitation of respiratory motor output, further supporting the notion that this important rhythm-generating network is also involved in the chemosensory response.⁴² Taken together, there is growing evidence that there is an important central component to hypoxia and that the PreBötC is an essential part of this response.⁷ Although we cannot exclude that the AEDs we investigated in our study will also have effects on other areas involved in autonomic control, it is in our view important to directly study the activity of the PreBötC and its hypoxic response⁷ in the presence of these drugs. It should also be emphasized that medical drugs and pharmacological agents can have distinct effects on other brain areas involved in respiratory activities. Such effects have been observed for doxapram, a respiratory stimulant used in the treatment of apnea of prematurity.⁴³

Due to technical difficulties in generating *ex vivo* preparations of the PreBötC for suction electrode recordings from older mice, we used neonatal mice in our study (P1-15). It is possible and indeed has been described previously, that functional differences in the PreBötC exist when the first and second postnatal week are analyzed¹⁹ or if prepared from male or female animals.⁴⁴ In our analysis, however, we could not detect such differences. In order to induce rhythmic activity in the brainstem preparation, we

increased the extracellular potassium concentration to 8 mM, which is used in the majority of studies investigating the PreBötC network *in vitro*.^{18,34,37,45,46} Although this is a standard procedure, we cannot exclude that the high extracellular concentration of potassium has altered the response of the PreBötC to the AEDs. One example could be the I_h current, which is present in the PreBötC and has been shown in rat hippocampal neurons to respond to LTG.^{47,48}

In this study, we have used wild-type mice in order to understand the effect of AEDs on the PreBötC in a healthy brainstem. In future studies it will be important to assess if epilepsy changes the activity of the PreBötC and the effects of AEDs described here. In fact, epilepsy can be associated with abnormalities of breathing, such as impaired breathing rate regulation of Dravet-syndrome mouse models when exposed to moderate exercise or temperature challenges.⁴⁹ Another study noted changes in subtype expression and accumulation of dysfunctional γ -aminobutyric acid (GABA) receptors in the PreBötC when comparing mice with severe mutations in GABA receptors leading to Dravet syndrome and SUDEP to mice with less severe mutations associated with absence seizures.⁵⁰

In conclusion, herein we describe the effect of AEDs on the activity of the PreBötC in an isolated slice preparation. Although AEDs blocking sodium currents such as LTG and CBZ dramatically impair the hypoxic response, non-sodium current blocking AEDs such as LEV do not have this effect. It is important to note that LTG reduced the persistent sodium current without affecting the rhythmic bursting behavior of neurons in the PreBötC. During hypoxia, LTG shifted the action potential rheobase in PreBötC neurons. Although the effects of these results on entire organisms in the context of epilepsy will have to be elucidated with further studies, this study helps us to understand the mechanism of action of AEDs on breathing generation.

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CONFLICT OF INTEREST


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

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