ORIGINAL ARTICLE

Modulation of TASK-1/3 channels at the hypoglossal motoneuron pool and effects on tongue motor output and responses to excitatory inputs in vivo: implications for strategies for obstructive sleep apnea pharmacotherapy

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Abstract

Obstructive sleep apnea (OSA) occurs exclusively during sleep due to reduced tongue motor activity. Withdrawal of excitatory inputs to the hypoglossal motor nucleus (HMN) from wake to sleep contributes to this reduced activity. Several awake–active neurotransmitters with inputs to the HMN (e.g. serotonin [5-HT]) inhibit K^+ leak mediated by TASK-1/3 channels on hypoglossal motoneurons, leading to increased neuronal activity in vitro. We hypothesize that TASK channel inhibition at the HMN will increase tongue muscle activity in vivo and modulate responses to 5-HT. We first microperfused the HMN of anesthetized rats with TASK channel inhibitors: doxapram (75 µM, $n = 9$), A1899 (25 µM, $n = 9$), ML365 (25 µM, $n = 9$), acidified artificial cerebrospinal fluid (ACSF, pH = 6.25, $n = 9$); and a TASK channel activator terbinafine (50 µM, $n = 9$); all with and without co-applied 5-HT (10 mM). 5-HT alone at the HMN increased tongue motor activity (202.8% ± 45.9%, $p < 0.001$). However, neither the TASK channel inhibitors, nor activator, at the HMN changed baseline tongue activity ($p > 0.716$) or responses to 5-HT ($p > 0.127$). Tonic tongue motor responses to 5-HT at the HMN were also not different ($p > 0.05$) between ChAT-Cre:TASK$^{f/f}$ mice ($n = 8$) lacking TASK-1/3 channels on cholinergic neurons versus controls ($n = 10$). In freely behaving rats ($n = 9$), microperfusion of A1899 into the HMN increased within-breath phasic tongue motor activity in wakefulness only ($p = 0.005$) but not sleep, with no effects on tonic activity across all sleep–wake states. Together, the findings suggest robust maintenance of tongue motor activity despite various strategies for TASK channel manipulation targeting the HMN in vivo, and thus currently do not support this target and direction for potential OSA pharmacotherapy.

Statement of Significance

The hypoglossal motor nucleus (HMN) innervates the tongue musculature. Strategies to modulate its activity may be beneficial to improve upper airway muscle tone and obstructive sleep apnea (OSA). Here we test the hypothesis that certain potassium "leak" (TASK-1/3) channels at the HMN contribute to resting tongue muscle tone in vivo, and that their targeted manipulation can modulate tongue motor activity. Studies were performed in (1) anesthetized rats, (2) chronically instrumented freely behaving rats awake and asleep, and (3) in mice lacking TASK-1/3 channels on cholinergic neurons. Together, the findings suggest robust maintenance of tongue motor activity despite various strategies for TASK channel manipulation targeting the HMN in vivo, and thus currently do not support this target and direction for potential OSA pharmacotherapy.

Key words: sleep; upper airway; genioglossus; obstructive sleep apnea; TASK channels; animal model

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Introduction

Obstructive sleep apnea (OSA) is a common breathing disorder with serious clinical, social, and economic consequences [1, 2]. OSA is caused by decreased tongue muscle tone in sleep leading to repeated episodes of upper airway closure, asphyxia, and sleep disturbance [3]. Identifying the neural mechanisms causing suppression of tongue muscle activity in sleep is a prerequisite to identifying a potential pharmacological strategy to prevent this suppression and treat OSA [4].

Serotonin (5-HT), noradrenaline, glutamate (via group I metabotropic receptors), thyrotropin-releasing hormone, and Substance P all lead to inhibition of K+ leak generated by TASK-1/3 channels that are highly expressed on pharyngeal motoneurons, leading to neuronal excitation in vitro [5–15]. In vivo, these neuromodulators are released from state-dependent cell groups that are active in wakefulness but are less active in sleep, especially rapid eye movement (REM) sleep [16, 17]. Together, these findings (1) implicate TASK1/3 channels in modulating the excitability of the hypoglossal motoneuron pool in vivo and (2) potentially constitute a *common* downstream mechanism impacted by the *collective* reductions in the wake–active neuromodulator inputs, thus contributing to decrease tongue muscle activity during sleep.

In the present study, we hypothesize that inhibition of TASK channels at the hypoglossal motoneuron pool will increase tongue muscle activity in vivo and modulate responses to applied 5-HT. To test this hypothesis, we first microperfused the hypoglossal motoneuron pool of anesthetized rats with three different TASK channel inhibitors, acidified artificial cerebrospinal fluid (ACSF) that also inhibits TASK1/3 channels, and a TASK channel activator, all with and without co-applied 5-HT. We also tested the effects on tongue motor activity of 5-HT applied to the hypoglossal motoneuron pool in ChAT-Cre:TASK$^{f/f}$ mice that lack TASK-1/3 channels on cholinergic neurons compared to controls. Finally, we also microperfused one of the TASK channel inhibitors into the hypoglossal motoneuron pool of freely behaving rats to test for activation of tongue muscle activity during sleep.

Overall, the findings suggest robust maintenance of tongue motor activity despite various strategies for TASK channel manipulation targeting the hypoglossal motor nucleus (HMN) in vivo, and thus currently do not support this target and direction for potential OSA pharmacotherapy.

Methods

Procedures conformed to the recommendations of the Canadian Council on Animal Care and the University of Toronto Animal Care Committee approved the protocols. Adult male Wistar rats (Charles River) were maintained on a 12/12-h light/dark cycle (lights on at 07:00 am) and had free access to food and water. The studies were performed in male mice to avoid the potential confounding effect of the estrus cycle on hypoglossal motor responses [18, 19]. The numbers used for each individual study and protocol are listed below.

Study 1: TASK channel inhibitors and an activator at the hypoglossal motoneuron pool in anesthetized rats

The first set of experiments was performed under general anesthesia in rats to identify the effects of microperfusion of selected TASK channel modulators into the hypoglossal motoneuron pool on tongue muscle activity and the responses to co-applied 5-HT in a controlled and systematic fashion. Study 1 under general anesthesia was performed prior to assessing the effects of a TASK channel modulator across natural sleep–wake states (Study 3). The choice of general anesthetic for these initial experiments was carefully considered (see Discussion section). Isoflurane is a TASK channel activator but the inhibitory effects of 5-HT and other neuromodulators and interventions on TASK channel activity modulate neuronal activity in vitro [10, 20–23] and in vivo [24].

Animal preparation

A total of 45 rats (mean body weight = 342.7 ± 5.1 [SEM] g; range, 273–455 g) were studied under general anesthesia induced (3%) and maintained (2%–2.5%) with isoflurane sufficient to abolish the corneal blink and pedal withdrawal reflexes, and to provide stable breathing and electroencephalogram (EEG) activity throughout. The level of isoflurane was set in each individual rat to maintain surgical levels of anesthesia and then held constant for the remainder of the experiments within an animal. For the duration of the experiments, the rats also spontaneously breathed 50% oxygen (balance nitrogen) and were kept warm with a heating pad (T-Pump Model #TP500, Gaymar Industries, Inc., Orchard Park, NY, USA). The rats were positioned supine and bipolar stainless steel electrodes (AS636, Cooner Wire, Chatsworth, CA, USA) were implanted onto the side of the diaphragm as it meets the abdominal wall to record diaphragm muscle activity. The rats were then positioned in a stereotaxic frame (Kopf model 962, Tujunga, CA, USA) using blunt ear bars, and with the snout secured in an anesthetic mask (Kopf model 923). Two platinum needle electrodes (Astro-Med Inc., Grass Instrument Division, West Warwick, RI, USA) were also inserted bilaterally into the tongue musculature via a per-oral approach to record tongue muscle activity.

Manipulations of the hypoglossal motoneuron pool

Microdialysis probes (CX-I-12-01, Eicom, San Diego, CA, USA) with a membrane length of 1 mm were placed through a small hole drilled at the junction of the interparietal and occipital bones. The probes were implanted into the hypoglossal motoneuron pool at the following coordinates: 14.2 ± 2.1 mm posterior to bregma, 1.0 mm lateral to the midline, and 9.9 ± 1.5 mm ventral to bregma. In each rat, a brief burst of tongue muscle activity was observed when the probe penetrated the motor pool. This burst of tongue muscle activity during probe insertion was transient (<5 min) and did not affect diaphragm activity or respiratory rate, and was useful as a preliminary indication of probe placement [25, 26]. The rats stabilized for at least 30 min before the onset of any interventions.

The microdialysis probes (outside/inside diameter of 220/200 μm) had a 1 mm membrane length and a 50,000 Dalton cutoff. The probes were connected to fluorinated ethylene propylene (FEP) Teflon tubing (inside diameter = 0.12 mm) in turn connected to 1.0 mL syringes via a zero dead space switch (Uniswitch, B.A.S., West Lafayette, IN, USA). The probes were continually flushed with ACSF at a flow rate of 2.1 μL/min using a syringe pump and controller (MD-1001 and MD-1020, B.A.S.). The lag time for fluid to travel to the tip of the probe at this flow rate was 2.4 min. The ACSF was made fresh each day for each experiment. The composition of the ACSF was 125 mM NaCl, 3 mM KCl, 2 mM CaCl$_2$, 1 mM MgSO$_4$, 25 mM

NaHCO$_3$, and 30 mM glucose. The ACSF was warmed to 37°C and bubbled with CO$_2$ to a pH of 7.4 unless otherwise indicated (see *Protocol 1d*).

Recordings and protocols

The electrophysiological signals were amplified and filtered (Super-Z head-stage amplifiers and BMA-400 amplifiers/filters, CWE Inc., Ardmore, PA, USA). The EEG was filtered between 1 and 100 Hz, and the tongue and diaphragm electromyograms (EMGs) between 100 and 1,000 Hz. The tongue and diaphragm signals were recorded at the same amplification across all experiments. The electrocardiogram was removed from the diaphragm signal using an electronic blanker (Model SB-1, CWE Inc.). The moving-time averages of the tongue and diaphragm EMGs were also obtained with a time constant of 100 ms. The signals were digitized at a sampling rate of 2,000 Hz using a data acquisition system (CED 1401 and Spike 2 software, version 6, Cambridge Electronic Design Ltd., Cambridge, UK).

Protocol 1a—repeatability of responses to ACSF and 5-HT: We first characterized the reproducibility of the tongue motor responses to applied ACSF and 5-HT to be able to identify the effects (if any) of the TASK channel modulators in the subsequent protocols (i.e. the inhibitors in *Protocols 1b–e* and the activator in *Protocol 1f*).

In nine rats, *Protocol 1a* consisted of insertion of the microdialysis probe into the hypoglossal motoneuron pool and stabilization of activity followed by (1) ACSF-1: baseline control period with microperfusion of ACSF for 30 min; (2) 5-HT-1: first application of 10mM 5-HT (Sigma-Aldrich, St. Louis, MO, USA) for 15min; (3) ACSF-2: switch back to ACSF (i.e. "washout") and continued ACSF microperfusion for 30 min after return to baseline activity and/or steady levels; (4) ACSF-3: continued ACSF for another 30 min (for comparison with the other ACSF periods, and to be consistent in timeline with *Protocols1b–e* applying the TASK channel modulators); and (5) 5-HT-2: second application of 10 mM 5-HT for 15 min. This dose and duration of 5-HT application are sufficient to produce robust hypoglossal motor responses followed by a return to baseline after returning to ACSF (see Results section). The timeline of the subsequent protocols was similar after insertion of the microdialysis probe into the hypoglossal motoneuron pool and stabilization of activity.

Protocol 1b: In nine rats, the protocol consisted of: (1) ACSF-1; (2) 5-HT-1; (3) ACSF-2; (4) TASK channel inhibitor A1899 (molecular weight [MW]: 500.5, catalogue number: AOB5851, Aobious, Gloucester, MA, USA) applied at 25 μM [27]; and (5) 5-HT-2 in the continuing presence of A1899 (i.e. co-application).

Protocol 1c: In nine rats, the protocol consisted of: (1) ACSF-1; (2) 5-HT-1; (3) ACSF-2; (4) TASK channel inhibitor doxapram (MW: 433.0, catalogue number: 7081-53-0, Sigma) applied at 75 μM [28]; and (5) 5-HT-2 in the continuing presence of doxapram.

Protocol 1d: In nine rats, the protocol consisted of: (1) ACSF-1; (2) 5-HT-1; (3) ACSF-2; (4) TASK channel inhibitor ML365 (MW: 360.4, catalogue number: 5337, Tocris, Bristol, UK) applied at 25μM [29]; and (5) 5-HT-2 in the continuing presence of ML365.

Protocol 1e: In nine rats, the protocol consisted of: (1) ACSF-1; (2) 5-HT-1; (3) ACSF-2; (4) acidified ACSF at a pH of 6.25; and (5) 5-HT-2 in the continuing presence of the acidified ACSF. The acidified pH was also used for TASK channel inhibition [30–32], with the selected pH expected to inhibit TASK-1/3 channels >90% [31].

Protocol 1f: In nine rats, the protocol consisted of: (1) ACSF-1; (2) 5-HT-1; (3) ACSF-2; (4) TASK channel activator terbinafine (MW: 327.9; catalogue number: 6484, Tocris) applied at 50 μM [33]; and (5) 5-HT-2 in the continuing presence of terbinafine. Terbinafine was recently identified as a TASK-3 channel activator with no effect on TASK-1 channels [33].

The dosages for A1899, doxapram, and ML365 were determined through extrapolation from in vitro dose–response relationships [28, 29, 34]. The aim was to choose a dose that was capable of inhibiting TASK-1/3 channels maximally within the limits of solubility. Unlike microinjections or in vitro studies, however, microdialysis perfusion results in delivery to the target of only 10%–20% of the concentration of the agent in the perfusate to the surrounding tissue [35, 36]. We have also shown that microdialysis perfusion of agents into the hypoglossal motoneuron pool typically requires approximately 10 times the dose used in in vitro studies to produce comparable effects [26, 37–40]. ML365, A1899, and terbinafine were each dissolved in dimethyl sulfoxide (DMSO), which was further dissolved in ACSF at a ratio of 1:99 (DMSO:ACSF). Doxapram and 5-HT were dissolved in ACSF.

Analyses

For each agent delivered to the hypoglossal motoneuron pool, measurements were taken over 1-min periods at the end of each intervention (i.e. periods (i) through (v) identified in each protocol). Breath-by-breath measurements of tongue and diaphragm activities were calculated and averaged in consecutive 5-s bins [36, 41]. All values were written to a spreadsheet and then matched to the corresponding intervention at the hypoglossal motoneuron pool to provide a grand mean for each variable, for each intervention, in each rat. The EMGs were analyzed from the respective moving average signal (above electrical zero) and were quantified in arbitrary units. Electrical zero was the voltage recorded with the amplifier inputs grounded. Tongue EMG was quantified as mean tonic activity (i.e. minimal activity in expiration), within-breath phasic activity (i.e. peak inspiratory activity—tonic activity), and peak activity (i.e. tonic + within-breath phasic activity). Respiratory frequency was not controlled under anesthesia. However, the respiratory rates in the presence of general anesthesia allow effects on respiratory-related and tonic tongue activities to be observed [42]. Mean diaphragm EMG amplitudes (i.e. phasic respiratory diaphragm activity) and respiratory rates were also calculated [36, 41].

The latency and duration of 5-HT responses were also measured. Within-breath phasic tongue muscle activity, rather than tonic, was used for the measures as the within-breath phasic response begins before, and ends after, any tonic response. The mean and *SD* of the within-breath phasic tongue muscle activity were calculated over 5-min before the switch to 5-HT. The latency of response was taken as the first 15 s (i.e. three consecutive 5-s bins) in which the within-breath phasic tongue muscle activity exceeded three *SD* above the preceding activity

(i.e. the designated "threshold" for a response). The lag time for fluid to travel from the switch to the tip of the probe (2.4 min) was subtracted from this calculated latency. The end of the response was taken as the first 15 s (i.e. three consecutive 5-s bins) when the within-breath phasic tongue muscle activity returned below the threshold for response. The duration of response was calculated from the difference between these two time-points. Occasionally, if the threshold criteria for the offset of a particular 5-HT response in an animal was not reached, then the duration of response was not calculated for that particular intervention.

Study 2: TASK channel deletion on cholinergic neurons and responses to 5-HT at the hypoglossal motoneuron pool in vivo

The second set of experiments was performed also under general anesthesia to identify the effects of microperfusion of 5-HT into the hypoglossal motoneuron pool on tongue muscle activity in ChAT-Cre:TASK$^{f/f}$ mice that lack TASK-1/3 channels specifically on cholinergic neurons (e.g. hypoglossal motoneurons) [23, 40].

Animal preparation
Eight ChAT-Cre:TASK$^{f/f}$ mice (mean body weight = 29.1 ± 0.5 [SEM] g, range, 27–31g) were studied under general anesthesia induced and maintained with isoflurane as identified above for the studies in anesthetized rats. Ten ChAT-IRES-Cre mice also on a C57BL/6J background (ChAT-Cre:Task$^{+/+}$) were used as controls (mean body weight = 29.3 ± 0.4 g; range, 28–31g). Genotyping was performed as previously described [23, 40]. Measures of respiratory motor activities were also performed as above for the studies in anesthetized rats.

Manipulations of the hypoglossal motoneuron pool
Microdialysis probes (CX-I-8-0.5, Eicom) with a membrane length of 0.5 mm were implanted into the hypoglossal motoneuron pool at the following coordinates: 7.46 ± 0.03 mm posterior to bregma, 0.06 ± 0.02 mm lateral to the midline, and 6.24 ± 0.05 mm ventral to bregma. Consistent with a reduction in sensitivity to the immobilizing effects of isoflurane in ChAT-Cre:TASK$^{f/f}$ mice that lack TASK-1/3 channels specifically on cholinergic neurons [23], there was a small but consistent increase in the concentration of isoflurane required to achieve similar levels of sedation (as judged by stable respiratory motor activities) compared to the controls (1.85% ± 0.03%; range, 1.8%–2.0% vs. 1.56% ± 0.02%; range, 1.5%–1.6%; $p < 0.001$, Mann–Whitney Rank Sum Test).

Recordings and protocol 2
The electrophysiological signals were amplified and filtered as identified above for the studies in anesthetized rats. In both groups of mice for these experiments, the protocol consisted of insertion of the microdialysis probe into the hypoglossal motoneuron pool and stabilization of activity, after which we applied an established protocol [38]. For the present study, this protocol consisted of applying the following concentrations of 5-HT, in ACSF, each for 30 min: 0, 0.001, 0.01, 0.1, 1, 10, 30, and 50 mM.

Analyses
For each concentration, measurements were taken over 1-min periods at the end of each intervention as identified above for the study in anesthetized rats.

Study 3: TASK channel inhibitor at the hypoglossal motoneuron pool in awake and sleeping rats

Animal preparation
Experiments were performed on nine rats (mean body weight = 326.4 ± 12.4 g; range, 280–398 g). General anesthesia was induced by inhaled isoflurane (3%–4%) with the animal in an induction chamber and anesthesia was then maintained via a mask placed over the snout (2.5%–4% isoflurane). Oxygen was administered to the inspired air (50% oxygen, balance air) throughout surgery. The rats were also given buprenorphine (1 mg/kg, subcutaneous) and meloxicam (2 mg/kg) for analgesia. Effective anesthesia was judged by abolition of the pedal withdrawal and corneal blink reflexes. During surgery, body temperature was maintained with a water pump and heating pad (T/Pump-Heat Therapy System, Gaymar, Orchard Park, NY, USA). Under sterile conditions, the rats were implanted with electrodes for the chronic recording of the EEG, and tongue, diaphragm neck EMGs as previously described [41, 43]. Microdialysis guides (CXG-8, Eicom) were targeted stereotaxically 4.5 mm above the hypoglossal motoneuron pool at the following coordinates: 13.8 ± 0.15 mm posterior to bregma (range, 12.8–14.2 mm), 0.03 ± 0.02 mm lateral to the midline (range, 0–0.1 mm), and 5.4 ± 0.06 mm ventral to bregma (range, 5.0–5.5 mm) [36]. An internal cannula (CXD-8, Eicom) was placed inside the guide to keep it patent until the experiments.

After surgery, the rats were transferred to a clean cage and kept warm under a heating lamp until full recovery as judged by normal motor activity, drinking, and eating. The rats were given soft food for the first day after surgery. The rats were then housed individually and recovered for a minimum of 7 days (range, 7–10 days) before the experiments were performed.

Recordings and protocol 3
For recordings, a lightweight shielded cable was connected to the plug on the rat's head. The recording environment consisted of a large open-topped bowl (Rodent Bowl, MD-1514, BAS) housed within an electrically shielded and soundproofed cubicle (EPC-010, BRS/LVE), with the animals free from any disturbance and supplied with fresh bedding, food, and water. A video camera inside the cubicle allowed for continuous monitoring. For habituation, the rats were connected to the cable and swivel apparatus the day before the experiments, typically in the late afternoon from 4:00 pm to 5:00 pm. At that time, the internal cannula was removed from the guide, and the microdialysis probe was inserted (CX-I-12-01, Eicom). The probe projected 4.5 mm from the tip of the guide and therefore targeted the hypoglossal motoneuron pool.

The probes were connected to FEP Teflon tubing (inside diameter, 0.12 mm) connected to 1.0 mL syringes via a zero dead space switch (Uniswitch, BAS). The probes were perfused with freshly made ACSF at a flow rate of 0.8 μL/min overnight (this flow rate was changed to 2.1 μL/min the morning of the experiments and maintained at that rate thereafter). The composition of the ACSF was as for the studies 1 and 2 and applied at a pH of 7.4. The signals were amplified and filtered as identified for studies 1 and 2 and as previously described [41, 43].

The experiments across natural sleep–wake states began at ~08:00 am and were performed during the day when the rats normally sleep. Signals were recorded during microperfusion of ACSF into the hypoglossal motoneuron pool (i.e. control condition) for at least 3 h across the naturally occurring sleep–wake

states. After this time, the perfusion medium was switched to A1899 at 25 μM, which was maintained for 4 h. Data were included for analysis 30 min after the switch to each perfusion medium.

Data analysis

Sleep–wake states were identified visually from inspection of the neck EMG and the EEG and classified using standard criteria, and data analyses were performed as previously described [36, 41, 44]. In summary, measurements of muscle activities within the identified sleep–wake states were made during all periods of wakefulness (>30 s in duration), non-REM sleep (>30 s duration), and REM sleep (>30 s duration). Data were included in the analyses of respiratory activity only if they were obtained during such unequivocal and clearly defined states. Data obtained during transitional states (e.g. drowsiness, arousals from sleep, and transitions from non-REM to REM sleep) were not included in the analyses.

As for the experiments under anesthesia in Studies 1 and 2, breath-by-breath measurements of tongue and diaphragm activities were calculated and averaged in consecutive 5-s bins as previously described [36, 41]. Mean neck muscle activity was also calculated [36, 41]. The EEG was also analyzed as previously described [36, 41]. The EEG was sampled at 500 Hz then analyzed on overlapping segments of 1,024 samples, windowed using a raised cosine (Hamming) function and subjected to a fast Fourier transform to yield the power spectrum [45]. The window was advanced in steps of 512 samples, and the mean power spectrum of the EEG signal for each 5 s epoch was calculated. The power contained within six frequency bands was recorded as absolute power and as a percentage of the total power of the signal. The band limits were δ_2 (0.5–2 Hz), δ_1 (2–4 Hz), θ (4–7.5 Hz), α (7.5–13.5 Hz), β_1 (13.5–20 Hz), and β_2 (20–30 Hz).

Histology

At the end of the study, the animals were overdosed with isoflurane. Upon the cessation of breathing, the animals were transcardially perfused with 30 mL of 0.9% saline, followed by 30 mL of 10% formalin, and the brainstem was extracted and stored in formalin at room temperature. Two days before sectioning, the brainstem was transferred to a 30% sucrose solution for cryoprotection and stored at 4°C. Brains were cut into coronal sections at a thickness of 50 μm for rats, and 40 μm for mice using a cryostat (Leica, CM 1850, Wetzlar, Germany) at –20°C, and sections were mounted on glass slides that were then sealed with Cytoseal 280 (Thermo Scientific, Waltham, MA, USA). Neutral red staining was used to locate the sites of microdialysis. Imaging was performed under bright field microscopy attached to a charge-coupled device camera (Infinity 1 and BX-41, Olympus, Center Valley, PA, USA), and images were captured using the Infinity Capture software (Lumenera, Ottawa, ON, Canada). Final probe placements were determined using atlases for the rat [46] and mouse [47] brains.

Statistics

The analyses performed for each statistical test are included in the text where appropriate. For all comparisons, differences were considered significant if the null hypothesis was rejected at $p < 0.05$ using a two-tailed test. Where post hoc comparisons were performed after analysis of variance with repeated measures (ANOVA-RM), an all-pairwise multiple comparison procedure (Holm–Sidak tests) was used to determine significant differences between conditions. When data were not normally distributed, the equivalent non-parametric test was performed as appropriate (e.g. one-way ANOVA-RM on ranks) and Dunn's Method used for the all-pairwise multiple comparison procedure. Analyses were performed using *Sigmaplot* version 11 (Systat Software Inc., San Jose, CA, USA). Data are presented as means ± SEM unless otherwise indicated.

Results

Study 1: TASK channel inhibitors and an activator at the hypoglossal motoneuron pool in anesthetized rats

Protocol 1a—repeatability of responses to ACSF and 5-HT

Figure 1 shows representative examples from three different rats to repeated applications of ACSF and 5-HT to the hypoglossal motoneuron pool via microdialysis perfusion in *Protocol 1a*. Note the consistency in latency, magnitude, and duration of responses with repeated interventions in the same animal. Individual and group data are shown in Figure 2.

Figure 2, A shows the distribution of microdialysis sites from each experiment in *Protocol 1a*. The sites of microdialysis perfusion were located within or immediately adjacent to the hypoglossal motoneuron pool in all experiments.

Figure 2, B and C identifies that there was a statistically significant effect of the interventions on both within-breath phasic and tonic tongue muscle activities (both $p < 0.001$, one-way ANOVA-RM and one-way ANOVA-RM on ranks, respectively). Within-breath phasic tongue muscle activity was significantly increased with 5-HT-1 and 5-HT-2 compared to all ACSF interventions (each $p < 0.001$, post hoc Holm–Sidak tests). Tonic tongue muscle activity was significantly increased with 5-HT-2 compared to all ACSF interventions (each $p < 0.005$) but not consistently with 5-HT-1 for tonic activity ($p > 0.237$).

Comparison of the consistency of the within-breath phasic, and tonic, tongue muscle activities across ACSF-1, ACSF-2, and ACSF-3 showed that these were statistically indistinguishable across the experiments (all $p \geq 0.936$). Comparison of the repeatability of the within-breath phasic and tonic tongue muscle responses to 5-HT-1 and 5-HT-2 showed that these responses were statistically similar to each other across interventions (each $p \geq 0.240$). In addition, neither the latency nor the duration of responses to 5-HT-1 and 5-HT-2 were different from each other ($p = 0.979$ and 0.937, respectively, paired *t*-tests, Figure 2, D and E).

There was generally a progressive reduction in respiratory rate observed from the beginning to the end of the experiment as indicated in Figure 2, F (both ACSF-1 and 5-HT-1 versus ACSF-3 and 5-HT-2, each $p \leq 0.018$, post hoc Holm–Sidak tests after the initial one-way ANOVA-RM indicated a significant effect of intervention: $p < 0.001$). There was no effect of the interventions on diaphragm amplitude in *Protocol 1a* ($p = 0.977$, one-way ANOVA-RM).
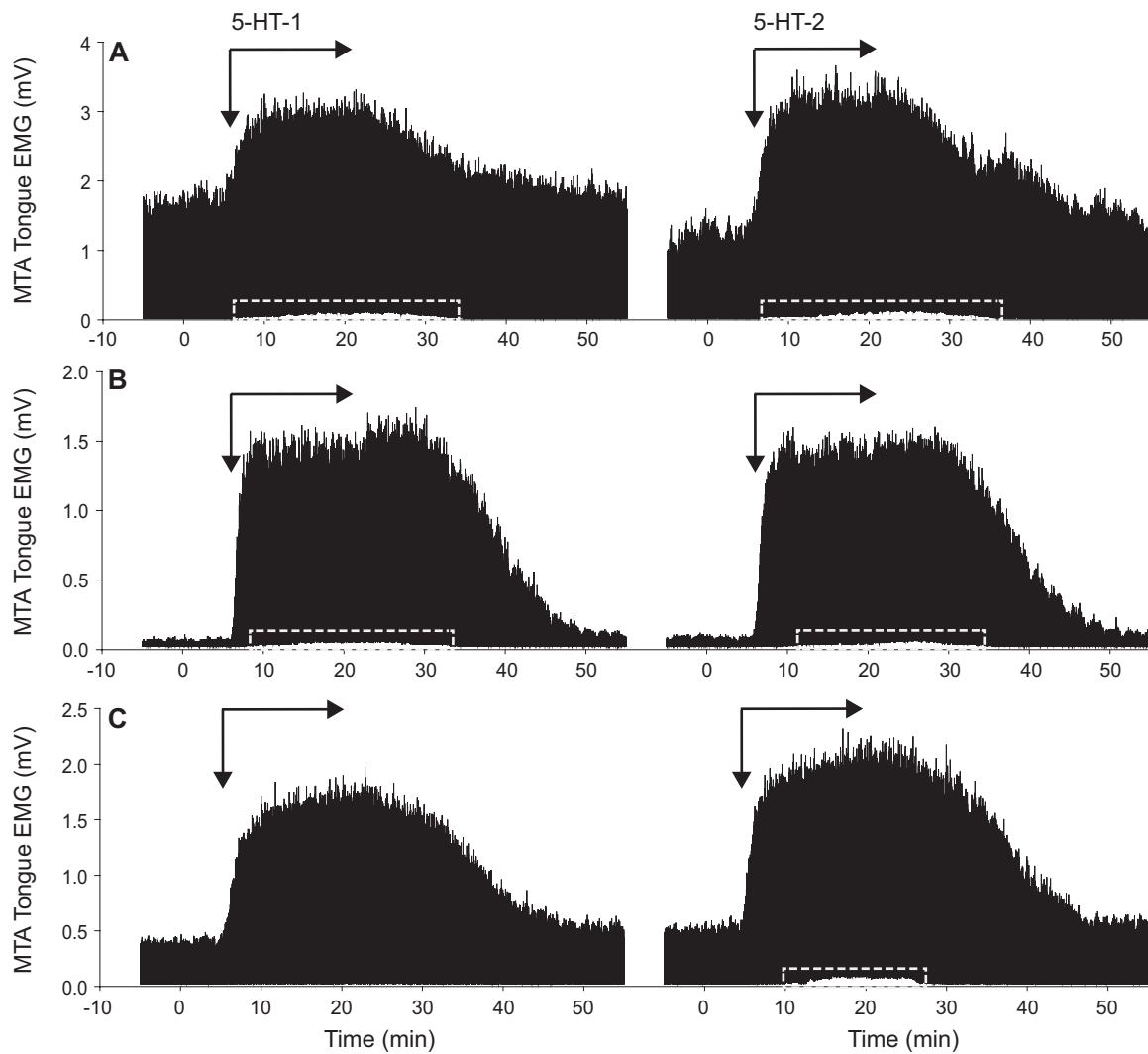
Figure 1. Repeatability of responses to 5-HT. Example responses showing the changes in the moving time average (MTA) signal of tongue EMG activity from three different rats (A–C). Responses are shown to repeated 15-min applications of 5-HT (1 and 2) to the hypoglossal motoneuron pool via microdialysis perfusion in *Protocol 1a*. Microperfusion of ACSF (vehicle control) occurs before and after 5-HT-1 and 5-HT-2 (see text for full protocol). Note the similar latency and magnitude of the tongue muscle responses to applied 5-HT. In rats A and B also note the small increase in tonic tongue muscle activity as indicated by the increase in baseline signal above electrical zero (see dashed grey rectangle), but this effect on tonic activity was inconsistent in rat C (i.e. was observed in response to 5-HT-2 but not 5-HT-1).

Protocols 1b–f—TASK channel inhibitors (A1899, doxapram, ML365, acidified ACSF) and activator (terbinafine):

Figure 3 shows representative traces from each of *Protocols 1b–f* and illustrates the stability and reproducibility of responses to ACSF and 5-HT with and without the various TASK channel interventions at the HMN. Group data from each of these protocols are shown in Figures 4–8.

Panel A of Figures 4–8 show the location of microdialysis sites were within or immediately adjacent to the hypoglossal motoneuron pool for each experiment across all protocols. Across all *Protocols 1b–f*, there was a statistically significant effect of the interventions on both within-breath phasic and tonic tongue muscle activities (all $p < 0.001$, one-way ANOVA-RM and one-way ANOVA-RM on ranks as appropriate). Post hoc tests and the panels B and C of Figures 4–8 identify that compared to the preceding baseline tongue muscle activity with ACSF, none of the various TASK channel inhibitors, or the activator, by themselves had an effect on either within-breath phasic (range of $p = 0.717$–0.971, post hoc Holm–Sidak tests) or tonic muscle activities

(all $p > 0.929$, post hoc Holm–Sidak tests or >0.05 Dunn's tests). Furthermore, there was no significant difference between the responses to 5-HT in the presence or absence of the various TASK channel inhibitors or activator for either within-breath phasic and tonic tongue muscle activity (all $p \geq 0.128$ post hoc Holm–Sidak tests or >0.05 Dunn's test).

Panels D and E of Figures 4–8 also show that none of the various TASK channel inhibitors, or the activator, had an effect of the duration of responses to 5-HT (range of $p = 0.178$–0.607 paired t-tests). Likewise, for A1899, doxapram and acidified ACSF (i.e. three of the four interventions for TASK channel inhibition) and for terbinafine, there was no effect on the latency of responses to 5-HT (range of $p = 0.243$–0.893 paired t-tests). However, the latency of response to 5-HT was increased with ML365 ($p = 0.039$, Wilcoxon Signed Rank Test).

The modest reduction in respiratory rate observed throughout the experiments in *Protocol 1a* (Figure 2, F) was also a general trend observed in *Protocols 1b–f* (see Figures 4, F–8, F with specific statistically significant differences indicated by
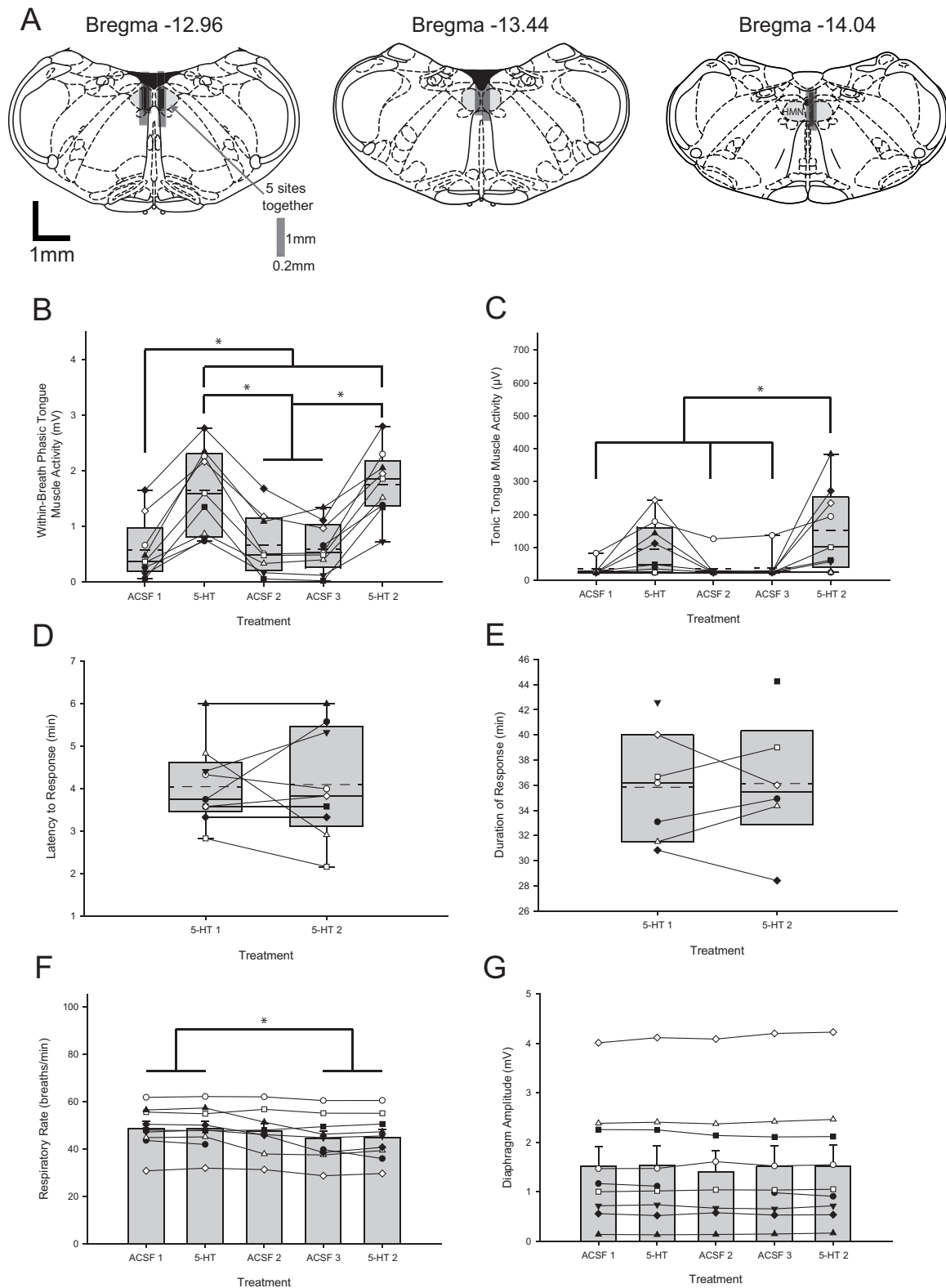
Figure 2. Group data from *Protocol 1a: Repeatability of responses to ACSF and 5-HT*. (A) Location of the microdialysis probes from all the experiments for *Protocol 1a* on coronal diagrams of the rat medulla [46]. Dark grey cylinders, drawn to scale, represent the microdialysis probe locations; overlap obscures some of the individual dialysis sites. The light grey shading represents the HMN. Box and whisker plots show the individual and group data (i.e. median [dashed line], mean [solid line], 25th and 75th percentiles) for within-breath phasic (B), and tonic (C) tongue muscle activity, as well as the latency (D) and duration (E) of the responses to 5-HT-1 and 5-HT-2. Also shown are the mean (+SEM) respiratory rate (F) and diaphragm amplitude (G) for the same periods as tongue muscle activities. Asterisk indicates *p* < 0.05 between the indicated pairs of treatments. See text for further details.
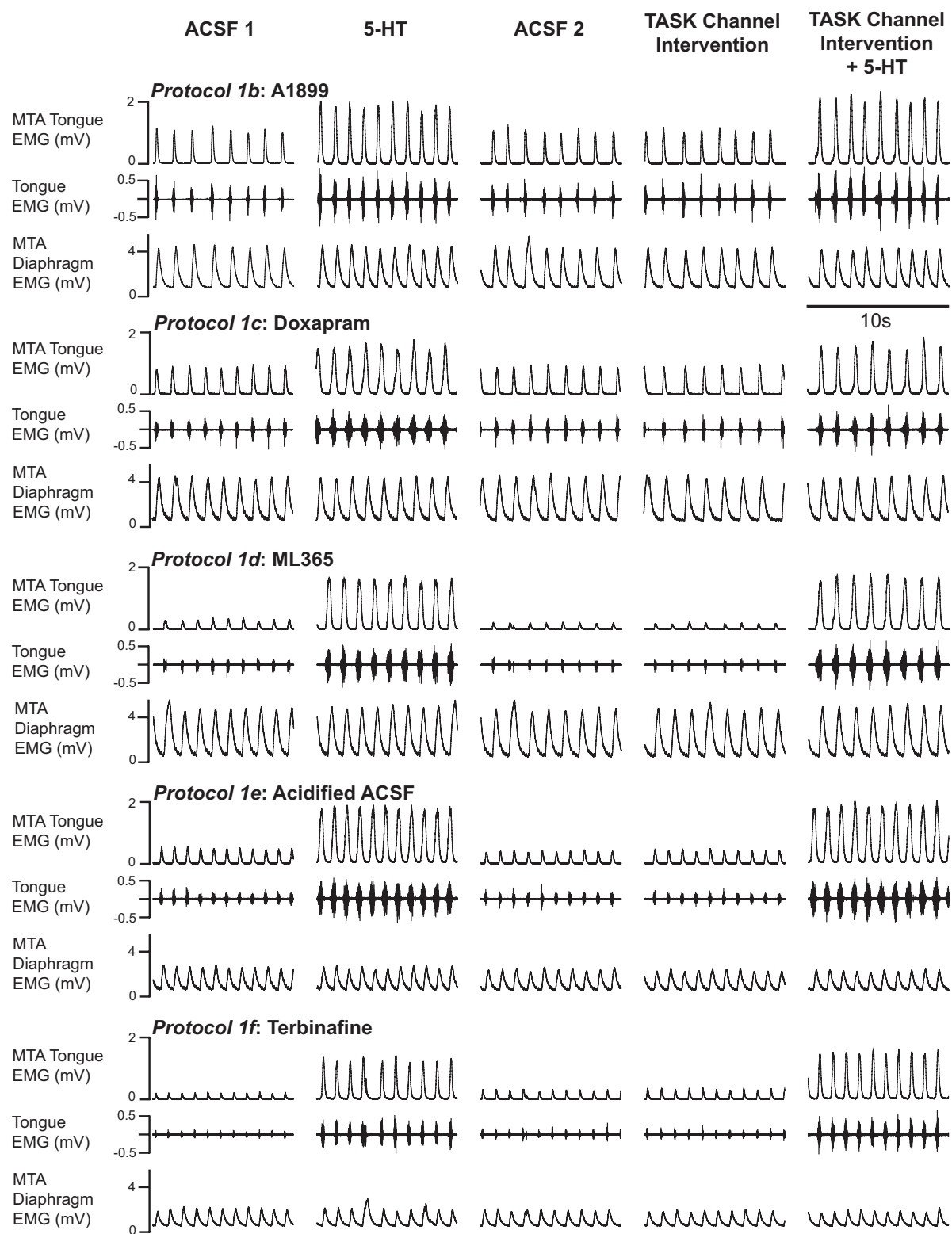
Figure 3. Repeatability of responses to ACSF and 5-HT with and without TASK channel interventions at the HMN across *Protocols 1b–f*. Example responses showing the raw and MTA tongue EMG signals and diaphragm MTA during microperfusion of ACSF and 5-HT into the HMN with and without the TASK channel inhibitors A1899, doxapram, ML365 and acidified ACSF, and the TASK channel activator terbinafine (i.e. *Protocols 1b–f*). Note the stability, reproducibility, and similarity of responses within and between protocols. For each agent delivered to the HMN the sample recordings are taken from the 1-min periods at the end of each intervention used for the data analyses.

the symbol "*"). Likewise, as also observed in *Protocol 1a*, there was no effect of intervention on diaphragm amplitude in each of the other protocols except for *Protocol 1d*, where there was a small but statistically significant increase in diaphragm

amplitude at the end of the experiment compared to the beginning ($p = 0.007$, post hoc Holm–Sidak test after the initial one-way ANOVA-RM indicated a significant effect of intervention: $p = 0.005$).

Study 2: TASK channel deletion on cholinergic neurons and responses to 5-HT at the hypoglossal motoneuron pool in vivo

Figure 9 shows an example and group data for the distribution of microdialysis sites from each experiment in *Protocol 2*. The sites of

microdialysis perfusion were located within or immediately adjacent to the hypoglossal motoneuron pool in all experiments.

Figures 10 and 11 show individual and group average responses to the different levels of 5-HT at the hypoglossal motoneuron pool in ChAT:Cre-TASK$^{f/f}$ mice lacking TASK-1/3 channels
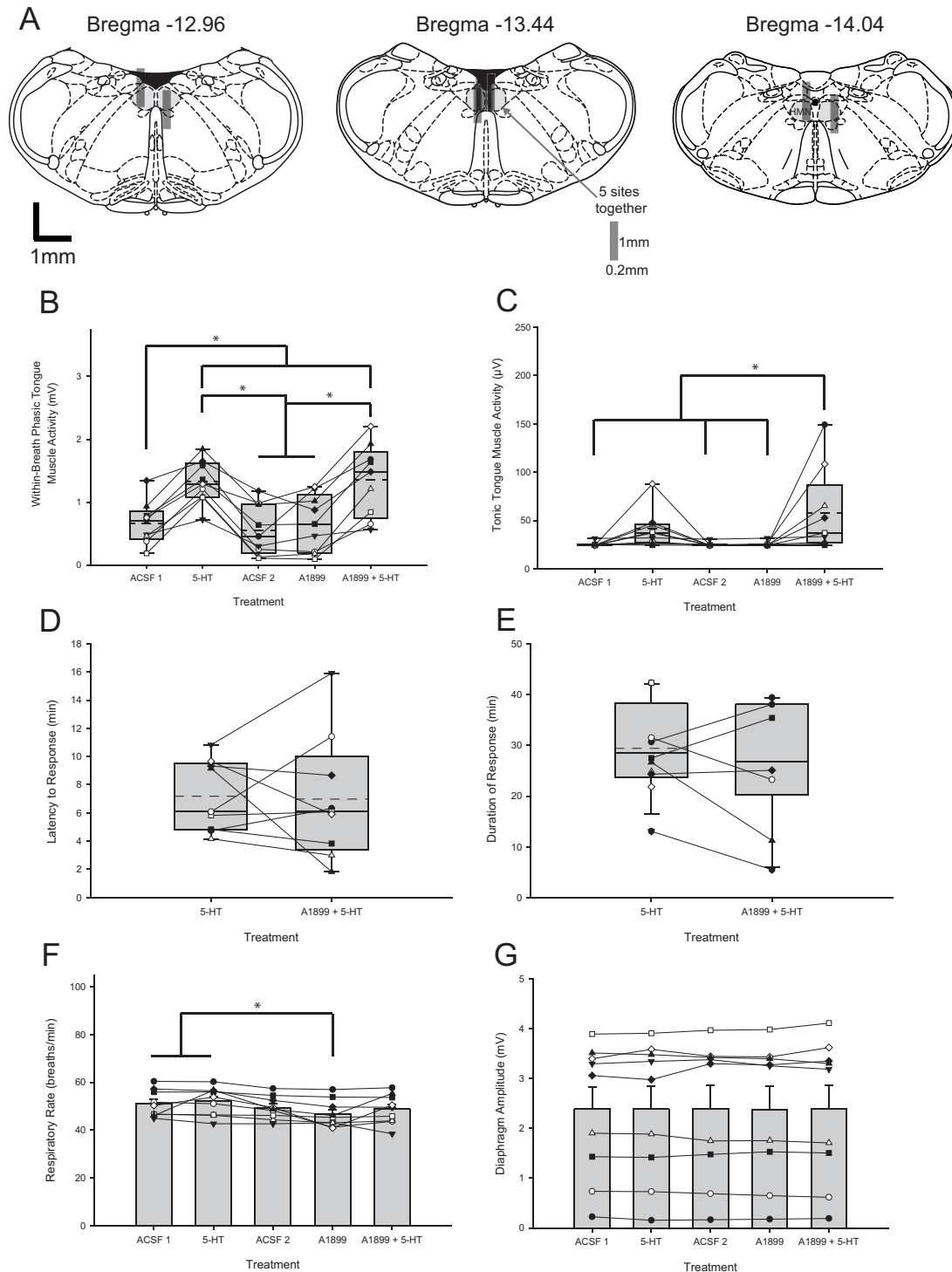

Figure 4. Group data from *Protocol 1b: Effects of the TASK channel inhibitor A1899 at the hypoglossal motoneuron pool on tongue muscle activity and the responses to 5-HT.* (A) Location of the microdialysis probes from all the experiments for *Protocol 1b*. Dark grey cylinders, drawn to scale, represent the microdialysis probe locations; overlap obscures some of the individual dialysis sites. The light grey shading represents the HMN. Box and whisker plots show the individual and group data (i.e. median [dashed line], mean [solid line], 25th and 75th percentiles) for within-breath phasic (B), and tonic (C) tongue muscle activity, as well as the latency (D) and duration (E) of the responses to 5-HT-1 and 5-HT-2. Also shown are the mean (+SEM) respiratory rate (F) and diaphragm amplitude (G) for the same periods as tongue muscle activities. Asterisk indicates $p < 0.05$ between the indicated pairs of treatments. See text for further details.
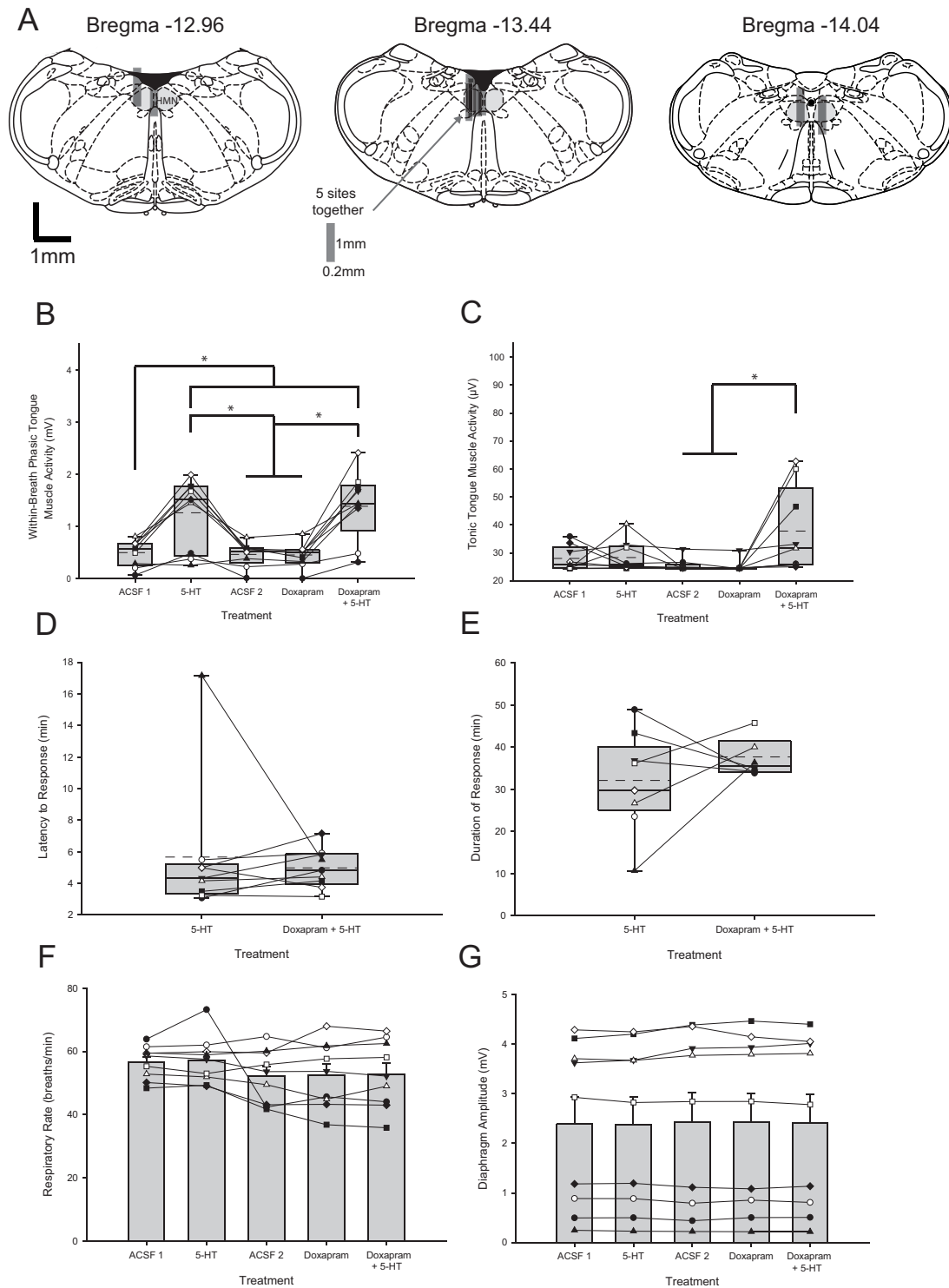
Figure 5. Group data from *Protocol 1c: Effects of the TASK channel inhibitor doxapram at the hypoglossal motoneuron pool on tongue muscle activity and the responses to 5-HT.* (A) Location of the microdialysis probes from all the experiments for *Protocol 1c*. Dark grey cylinders, drawn to scale, represent the microdialysis probe locations; overlap obscures some of the individual dialysis sites. The light grey shading represents the HMN. Box and whisker plots show the individual and group data (i.e. median [dashed line], mean [solid line], 25th and 75th percentiles) for within-breath phasic (B), and tonic (C) tongue muscle activity, as well as the latency (D) and duration (E) of the responses to 5-HT-1 and 5-HT-2. Also shown are the mean (+SEM) respiratory rate (F) and diaphragm amplitude (G) for the same periods as tongue muscle activities. Asterisk indicates $p < 0.05$ between the indicated pairs of treatments. See text for further details.

on cholinergic neurons and controls (*Protocol 2*). These data show that 5-HT had effects on tonic, within-breath phasic, and peak tongue muscle activities depending on the dose applied.

Tonic tongue muscle activity

There was a significant effect of 5-HT at the hypoglossal motoneuron pool on raw tonic tongue muscle activity ($p < 0.001$,
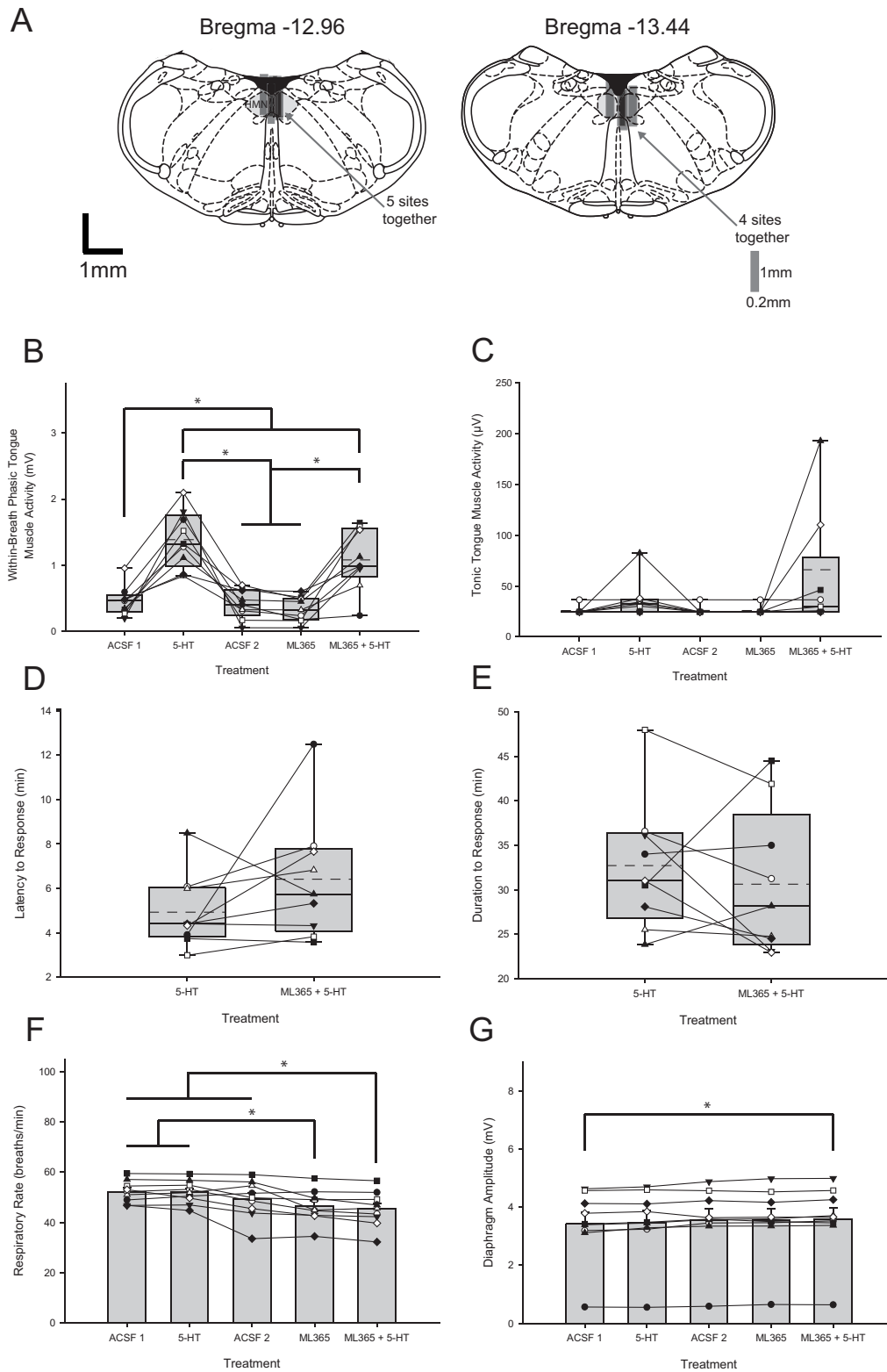
Figure 6. Group data from *Protocol 1d: Effects of the TASK channel inhibitor ML365 at the hypoglossal motoneuron pool on tongue muscle activity and the responses to 5-HT*. (A) Location of the microdialysis probes from all the experiments for *Protocol 1d*. Dark grey cylinders, drawn to scale, represent the microdialysis probe locations; overlap obscures some of the individual dialysis sites. The light grey shading represents the HMN. Box and whisker plots show the individual and group data (i.e. median [dashed line], mean [solid line], 25th and 75th percentiles) for within-breath phasic (B), and tonic (C) tongue muscle activity, as well as the latency (D) and duration (E) of the responses to 5-HT-1 and 5-HT-2. Also shown are the mean (+SEM) respiratory rate (F) and diaphragm amplitude (G) for the same periods as tongue muscle activities. Asterisk indicates $p < 0.05$ between the indicated pairs of treatments. See text for further details.
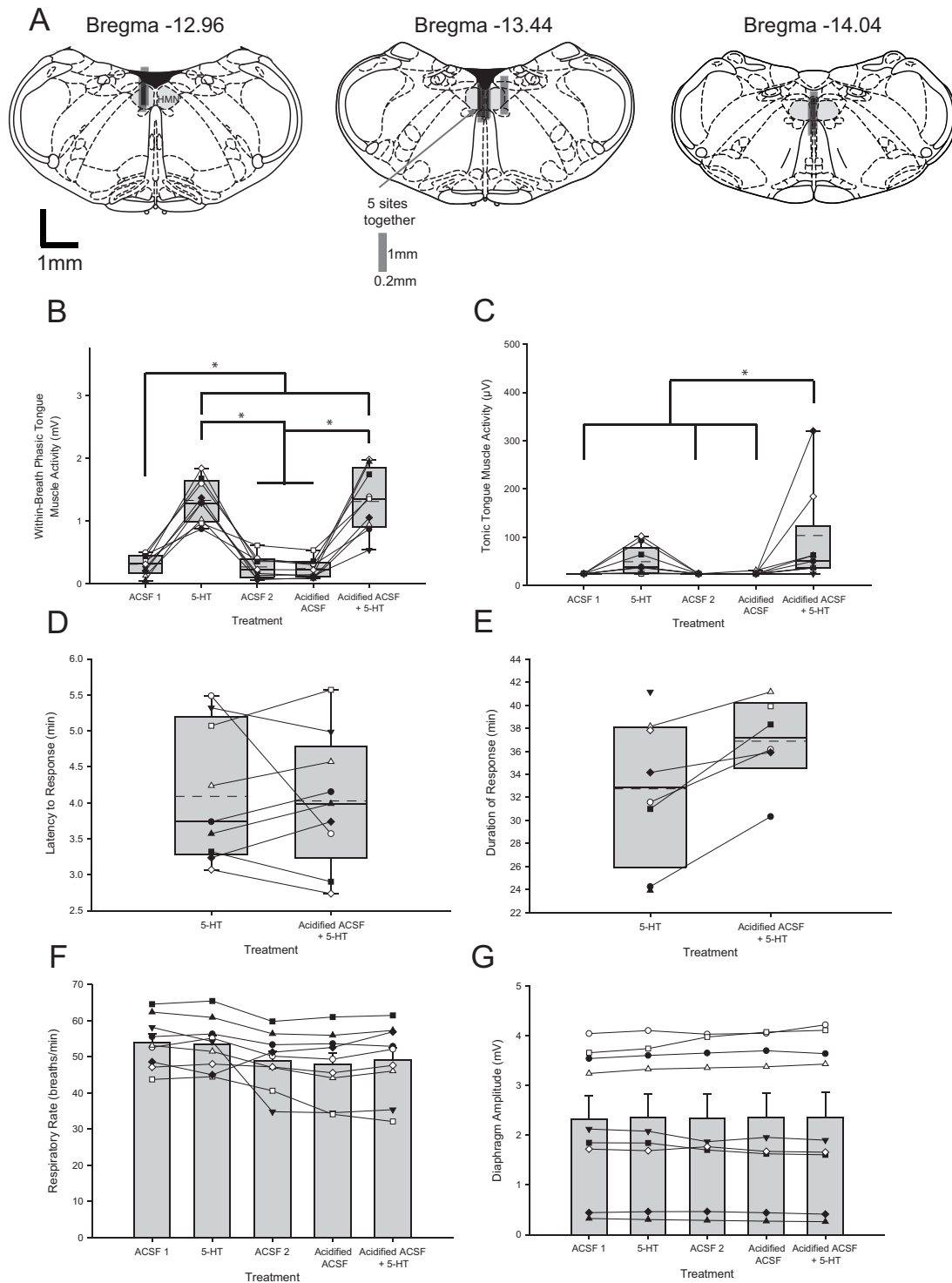
Figure 7. Group data from *Protocol 1e: Effects of acidified ACSF at the hypoglossal motoneuron pool on tongue muscle activity and the responses to 5-HT*. (A) Location of the microdialysis probes from all the experiments for *Protocol 1e*. Dark grey cylinders, drawn to scale, represent the microdialysis probe locations; overlap obscures some of the individual dialysis sites. The light grey shading represents the HMN. Box and whisker plots show the individual and group data (i.e. median [dashed line], mean [solid line], 25th and 75th percentiles) for within-breath phasic (B), and tonic (C) tongue muscle activity, as well as the latency (D) and duration (E) of the responses to 5-HT-1 and 5-HT-2. Also shown are the mean (+SEM) respiratory rate (F) and diaphragm amplitude (G) for the same periods as tongue muscle activities. Asterisk indicates $p < 0.05$ between the indicated pairs of treatments. See text for further details.

two-way ANOVA with one repeated measure). Compared with the baseline ACSF control, tonic tongue muscle activity increased significantly at 1 mM 5-HT ($p < 0.001$, post hoc Holm–Sidak tests) and remained elevated above ACSF for the remaining doses (i.e.

1–50 mM, see symbols "*" in Figure 11, A). The response to 5-HT did not significantly depend on the presence/absence of TASK-1/3 channels on cholinergic neurons, that is, the genotype of the mice ($p = 0.057$).
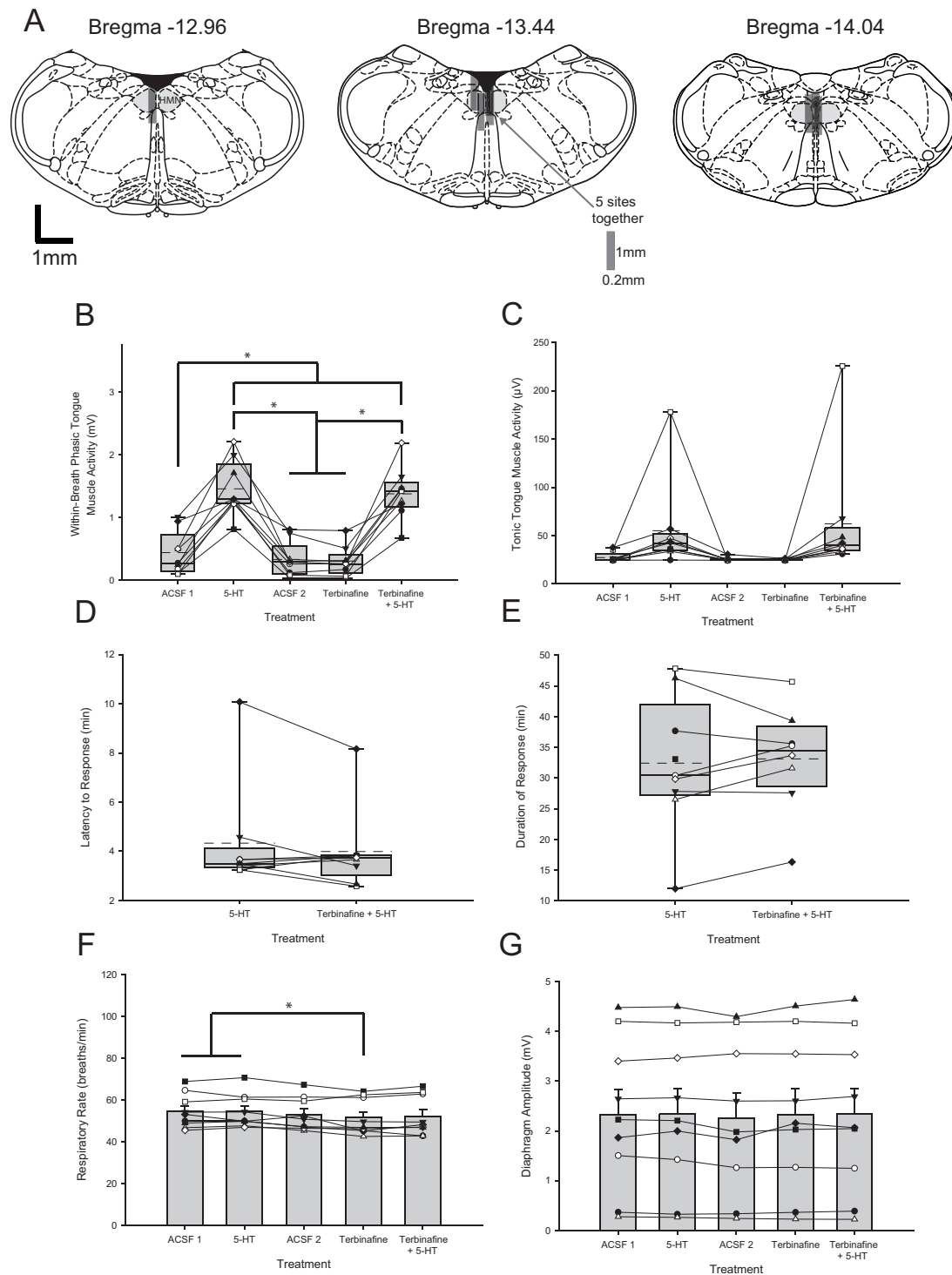
Figure 8. Group data from *Protocol 1f: Effects of the TASK channel activator terbinafine at the hypoglossal motoneuron pool on tongue muscle activity and the responses to 5-HT*. (A): Location of the microdialysis probes from all the experiments for *Protocol 1f*. Dark grey cylinders, drawn to scale, represent the microdialysis probe locations; overlap obscures some of the individual dialysis sites. The light grey shading represents the HMN. Box and whisker plots show the individual and group data (i.e. median [dashed line], mean [solid line], 25th and 75th percentiles) for within-breath phasic (B), and tonic (C) tongue muscle activity, as well as the latency (D) and duration (E) of the responses to 5-HT-1 and 5-HT-2. Also shown are the mean (+SEM) respiratory rate (F) and diaphragm amplitude (G) for the same periods as tongue muscle activities. Asterisk indicates $p < 0.05$ between the indicated pairs of treatments. See text for further details.

These effects of 5-HT were also identified when analyses were performed on tonic tongue muscle activity normalized to the control baseline activity with ACSF, with again the stimulating effect of 5-HT on tonic motor activity (see symbols "*" in Figure 11, B) not depending on genotype ($p = 0.306$).

Within-breath phasic tongue muscle activity

There was a significant effect of 5-HT at the hypoglossal moto-neuron pool on raw within-breath phasic tongue muscle activity that depended on genotype ($p < 0.001$, two-way ANOVA with one repeated measure). Within-breath phasic tongue muscle activity
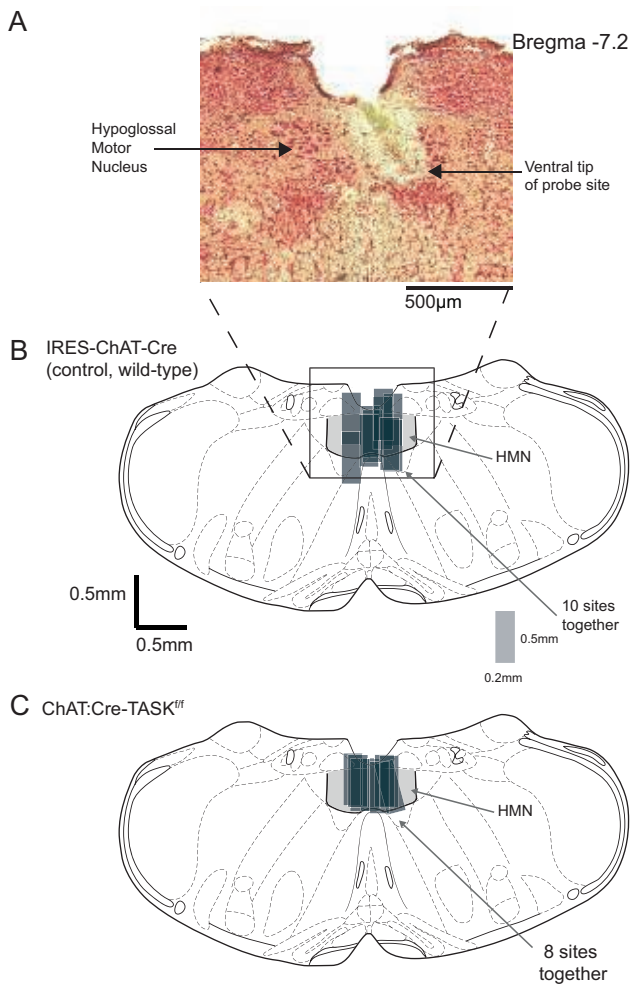
Figure 9. Location of microdialysis probes in Study 2: TASK channel deletion on cholinergic neurons and responses to 5-HT at the hypoglossal motoneuron pool. Example (A) and group (B–C) data showing the location of the microdialysis probes on coronal diagrams of the mouse medulla [47] from all the experiments from *Protocol 2*. The probe locations are shown separately for the controls and the ChAT:Cre-TASK$^{f/f}$ mice that lack TASK-1/3 channels on cholinergic neurons (B and C, respectively). Dark grey cylinders, drawn to scale, represent the microdialysis probe locations; overlap obscures some of the individual dialysis sites. The light grey shading illustrates the HMN.

in the presence of ACSF, 0.001 and 0.01mM 5-HT at the hypoglossal motoneuron pool was lower in the control mice compared to the mice lacking TASK-1/3 channels on cholinergic neurons (each $p < 0.045$, post hoc Holm–Sidak tests, see symbols "#" in Figure 11, C), whereas it was not significantly different between genotypes with 0.1 through 50 mM 5-HT (each $p > 0.185$, Figure 11, C). Consistent with higher levels of 5-HT increasing tonic motor activity and decreasing phasic activity as previously observed in rats [38], there was a reduction in within-breath phasic tongue muscle activity compared to the ACSF controls with 10 through 50 mM in the knock-out mice and 50 mM 5-HT in the control mice (all $p < 0.031$, see symbols "‡" in Figure 11, C).

When normalized to the control baseline activity with ACSF, within-breath phasic tongue muscle activity was reduced with 0.1–30 mM 5-HT in the mice lacking TASK-1/3 channels on cholinergic neurons compared to controls (each $p < 0.020$, post hoc Holm–Sidak tests, see symbols "#" in Figure 11, D). There were also genotype-specific reductions in normalized within-breath

phasic tongue muscle activity with 5-HT compared to the respective ACSF controls (see symbols "‡" in Figure 11, D (each $p < 0.001$, post hoc Holm–Sidak tests).

Peak tongue muscle activity
The effects of 5-HT at the HMN on both raw and normalized peak tongue muscle activity were similar to those identified for within-breath phasic tongue muscle activity (compare panels E and F in Figure 11 with panels C and D, respectively, with significant differences likewise indicated by the symbols "#" and "‡").

Other respiratory parameters
There was a significant decline in respiratory rate observed throughout the experiment ($p < 0.001$, see symbols "*" in Figure 11, G) that did not depend on genotype ($p = 0.4189$). An effect on diaphragm amplitude was also observed that did depend on genotype ($p < 0.001$, Figure 11, H). Compared to the baseline ACSF controls within each group of mice, post hoc testing identified a small but consistent increase in diaphragm amplitude at the end of the experiment when 50 mM 5-HT was applied to the hypoglossal motoneuron pool in the knock-out mice ($p = 0.036$) and decreases in amplitude when 10 through 50 mM was applied in the control mice ($p < 0.018$, see symbols "‡" in Figure 11, H). Un-paired post hoc comparisons between genotypes, however, identified no significant differences throughout the experiments (all $p > 0.177$).

Study 3: TASK channel inhibitor at the hypoglossal motoneuron pool in awake and sleeping rats

Figure 12 shows example traces with microperfusion of ACSF and the TASK channel inhibitor A1899 into the HMN in an awake and sleeping rat. Note the lack of effect of A1899 except for an increase in tongue EMG activity observed in wakefulness.

Figure 13, A shows the distribution of microdialysis sites from each experiment in *Protocol 3*. The sites of microdialysis perfusion were located within or immediately adjacent to the hypoglossal motoneuron pool in all experiments. Figure 13, B–F shows the group average responses to microperfusion of the TASK channel inhibitor A1899 into the hypoglossal motoneuron pool in awake and sleeping rats.

For within-breath phasic tongue muscle activities, the statistical analyses identified that the effects of A1899 on motor activity depended on sleep–wake state ($p = 0.044$, two-way ANOVA-RM), with activity in wakefulness with A1899 being increased compared to ACSF ($p = 0.005$, post hoc Holm–Sidak tests, see symbol "*" in Figure 13, B). However, for both tonic tongue muscle activity, neck muscle activity, respiratory rate, and diaphragm activity, there was no main effect of drug treatment (i.e. ACSF vs. A1899, range of $p = 0.912$–0.213, Figure 13, C–F), nor an interaction effect (i.e. no effect of drug treatment that depended on sleep–wake state, range of $p = 0.911$–0.083), such that further post hoc testing was not performed.

The significant effects of sleep–wake state *per se* on within-breath phasic and tonic tongue muscle activities, neck muscle activity, and respiratory rate (all $p < 0.001$, two-way ANOVA-RM) are identified in Figure 13, B–D and F (see symbols "#").

For total EEG power and EEG power within the specific frequency bands (δ_2, δ_1, θ, α, β_1, and β_2), there was no effect of drug treatment and no statistical interaction (range of $p = 0.947$–0.129).
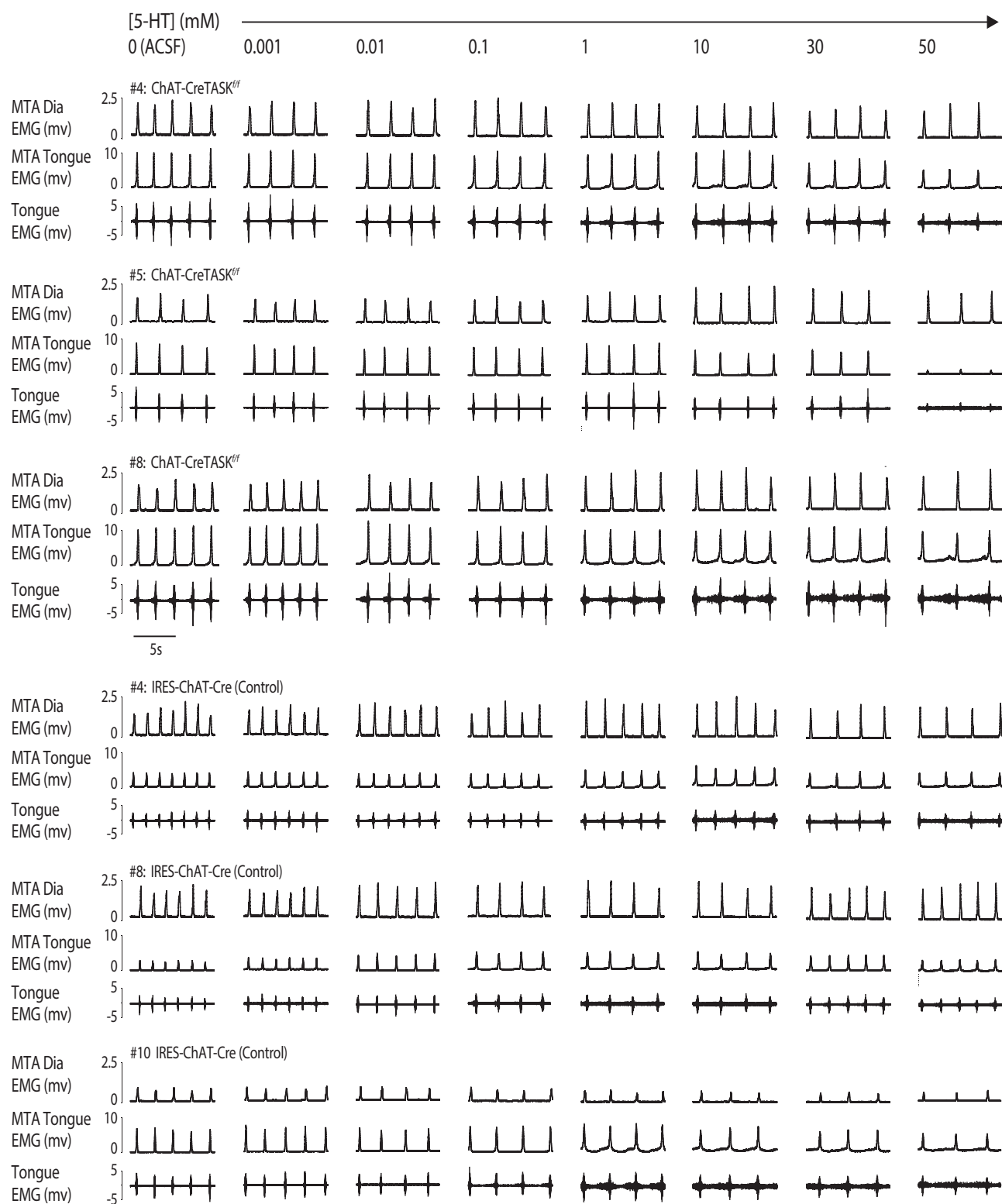
Figure 10. Example traces from Study 2: TASK channel deletion on cholinergic neurons and responses to 5-HT at the hypoglossal motoneuron pool. Example responses showing the raw and MTA tongue EMG signals and diaphragm (Dia) MTA during microperfusion of ACSF and 5-HT into the HMN in ChAT:Cre-TASK$^{f/f}$ mice lacking TASK-1/3 channels on cholinergic neurons and controls. Sample recordings are taken from the 1-min periods at the end of each intervention used for the data analyses. Examples are shown for three mice in each group to illustrate similarities and qualitive differences between mice and across genotypes that are represented in the group data. See text for further details.

Discussion

There is strong evidence that K$^+$ "leak" (TASK-1/3) channels are important determinants of resting membrane potential and excitability of hypoglossal motoneurons in vitro [8, 10, 20–23]. The results of the present in vivo study are *not* taken to contest or challenge the role of such channels in modulating neuronal activity. Rather, the aim of this study was to identify the potential of TASK channel-modifying interventions at the hypoglossal
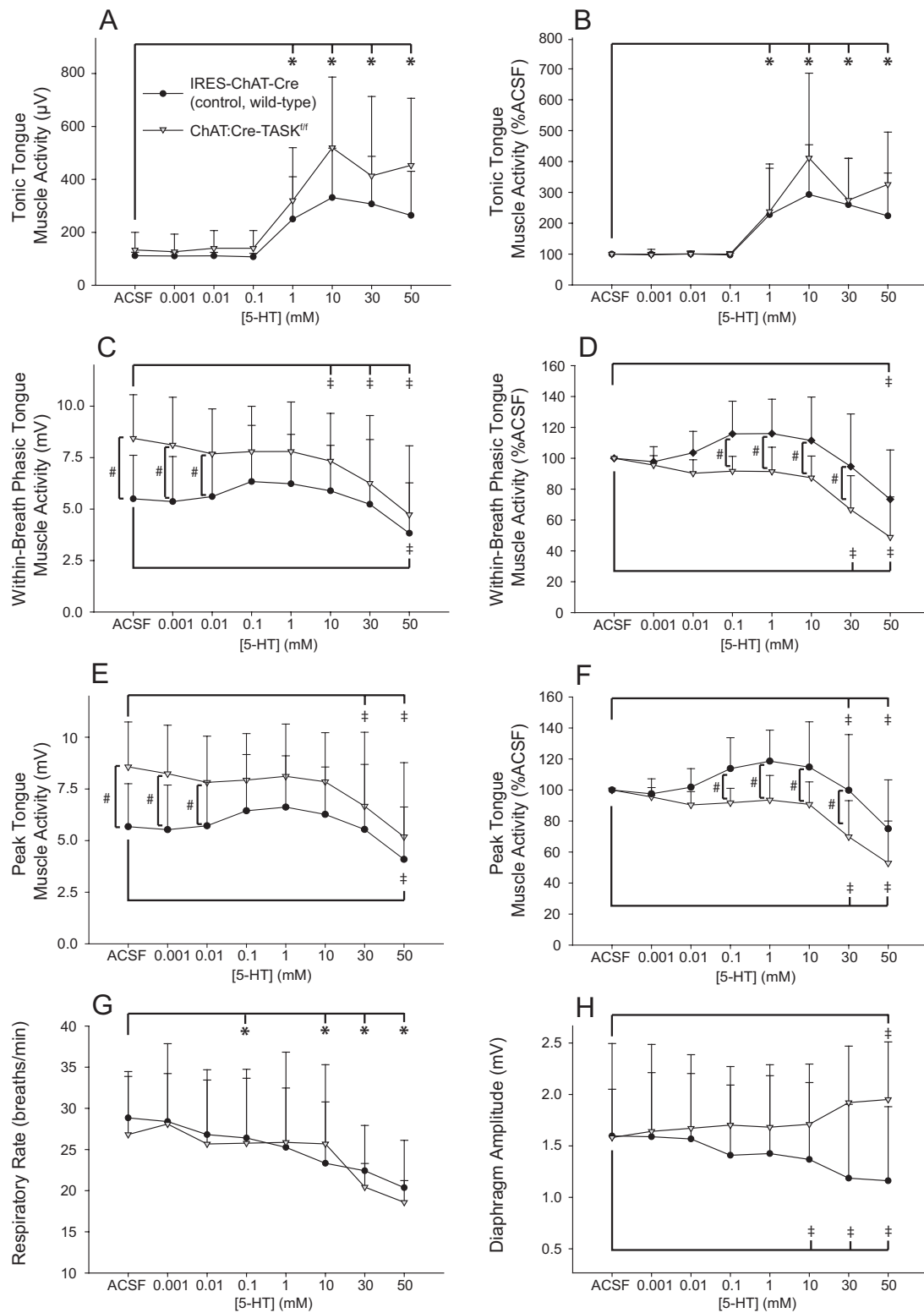
Figure 11. Group data from Study 2: TASK channel deletion on cholinergic neurons and responses to 5-HT at the hypoglossal motoneuron pool. The data show mean (+SEM) responses to microperfusion of 5-HT into the HMN in the controls and the ChAT:Cre-TASK$^{f/f}$ mice that lack TASK-1/3 channels on cholinergic neurons. Data show non-normalized and normalized tonic tongue muscle activity (A and B, respectively), within-breath phasic tongue muscle activity (C and D), and peak tongue muscle activity (E and F), as well as respiratory rate (G) and diaphragm amplitude (H). $p < 0.05$ are shown by the symbols: (i) "Asterisk" for the indicated [5-HT] vs. baseline ACSF control independent of genotype; (ii) "Number sign" for control vs. ChAT:Cre-TASK$^{f/f}$ mice independent of drug treatment; and (iii) "Double dagger" for the indicated [5-HT] vs. ACSF specific to the indicated genotype. See text for further details.

motor pool to increase tongue muscle activity and responsivity in vivo. Such potential has not been tested previously, but is of relevance given the interest in TASK channel blockers for the pharmacological treatment of OSA [4, 48, 49]. The latter includes the SANDMAN trial (ClinicalTrials.gov Identifier: NCT03603678) that tests nasal administration of BAY2253651, a TASK-1 channel inhibitor. The nasal administration is presumably aimed at augmenting the local negative pressure reflex to drive upper airway muscle activity, as has been tested in pre-clinical models using a different propriety agent that targets TASK-1/3 channels [50]. At the hypoglossal motoneuron pool, K+ channel subfamily K member 9 (TASK-3) channel RNA shows a high degree of differential expression, being 12.7-fold higher compared to the rest of the brain [4].

The present study used a combination of electrophysiological and pharmacological approaches in rats and genetically modified mice to test the effects on tongue muscle activity and responsivity of selected TASK channel interventions at the hypoglossal motoneuron pool under anesthesia and across sleep–wake states. The findings identified robust maintenance of tongue motor activity despite the various strategies for TASK channel manipulation. The difficulty to increase tongue muscle activity by targeting TASK channels at the hypoglossal motoneuron pool, despite these channels being abundant at this and other cranial motoneuron pools relevant to upper airway patency and the pathogenesis of OSA [4, 32, 51], suggests that this pharmacological target (i.e. the hypoglossal motoneuron pool) and direction for potential OSA pharmacotherapy is not currently supported, at least with the agents currently available in the public domain.

There are several K+ channel families with numerous subtypes expressed at the HMN, modulation of which can alter tongue motor activity [36, 52], including during sleep [41]. These K+ channel families encompass voltage-gated, Ca2+-dependent, inward rectifier, and tandem pore domain [8, 52], the latter including TASK-1/3 channels. We are not aware of specific evidence for a hypothesized mechanism of action of wake–active neurotransmitters on tongue motor activity being linked to voltage-gated, Ca2+-dependent, or inward rectifier K+ channels that could be responsible for changes in motor activity from wake to sleep. However, for many of those K+ channels, the nature of the endogenous modulation and links to receptors at the HMN is not well known [52]. Nevertheless, although several awake–active neurotransmitters with inputs to the HMN lead to inhibition of K+ leak mediated by TASK-1/3 channels and increased neuronal activity, TASK channels are not the exclusive pathway by which these neurotransmitters can activate neurons, that is, non-TASK channel mechanisms are also involved [6, 8, 11, 22, 53, 54].

The results of the present study showed that neither the various TASK channel inhibitors, nor an activator, at the HMN changed baseline tongue motor activity or responses to 5-HT in anesthetized rats, and that a TASK channel inhibitor also did not increase tongue motor activity during natural sleep. Together these data suggested robust maintenance of tongue motor activity despite the various strategies for TASK channel manipulation. These data also suggest that the aforementioned non-TASK channel pathways can contribute to the motor responses to 5-HT and imply that other intracellular signaling components linked to the receptors modulated by monoamines may be more strongly linked in the state-dependent modulation of tongue motor activity. Such candidates include phospholipase C and inositol triphosphate that are impacted by 5-HT and other awake–active monoamines via their associated receptor activation [54]. Given that some of these other cellular signaling cascades that are linked to the receptors modulated by the monoamines are ubiquitous, they do not appear to be strong targets for OSA pharmacotherapy at this time.

Study 1: responses under general anesthesia and their interpretation

The initial studies under general anesthesia in rats (Study 1) were performed before the studies across natural sleep–wake states (Study 3) because the condition of anesthesia allowed

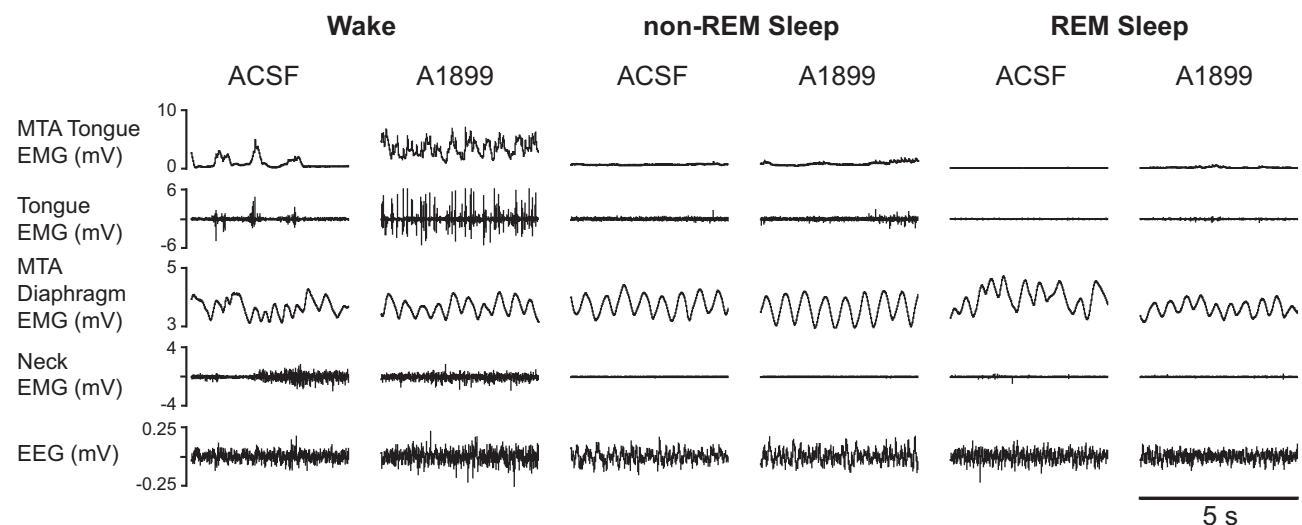**Figure 12.** Example traces from Study 3: TASK channel inhibitor at the hypoglossal motoneuron pool across sleep–wake states. Example responses show the raw and MTA of the tongue EMG, diaphragm MTA, neck EMG and EEG with microperfusion of ACSF and the TASK channel inhibitor A1899 into the HMN in an awake and sleeping rat. Note the lack of effect of A1899 except for an increase in tongue EMG activity in wakefulness.
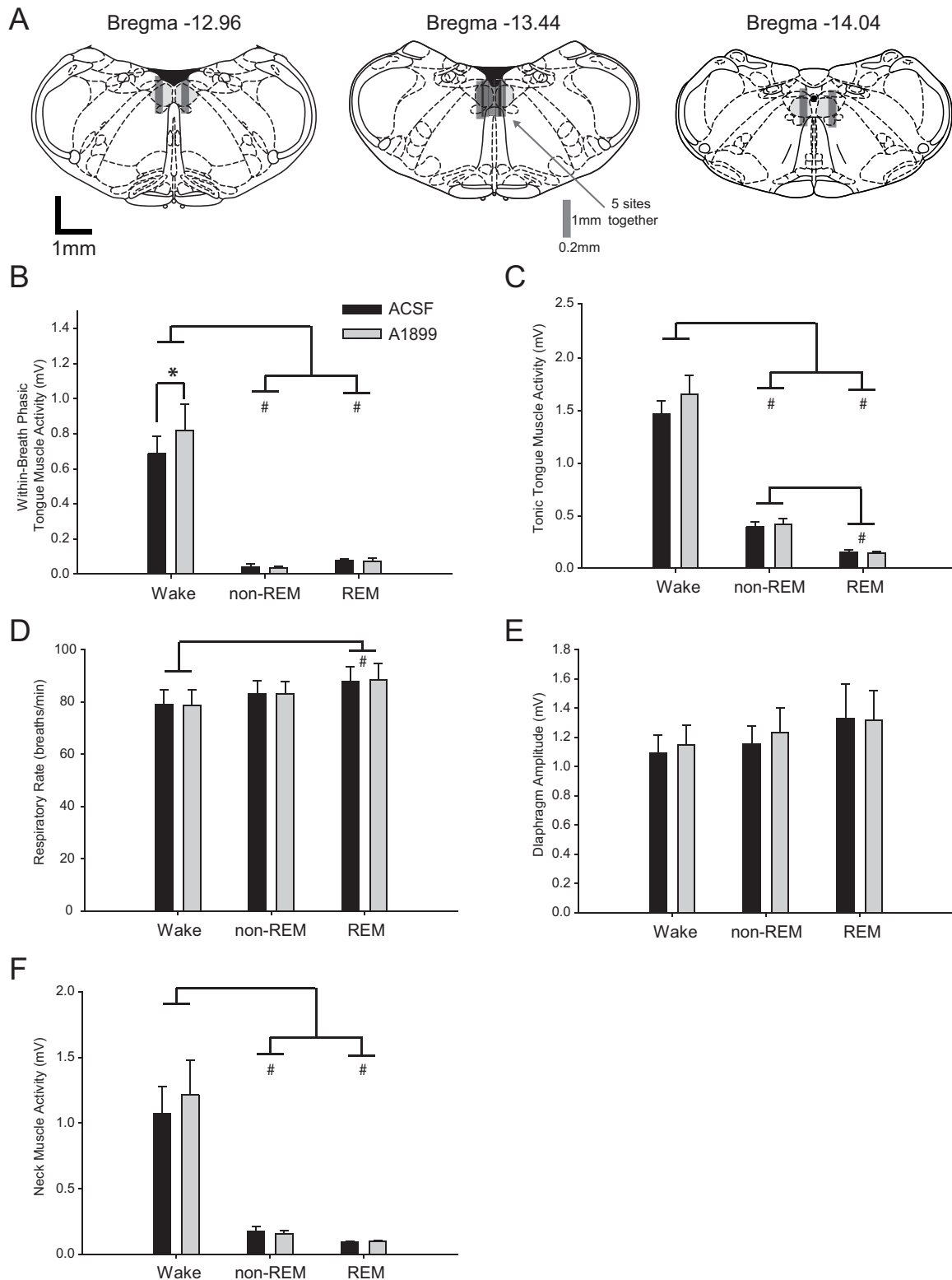
Figure 13. Group data from Study 3: TASK channel inhibitor at the hypoglossal motoneuron pool in awake and sleeping rats. (A) Location of the microdialysis probes from all the experiments for *Protocol 3* on coronal diagrams of the rat medulla [46]. Dark grey cylinders, drawn to scale, represent the microdialysis probe locations; overlap obscures some of the individual dialysis sites. The light grey shading represents the HMN. The group data (mean +SEM) show the effects of microperfusion of ACSF and the TASK channel inhibitor A1899 into the hypoglossal motoneuron pool on within-breath phasic and tonic tongue muscle activities (B–C), respiratory rate (D), diaphragm amplitude (E), and neck muscle activity (F). Asterisk indicates $p < 0.05$ for the effect of A1899 vs. ACSF that was specific to within-breath phasic tongue muscle activity in wakefulness only (panel B). Number sign indicates $p < 0.05$ for differences between the indicated sleep–wake states. See text for further details.

for: (1) controlled time-dependent sequential delivery of ACSF and the selected TASK channel interventions into the hypoglossal motoneuron pool to characterize their effects on tongue muscle activity, (2) across the six parallel time-aligned protocols (*Protocols 1a–f*), (3) in both the presence and absence of applied 5-HT, and (4) all without the confound of changes in motor

activity elicited by spontaneous changes in behaviors and sleep–wake states that occurs in the freely behaving preparation.

The choice of general anesthetic for these initial experiments was carefully considered. We chose to use an inhalational anesthetic because it provides reliable and stable preparations over time [44, 55], without the requirement for bolus does of supplemental injectable anesthetics, as with urethane-induced anesthesia, for example, that can transiently alter respiratory motor activities and electrocortical activity. Such additional bolus does of anesthetic complicate the comparison of the primary outcome variable (respiratory motor activity) within and between animals in a repeated-measures design. In contrast, volatile anesthetics can be maintained at stable levels within an animal such that no further adjustments are typically necessary across the experiments to maintain stable tongue motor activity, diaphragm activity, and electrocortical activity [44, 55].

Isoflurane was chosen as the inhalational anesthetic given our experience of reliable and stable preparations to characterize tongue motor responses to controlled time-dependent sequential delivery of selected interventions into the hypoglossal motoneuron pool in vivo [44, 55]. Volatile anesthetics also increase the respiratory-related component of hypoglossal motor activity in rodents, unlike in some other species [24], due to augmentation of hypoglossal pre-motor respiratory inputs compared to the non-anesthetized awake preparation [56], thus facilitating the identification of potential effects of the interventions on tongue motor activity. Direct exposure of hypoglossal motoneurons to inhalational anesthetics in tissue slices in vitro causes membrane hyperpolarization and decreased input resistance due to augmentation of K^+ leak, an effect that can be reversed by acidifying the bathing medium [20–22]. The K^+ current properties of the responses in those in vitro studies were identical to those mediated by TASK channels [20–22]. In vitro, inhalational anesthetics and 5-HT target the same TASK channels on hypoglossal motoneurons, with the K^+ current blocked by 5-HT [10, 22]. Overall, the rationale for the use of isoflurane in the present study was that under baseline conditions in the presence of general anesthesia the effects of the various interventions to inhibit TASK channels using A1899, doxapram, ML365, and acidified ACSF [27–32], and activate TASK channels using terbinafine [33], could be observed in the presence and absence of applied 5-HT in vivo [10, 22].

For these experiments, the hypoglossal motor responses were measured across parallel time-dependent protocols (*Protocols 1a–f*). The repeatability and stability of responses were identified in *Protocol 1a* and these characteristics formed the basis for the interpretations of responses in *Protocols 1b–f*. In this context, we used various strategies to modulate TASK channel activity at the hypoglossal motoneuron pool to identify effects on baseline tongue muscle activity. However, we consistently used 5-HT to test for effects of such TASK channel modulation on the evoked motor responses to applied excitatory neurotransmitter inputs. The choice of 5-HT for these experiments was also carefully considered over other potential options. Specifically, we identified previously that an endogenous α_1 receptor mechanism at the hypoglossal motoneuron pool is a major determinant of tongue motor activity across sleep–wake state in vivo [57] compared to, for example, endogenous 5-HT receptor mechanisms [58–60]. However, the motor responses to noradrenergic stimulation involve an interaction with glutamatergic inputs [8], an effect that can lead to more variable responses to noradrenergic stimulation whether released endogenously or applied exogenously

[61, 62]. In contrast, the responses to applied 5-HT are reliably stable and repeatable as identified from previous experiments [38] and as confirmed in Figures 1–8. It is for that reason that 5-HT was chosen for the present study as it was suited to test the hypothesis and detect any potential changes in response to the interventions.

Repeatability
The repeatability of the latency, magnitude, and duration of the responses to 5-HT-1 and 5-HT-2 under baseline conditions (i.e. without the TASK channel interventions) in *Protocol 1a* (Figures 1 and 2) was a necessary pre-requisite for interpretation of the potential effects on the responses to 5-HT and to the TASK channel inhibitors and activator in *Protocols 1b–f* (Figures 3–8). There were no significant differences between 5-HT-1 and 5-HT-2.

Stability
Testing for the stability of tongue motor activity under baseline conditions (i.e. during ACSF-1, ACSF-2, and ACSF-3 without 5-HT or the TASK channel interventions) in *Protocol 1a* (Figures 1 and 2) was also a necessary pre-requisite for interpretation of the potential effects of 5-HT with and without the TASK channel inhibitors and activator in *Protocols 1b–f* (Figures 3–8). There were no significant differences between ACSF-1, ACSF-2, and ACSF-3.

Maintenance of tongue motor activity and responses despite the interventions at the hypoglossal motoneuron pool to modulate TASK channels
There was robust maintenance of tongue motor activity at control levels (i.e. ACSF-1 and ACSF-2) despite the presence of the TASK channel inhibitors or activator at the hypoglossal motoneuron pool (*Protocols 1b–f*, Figures 3–8). Likewise, the tongue motor responses to 5-HT were unaffected by the presence of the TASK channel inhibitors or activator (*Protocols 1b–f*, Figures 3–8). There were no significant differences across any of these conditions.

We do not conclude from these results, however, that TASK channels exert no influence on the resting membrane potential and excitability of hypoglossal motoneurons. The evidence is overwhelmingly strong that this is the case in vitro [8, 10, 20–23]. Although we did not perform parallel in vitro studies in the present article, the doses for each intervention were determined through extrapolation from in vitro dose–response relationships [28, 29, 34] as detailed in the Methods. Rather, we suggest that the lack of effect of the TASK channel inhibitors or activator at the hypoglossal motoneuron pool on tongue motor activity may reflect robust homeostatic maintenance of resting membrane potential in vivo [63]. Regardless of the ionic source of such possible homeostatic compensation, its presence would provide a challenge for the clinical potential of TASK channel modulators acting at the hypoglossal motoneuron pool to increase tongue muscle activity and responsivity. The potential beneficial responses of upper airway muscle activity and responsivity to systemic administration of TASK channel blockers, for example, to augment the negative pressure reflex [50], was not the focus of this study.

Study 2: responses in mice lacking TASK-1/3 channels on cholinergic neurons

Additional studies were performed in ChAT-Cre:TASK$^{f/f}$ mice lacking TASK-1/3 channels on cholinergic neurons [23, 40]. It was noted that there was a consistent and statistically

significant increase in the level of isoflurane required to achieve similar levels of sedation, as judged by stable respiratory motor activities, in the ChAT-Cre:TASK$^{f/f}$ mice compared to the controls (1.85% ± 0.03% vs. 1.56% ± 0.02%). This difference is consistent with a reduction in sensitivity to the immobilizing effects of isoflurane in ChAT-Cre:TASK$^{f/f}$ mice [23]. Although the response of tonic tongue motor activity to 5-HT at the hypoglossal motoneuron pool was not significantly different in the ChAT-Cre:TASK$^{f/f}$ mice compared to the controls (Figure 11, A–B, *p* = 0.057 and 0.360 for non-normalized and normalized data, respectively), we do not know if the difference in required levels of isoflurane contributed to some of the differences between genotypes observed in Figure 11 and discussed later. We did not further increase the levels of isoflurane in the control mice to match that of the ChAT-Cre:TASK$^{f/f}$ mice due to concerns regarding excessive anesthesia and effects on breathing. Baseline respiratory rates and diaphragm amplitudes were similar in the ChAT-Cre:TASK$^{f/f}$ mice and controls suggesting otherwise similar initial conditions. Once the level of isoflurane was set in each individual mouse to maintain surgical levels of anesthesia and stable respiratory motor activities, it was then held constant for the remainder of the experiments within an animal.

Within-breath phasic tongue motor activity and responses to 5-HT in ChAT-Cre:TASKf/f mice

The ChAT-Cre:TASK$^{f/f}$ mice exhibited greater within-breath phasic tongue muscle activity with ACSF and at low levels of 5-HT compared to the controls (Figure 11, C). The aforementioned higher levels of isoflurane required in the ChAT-Cre:TASK$^{f/f}$ mice during the experiments (mean increase of 18.6% compared to controls) may have contributed to this effect, given that volatile anesthetics increase the respiratory-related component of tongue muscle activity due to augmentation of hypoglossal premotor respiratory inputs [56].

In vitro studies also identify that hypoglossal motoneurons are directly hyperpolarized by volatile anesthetics via TASK channels [21], an effect reduced in the absence of TASK channels [23]. The resting membrane potential of hypoglossal motoneurons in vitro is more depolarized in mice lacking TASK-1/3 channels [23]. Such an effect may contribute to an increase in within-breath phasic tongue muscle activity in response to a given incoming respiratory drive potential (Figure 11, C) although it did not change resting tonic motor tone (Figure 11, A).

A decrease in within-breath phasic and peak tongue motor responses to 5-HT was observed in the ChAT-Cre:TASK$^{f/f}$ mice compared to the control mice for the normalized data, although not in the non-normalized data where the opposite directional difference between mice was observed (Figure 11, D and F vs. Figure 11, C and E, respectively). As expected, microperfusion of 5-HT into the hypoglossal motoneuron pool caused increases in tonic tongue motor activity and a concomitant decrease in the respiratory-related component tongue motor activity (Figure 11, A–D). Such an effect has also been observed in other studies [38, 64, 65]. This activation of tonic motor activity occurred in both the ChAT-Cre:TASK$^{f/f}$ mice and controls. Importantly, however, a decrease in within-breath phasic and peak tongue motor responses to 5-HT at the hypoglossal motoneuron pool with TASK channel knock-out (Figure 11, D and F) would not translate to being helpful in the context of potential OSA pharmacotherapy via TASK channel blockade at the HMN, that is, this finding also does not support that direction for potential OSA pharmacotherapy.

Tonic tongue motor responses to 5-HT in the ChAT-Cre:TASKf/f mice

If a reduction in K$^+$ leak mediated via 5-HT receptor activation is a major determinant of the tonic motor responses to applied 5-HT at the HMN (as suggested from in vitro studies [8–10, 14]), then the tonic motor responses to 5-HT should be reduced (or even absent) in the TASK1/3 deficient mice compared to the controls. However, the significant activating effects of 5-HT on tonic tongue motor activity were statistically independent of genotype as analyzed from both the non-normalized data and the data normalized to the control baseline activity with ACSF (Figure 11, A and B, *p* = 0.057 and *p* = 0.360, respectively). Moreover, although the tonic motor responses were not statistically significant between genotypes, there was even a trend to increased rather than decreased responses in the ChAT-Cre:TASK$^{f/f}$ mice compared to controls as observed in both the non-normalized and normalized data (Figure 11, A–B). These data indicate that other (i.e. non-TASK channel) mechanisms are involved in mediating and contributing to the hypoglossal motor responses to 5-HT (e.g. phospholipase C and inositol triphosphate that are coupled to monoamine receptor activation as discussed above). Such non-TASK channel mechanisms may even have increased their contribution to neuronal activity in the ChAT-Cre:TASK$^{f/f}$ mice to compensate for the lack of TASK-1/3 channels. The involvement of non-TASK channel mechanisms in mediating and contributing to the hypoglossal motor responses to 5-HT is also supported by the extensive data from the five protocols (b–f) in Study 1 involving three different TASK channel inhibitors, plus acidified ACSF, and a TASK channel activator. Again, overall these results do not support TASK channel blockade at the HMN as a strategic direction in the context of potential OSA pharmacotherapy.

The subsequent experiments across natural sleep–wake states (Study 3) were performed in rats rather than ChAT-Cre:TASK$^{f/f}$ mice and controls. This choice was made because, in our experience, baseline tongue motor activity is typically tonic and otherwise minimal in chronically instrumented mice such that application of other techniques such as "chemogenetics" [66] or "optogenetics" [67] are necessary to evoke hypoglossal motor responses to test for changes in motor excitability across sleep–wake states. The addition of such methodology in addition to microdialysis probes at the HMN was beyond the scope of the present study, and also not supported by the findings from Studies 1–3 inclusive.

Study 3: TASK channel inhibitor at the hypoglossal motoneuron pool in awake and sleeping rats

The studies in freely behaving rats across states of wakefulness and natural sleep were performed because TASK-1,3 channels may constitute a common downstream mechanism impacted by the collective reductions in the wake–active neuromodulator inputs (e.g. such as noradrenaline and 5-HT), thus contributing to decreased tongue muscle activity in sleep [10, 52]. As such, it was hypothesized that if TASK-1,3 channel activation at the hypoglossal motoneuron pool mediate decreased tongue motor activity in sleep, then their inhibition via microperfusion of A1899 would increase tongue muscle activity during sleep.

This did not occur. Rather, the effect observed with A1899 at the hypoglossal motoneuron pool was increased within-breath phasic tongue muscle activity that occurred in wakefulness only (Figure 13). Interestingly, the same effect was observed in a previous study during microperfusion of methanandamide, a cannabinoid receptor agonist and TASK channel inhibitor [68], into the hypoglossal motoneuron pool at a concentration that aimed to target TASK channels [36].

This wakefulness-dependent effect of TASK channel inhibition on motor activity is consistent with a previous study showing increased motor activity in the active (i.e. waking) phase of the light–dark cycle in TASK-3 knockout mice but not in the inactive (sleeping) phase [69]. The lack of other observable motor or behavioral phenotypes in mice lacking TASK channels may be due to compensatory increases in inhibitory γ-amino-butyric acid type-A ($GABA_A$) receptor activity [63, 70]. As discussed above for Study 1, it is possible, therefore, that in the present study the physiological significance of TASK channel modulation is effectively masked by a homeostatic regulation of membrane potential by other systems. Even if this is not the case, the effects of targeting TASK channel blockers to the hypoglossal motor pool may not be an effective strategy to reactivate pharyngeal motor activity in sleep (as the effects are confined to wakefulness) such that targeting these channels may also not be effective for OSA pharmacotherapy.

Overall, despite the interest and theoretical potential for TASK channel blockers acting at the hypoglossal motoneuron pool to increase tongue motor tone and provide a new strategic direction of OSA pharmacotherapy, the results of the present studies performed across a range or preparations suggest that this direction is not currently supported, at least with the agents currently available in the public domain. Other strategies also rooted in basic mechanistic studies in pre-clinical animal models have recently proved more effective for OSA pharmacotherapy [71, 72].

Funding

Disclosure Statement

Financial disclosure: none.
Non-financial disclosure: none.
Conflict of interest statement. None declared.

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