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Ventilation and Neurochemical Changes During μ-Opioid Receptor Activation Or Blockade of Excitatory Receptors In The Hypoglossal Motor Nucleus Of Goats

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Abstract

Neuromodulator interdependence posits that changes in one or more neuromodulators are compensated by changes in other modulators to maintain stability in the respiratory control network. Herein, we studied compensatory neuromodulation in the hypoglossal motor nucleus (HMN) after chronic implantation of microtubules unilaterally (n = 5) or bilaterally (n = 5) into the HMN. After recovery, receptor agonists or antagonists in mock cerebrospinal fluid (mCSF) were dialyzed during the awake and non-rapid eye movement (NREM) sleep states. During day studies, dialysis of the μ-opioid inhibitory receptor agonist [d-Ala^2, *N*-MePhe^4, Gly-ol]enkephalin (DAMGO; 100 μM) decreased pulmonary ventilation ($\dot{V}i$), breathing frequency (f), and genioglossus (GG) muscle activity but did not alter neuromodulators measured in the effluent mCSF. However, neither unilateral dialysis of a broad spectrum muscarinic receptor antagonist (atropine; 50 mM) nor unilateral or bilateral dialysis of a mixture of excitatory receptor antagonists altered $\dot{V}i$ or GG activity, but all of these did increase HMN serotonin (5-HT) levels. Finally, during night studies, DAMGO and excitatory receptor antagonist decreased ventilatory variables during NREM sleep but not during wakefulness. These findings contrast with previous dialysis studies in the ventral respiratory column (VRC) where unilateral DAMGO or atropine dialysis had no effects on breathing and bilateral DAMGO or unilateral atropine increased $\dot{V}i$ and f and decreased GABA or increased 5-HT, respectively. Thus we conclude that the mechanisms of compensatory neuromodulation are less robust in the HMN than in the VRC under physiological conditions in adult goats, possibly because of site differences in the underlying mechanisms governing neuromodulator release and consequently neuronal activity, and/or responsiveness of receptors to compensatory neuromodulators.

NEW & NOTEWORTHY

Activation of inhibitory μ-opioid receptors in the hypoglossal motor nucleus decreased ventilation under physiological conditions and did not affect neurochemicals in effluent dialyzed mock cerebral spinal fluid. These findings contrast with studies in the ventral respiratory column where unilateral [d-Ala^2, *N*-MePhe^4, Gly-ol]enkephalin (DAMGO) had no effects on ventilation and bilateral DAMGO or unilateral atropine increased ventilation and decreased GABA or increased serotonin, respectively. Our data support the hypothesis that mechanisms that govern local compensatory neuromodulation within the brain stem are site specific under physiological conditions.

The importance of maintaining a balance between excitatory and inhibitory neuromodulation in neural networks is illustrated by the severity of pathological conditions such as Parkinson's disease (44), epilepsy (37), schizophrenia (40), and depression (38). It is also widely accepted that an imbalance in neuromodulators contributes to dysfunction in diseases of respiratory control such as sudden infant death and opioid-induced

respiratory depression (10, 13, 17). Accordingly, to develop more effective treatments for these pathologies, there is a need to develop a better understanding of the fundamental mechanisms that govern the neuromodulation and thus the excitability of neural networks.

Important studies aiming to better understand the fundamental role of excitatory and inhibitory modulation have been focused on the neural network that generates respiratory rhythm and/or pattern, which includes a medullary site critical for rhythm generation, the pre-Bötzinger complex (preBötC; 7, 8). The alterations of various excitatory neuromodulatory receptors in this region suggested that no single excitatory neuromodulatory receptor, when blocked, could lead to ventilatory depression in vitro and in vivo. Instead, changes in ventilation required multiple receptor antagonists simultaneously, which led to the concept of neuromodulator interdependence whereby perturbations in the activity of one or more neuromodulator receptors is compensated by changes in other neuromodulators to maintain stable ventilation (7, 8). This hypothesis has been further tested by unilateral microdialysis of antagonists to excitatory neuromodulator receptors in the preBötC region of the ventral respiratory column (VRC) of awake and sleeping goats (32–35). Unexpectedly, VRC dialysis of the broad spectrum muscarinic receptor antagonist atropine (50 mM) increased pulmonary ventilation ($\dot{V}i$) and breathing frequency (*f*) during wakefulness and to a lesser extent during non-rapid eye movement (NREM) sleep, with concomitant, large, and local increases in serotonin (5-HT) and substance P (SP) in effluent dialysate (32). Lower concentrations of atropine alone or in combination with neurokinin-1 (NK_1) and/or 5-HT receptor blockers dialyzed in this same region did not affect $\dot{V}i$ but still elicited compensatory increases in excitatory neuromodulators 5-HT and SP (32–35). These local increases in the excitatory neuromodulators SP and 5-HT may conceivably compensate for the induced antagonism of excitatory receptors (32).

Increased activity of inhibitory neuromodulatory receptors in the VRC of awake and sleeping goats does not elicit local increases in excitatory neuromodulators but still provides another example of compensatory neuromodulation. For example, dialysis of the μ-opioid receptor agonist [d-Ala^2, *N*-$MePhe^4$, Gly-ol]enkephalin (DAMGO) led to a dose-dependent decrease in the local, endogenous GABA in the effluent dialysate (20). The reduced GABA levels were concomitant with transient decreases in $\dot{V}i$ with unilateral DAMGO, whereas bilateral dialysis of 100 μM DAMGO increased $\dot{V}i$ and *f* and increased the variability of $\dot{V}i$ (20), indicative of local neuromodulator compensation for the increased inhibition by the exogenously delivered opioid. Thus, collectively, the summarized results support the concept of neuromodulator interdependence in respiratory rhythm- and pattern-generating nuclei such as the preBötC of the VRC (7, 8, 20, 21, 32–35). The mechanism of compensation is unknown, but the changes in neuromodulators are local; thus compensation may occur within individual ionic currents of a single neuron. It is also unclear whether these compensatory mechanisms exist at other sites within the respiratory control system or whether pathophysiological shifts in the balance of excitation and inhibition in disorders of respiratory control represent failures or exacerbations of such neuromodulatory compensatory mechanisms.

Accordingly, the objective herein was to determine whether similar mechanisms of compensatory neuromodulation occur at another site within the neural respiratory control network that has distinctly different functions and cell composition from those of the VRC, such as the hypoglossal motor nucleus (HMN). Largely composed of large motor neurons that innervate the genioglossus (GG) muscle, the HMN motor neurons direct muscular GG contraction during inspiration to maintain a patent upper airway and minimize airway resistance (41–43). The GG is a main muscle in swallowing behaviors. Abnormal function of the HMN can lead to airway constriction, reduced airflow, obstructive apneas, and dystussia/aspiration, suggesting that a better understanding of potential mechanisms of neuromodulator compensation in the HMN has major clinical relevance, particularly during sleep when hypoglossal muscle activity is reduced from the awake state more than diaphragm activity (10, 15, 41).

Our overall hypothesis was that mechanisms of compensatory neuromodulation are not uniform throughout the respiratory control network. Accordingly, since others (10, 13, 48) found that dialysis of various μ-opioid receptor agonists into the HMN depressed respiratory output and GG muscle activity, we hypothesized that unilateral and bilateral dialysis of DAMGO in the HMN would depress respiratory output and GG muscle activity in awake and sleeping goats and would not elicit local changes in neuromodulators In addition, since others (1, 48) have shown that acetylcholine modulation of the HMN is via the inhibitory M2 receptor and since there appears to be minimal stimulation of the HMN by 5-HT (15), we hypothesized that dialysis of antagonists to muscarinic, NK_1, and 5-HT receptors would not depress ventilation and there would be no compensatory increase in neuromodulators.

METHODS

Goats.

Ten adult, female, nonpregnant goats with an average body weight of 44.5 ± 2.6 (SE) kg were housed and studied in an environmental chamber with a fixed ambient temperature and alternating 12-h light-dark cycles set between 7 AM and 7 PM. The goats had free access to food and water except for study periods and the 24-h preceding surgeries. Protocols and procedures utilized in this study were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Surgical procedures.

Prior to surgery, the goats were anesthetized with ketamine (intravenous), intubated, and mechanically ventilated with isoflurane in 100% oxygen throughout surgery. To minimize sensation of pain, flunixin meglumine (2 mg/kg im) was administered once preoperatively and for 2 days postoperatively. To prevent infections, ceftriaxone (25 mg/kg iv) in 100-ml saline was administered over 30 min twice daily at 8–12-h intervals for 3 days starting the morning of a craniotomy surgery. Thereafter, ceftiofur sodium (4 mg/kg im) and gentamicin (6 mg/kg im) were administered once daily for the remainder of the study period. To minimize intracranial swelling following the craniotomy, dexamethasone (4 mg/ml iv) was administered three times daily and tapered for a week following surgery. After surgeries, sites of surgical incisions were treated daily for 7 days with triple-antibiotic ointment. Rectal temperature (TR), heart rate (HR), and oxygen saturation were continuously monitored during surgery and at regular intervals over 24 h postsurgery.

Two sterile surgeries were performed separated by 2 wk. For the first, an incision was made in the goat's neck to locate the carotid artery, which was subsequently dissected out, elevated to beneath the skin, and sutured to the overlaying skin before closing the incision. In addition, electroencephalographic (EEG) and electrooculographic (EOG) electrodes were inserted into the midline cranium and the superior orbital ridge for subsequent recordings to score wakefulness/sleep during night studies. The electrodes were attached to screws and secured with dental acrylic, and the wires were pulled through the skin before closure of the incision. Finally, bipolar electromyographic (EMG) electrodes were implanted into the diaphragm (DIA), abdominal, and genioglossus (GG) muscles and tunneled to the exterior for recording of inspiratory, expiratory, and airway muscle activity.

Two weeks later, stainless steel microtubules (70.0-mm length, 1.27-mm OD, and 0.84-mm ID) were chronically implanted unilaterally (n = 5) or bilaterally (n = 5) into the HMN. An incision bisected the nuchal ligament along the dorsal midline of the skull and neck, and then a rotary drill was used to create an occipital craniotomy for visualization of the dura, which, when opened, exposed the dorsal medulla. Obex was the anatomical reference for micromanipulator-controlled microtubule placement into the HMN, which in goats extends from ~3 mm caudal to 3.5 mm rostral to obex, 0.5–1.5 mm lateral to the midline, and 2–3.5 mm from the dorsal surface. In some goats, adjustments in microtubule placements had to be made to avoid blood vessels. Screws were

inserted into the skull to which the microtubules were secured using dental acrylic. Stainless steel stylets slightly shorter than the microtubules were inserted into the microtubules, and the incisions were sutured closed. Following the procedure, postoperative monitoring and medications were administered as described above.

Physiological data collection.

Following the second surgery, the goats were allowed a minimum of 2 wk to recover before the initiation of studies. During this time, the goats were accustomed to the study apparatus and protocol. To obtain ventilatory data, a custom mask was specially fitted to each animal and during studies secured to their muzzles. A two-way valve was inserted into the end of the mask, and tubing was attached to inspiratory and expiratory sides of the valve. The inspiratory tubing was attached to a pneumotachograph connected to a Windaq data-recording system and used to obtain breath-by-breath inspiratory flow ($\dot{V}i$, l/min), breathing frequency (*f*, breaths/min), tidal volume (V_T, l/breath), inspiratory (T_I) and expiratory (T_E) time(s), and inspiratory drive (V_T/T_I). During daytime studies, the expiratory tubing was attached to a Tissot spirometer. The expired air was collected at 5-min intervals and analyzed for O_2 and CO_2 concentration for calculation of O_2 consumption. Every 30 min of several studies, arterial blood was withdrawn from a catheterized carotid artery for analysis of pH, Pco_2, and Po_2. The arterial catheter line was attached to a transducer to obtain blood pressure (BP) and HR using the Windaq data analysis system. Respiratory muscle activity was recorded via Windaq using the chronically implanted electrodes. To determine and score sleep state during night studies, signals from EEG and EOG electrodes were recorded via Windaq and later scored always by the same investigator. Finally, TR was recorded throughout the studies herein.

Dialysis probes [Harvard Apparatus (formerly CMA Microdialysis), Holliston, MA] inserted into the microtubules were 72 mm in length, 70 mm of which is a stainless steel tube and the final 2 mm of which is a semipermeable membrane (membrane diameter 0.5 mm, 20-kDa cutoff, 3-µl internal volume). Only the 2-mm membrane penetrated the brain tissue. During studies, the perfusate was either mock cerebrospinal fluid (mCSF: 124 mM NaCl, 2.0 mM KCl, 2.0 mM $MgCl_2$, 1.3 mM K_2PO_4, 2.0 mM $CaCl_2$, 11 mM glucose, and 26 mM NaHCO−3HCO3−, pH 7.32 in sterile distilled H_2O) alone or mixed with receptor agonist or antagonists described below. The solutions were prepared in a tonometer flask, warmed to 39°C, and equilibrated with 6.4% CO_2 and 12% O_2 balance N_2. A syringe pump (Harvard Apparatus) delivered the dialysate to the dialysis probe at a flow rate of 25 µl/min. To minimize distraction of the goat, the pump was outside of the chamber; thus a 150-cm length of polyethylene tubing (PE50) was needed to connect the syringe to the probe. The length of the tubing caused a delay of ~20 min between the start of dialysis and the arrival of perfusate at the probe tip. As a result of this delay, the first 15 min of *hour 2* of dialysis were omitted from statistical analysis. Moreover, during the initial 15–20 min of *hour 3,* drug delivery to the tissue would continue as in *hour 2*. In addition, thereafter, washout of the drug from the tissue would be time dependent. As a result, the reported ventilatory responses herein would likely reflect these drug delivery and washout characteristics. To collect effluent dialysate, a short length of tubing was attached to the outlet end of the probe, which was then attached to a modified cryotube. Separate tubes were used for effluent collection during each hour of dialysis. The effluent was then aliquoted and frozen at −80°C for subsequent analyses.

Three continuous hours of dialysis began 60 min after probe insertion. *Hour 1* was mCSF alone, *hour 2* was mCSF alone or mCSF mixed with different drugs, and *hour 3* was mCSF alone. One dialyzed drug was the µ-opioid agonist DAMGO at concentrations of 10 or 100 µM, which is over the range and duration others used in dialysis studies on rats or dogs (29, 36) and the same as in previous studies in the VRC (20). The second drug was 50 mM atropine, which was at the same concentrations during dialysis into the VRC (21, 32). The third drug study was a mixture of the 5-HT_2 receptor antagonist α-phenyl-1-(2-phenylethyl)-4-piperidine methanol (MDL 11,939; 0.5 mM), the NK_1 receptor antagonist spantide (500 µM), and the muscarinic receptor antagonist atropine (5 mM). These antagonists were the same as used in VRC studies (21, 35). The day studies were completed between 9

AM and 2 PM. Night studies were completed between 7 PM and 2 AM. A minimum of 36 h were allowed between consecutive studies on individual goats.

Neurochemical analyses.

To measure the amino acids glutamine, glycine, and GABA present in effluent dialysate, reverse phase high-performance liquid chromatography was used as published by Muere et al. with fluorescent detection using a Waters Resolve C18 column (150 × 3.9 mm) and a fluorescent detector with excitation at 229 nm and emission at 470 nm; β-alanine internal standard was used with *o*-phthaldialdehyde derivatization (32). To measure 5-HT and its metabolite hydroxyindoleacetic acid (HIAA), an identical column was used; however, the potential was set at 0.6 V vs. an Ag/AgCl reference electrode, and an *N*-methylserotonin internal standard was used. A commercially available assay (Assay Designs 900-018, range 9.76–10,000 pg/ml; Enzo Life Sciences) and a microplate reader at 405 nm were used to determine SP levels. Measurement of norepinephrine, dopamine, and their metabolites [3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)] was accomplished by using a Waters μBondapak C18 column (300 × 3.9 mm), a potential setting of 0.65 V vs. Ag/AgCl reference electrode, and a 2,5-dihydroxybenzylamine hydrobromide internal standard. The minimum detectable values for our analyses of neurochemicals are as follows: glutamine and glycine, <0.04 μM; GABA, <0.02 μM; 5-HT, <0.01 nmol/l; SP, <6.9 pg/ml; and norepinephrine, dopamine, HIAA, and HVA, <0.02 ng/ml.

Brain stem tissue analyses.

Following euthanasia, the cerebral circulation was isolated to flush the brain stems first with phosphate-buffered saline followed by 4% paraformaldehyde. The brain stems were then extracted, dehydrated in successive 20 and 30% sucrose solutions, and frozen and serially sectioned at 25-μm thickness in the transverse plane. One series was used for Nissl (cresyl violet) staining to facilitate measurements of microtubule placement. Images of Nissl-stained (4,000 dots/in.-scanned images) of the entire microtubule tract were captured (Nikon Super Coolscan 9000). Image software (Metamorph) was used to calibrate and measure microtubule placement in millimeters (relative to the midline, the obex, and the ventral medullary surface) near the middle of the rostral-caudal microtubule damage range.

Data and statistical analyses.

A pump with a known airflow rate was used to calibrate the inspiratory flow signal for breath-by-breath calculation of $\dot{V}i$, *f,* V_T, T_I, T_E, and V_T/T_I, using custom-designed software programs.

The EEG and EOG signals were utilized by a single investigator to score sleep state for each breath for night studies. Wakefulness was characterized by a high-frequency, low-voltage EEG, with variable EOG activity. NREM sleep was characterized by a low-frequency (<2 Hz), high-voltage (2–3 times greater than wakefulness) EEG with absent EOG activity. REM sleep was characterized by a high-frequency, low-voltage EEG, similar to wakefulness but concurrent with frequent and distinct bursting (>30 μV) in the EOG. For *hour 1* of the protocol when mCSF was dialyzed, the average percentages of time awake and in NREM were 81.8 ± 7.2 and 18.2 ± 6.5, respectively. For *hour 2*when 100 μM DAMGO was dialyzed, these percentages were 70.8 ± 4.9 and 28.8 ± 4.1, respectively. For *hour 3,* when mCSF was dialyzed, these percentages were 68.8 ± 7.4 and 31.0 ± 7.3, respectively. The percentages were nearly the same during the studies when the mixture of excitatory receptor antagonists was dialyzed during *hour 2*. These values for awake and NREM sleep are comparable to previous studies during which there was no dialysis (27). Similar to our previous studies, there was minimal/no REM sleep during any hour of dialysis. EEG and EOG were recorded only in night studies, and these signals did not change during the periods of drug dialysis.

The EMG signals were zeroed at baseline and then rectified and time averaged, after which the signals were recalibrated with the assignment of arbitrary peak (1) and baseline (0) values (2). These recalibrated EMG signals

were again rectified and time averaged (2). This processing was necessary because of the absence of known values and provided a measure of relative magnitude within studies (2). The signals were finally converted to a .txt file, and a custom-designed program outputted all parameters on a breath-by-breath basis (2).

Individual breath ventilatory and EMG data were averaged into 5- and 15-min bins. To restrict statistical analyses to stable conditions, statistical treatment was only for the last 15 min of *hour 1,* the final 45 min of *hour 2,* and all but the first 5 min of *hour 3*. For all ventilatory and EMG variables, metabolic rate ($\dot{V}o_2$), and TR, a two-way repeated-measures (RM) ANOVA compared each drug study to mCSF alone (1-factor repetition with dose and time as factors). The interaction *P* values indicated whether there were statistically significant differences between mCSF alone and mCSF mixed with drugs. For night studies, interaction term *P*values from two-way RM ANOVAs (2-factor repetition, state and time as factors with Holm-Sidak post hoc test used when appropriate) performed on 15-min bins alone were used to determine the effect of sleep state on ventilatory variables. These statistical analyses were on absolute values for each variable and again after normalizing each variable during *hours 2* and *3* of the protocol to the variable over the last 15 min of *hour 1*. Variability in all ventilatory parameters was quantified using the coefficient of variation (CV). The CV was determined for all breaths during 5-min intervals. A two-way RM ANOVA (with dose and time as factors) was used to determine whether significant interactions occurred as a result of dialysis (Holm-Sidak post hoc test used where appropriate).

For statistical analyses of neurochemical data, two-way RM ANOVAs (1-factor repetition, dose and time as factors) were used. The *P* values of the interaction term of ANOVA indicated whether the effect on effluent neurochemical concentration over time of antagonist dialysis was significantly different from that of the time-control studies. This analysis was performed for SP, 5-HT, glutamate, glycine, GABA, dopamine, and norepinephrine.

RESULTS

Placement of microtubules.

In Fig. 1, *left,* are representative transverse sections and graphical sketches of multiple rostrocaudal levels of the goat medulla, in which the location of the distal end of the histologically identified microtubule tract is shown for each goat (represented by number). On the *right* are shown Nissl-stained sections from four different goats in which the disrupted tissue indicates the site of microtubule implantation. The tissue damage shown on the *right* likely reflects a combination of microtubule and dialysis probe damage, tissue adherence during microtubule removal postmortem, and loss of structural support during processing. Even though the 1.27-mm-wide microtubule potentially destroyed the nucleus at the insertion site, drugs would diffuse rostrally and caudally from the dialysis probe (inserted 2 mm beyond the distal end of the microtubule). We did not measure diffusion of the drugs in this study, but on the basis of measurements described previously (20, 21) we conservatively estimate diffusion of 0.25 mm from the 1-mm-wide probe, which represents a minimal estimate of the affected brain stem region in each individual goat. On the basis of calculations of others (29) on drug diffusion in rats, it is likely that our estimates of diffusion indicated that the drugs were dialyzed into but were not restricted to the HMN.

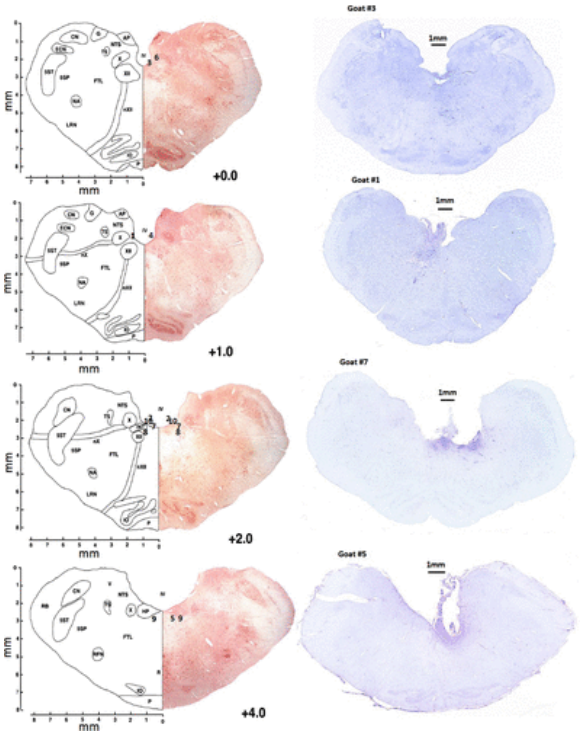

Fig. 1.*Left*: representative transverse sections of the goat medulla, at obex (0.0), 1.0 mm rostral (+1.0) to obex, 2.0 mm rostral (+2.0) to obex, and 4.0 mm rostral (+4.0) to obex, in which the location of the distal end of the histologically identified microtubule tract is shown for each goat (represented by number). Medial-lateral and dorsal-ventral coordinates on the axes are given in mm. *Right*: Nissl-stained sections from four different goats in which the disrupted tissue indicates the microtubule tracts. The dialysis membrane extended 2 mm beyond (ventrally) the microtubule. As detailed in the text, it is likely that for each goat, the dialyzed drugs diffused to a part of the HMN but that diffusion was not restricted to this nucleus. AP, area postrema; CN, cuneate nucleus; ECN, external cuneate nucleus; FTL, lateral tegmental field; G, gracile nucleus; HP, nucleus praepositus hypoglossi; INT, nucleus intercalatus; IO, inferior olivary complex; IV, trochlear nucleus; LRN, lateral reticular nucleus; NA, nucleus ambiguus; NTS, nucleus tractus solitarii; nX, vagus nerve; nXII, hypoglossal nerve; P, pyramidal tract; R, raphe nucleus; RB, restiform body; RFN, retrofacial nucleus; 5SP, spinal trigeminal nucleus; 5ST, spinal trigeminal tract; TS, tractus solitaries; V, vestibular nucleus; X, dorsal motor nucleus of the vagus; XII, hypoglossal nucleus.

Effects of DAMGO dialysis in the HMN.

Unilateral dialysis of 10 µM DAMGO during the day did not significantly ($P > 0.05$) change any ventilatory variable compared with control values obtained during unilateral dialysis of only mCSF (2-way RM ANOVA, time and dose as factors), either when the data were expressed in absolute values or when they were normalized to control ($n = 8$). However, unilateral 100 µM DAMGO dialyzed during the day significantly decreased $\dot{V}i$ ($P = 0.016$) and significantly increased T_E ($P < 0.05$) during and after DAMGO dialysis (2-way RM ANOVA, time and dose as factors; $n = 8$). This difference was apparent in the absolute values of $\dot{V}i$ and when $\dot{V}i$ was normalized to control dialyses (Fig. 2). On the other hand, *f* during DAMGO dialysis significantly differed from mCSF when expressed as absolute values but not when normalized (Fig. 2). Unilateral dialysis of 10 and 100 µM DAMGO did not significantly alter absolute or normalized V_T, T_I, or V_T/T_I.

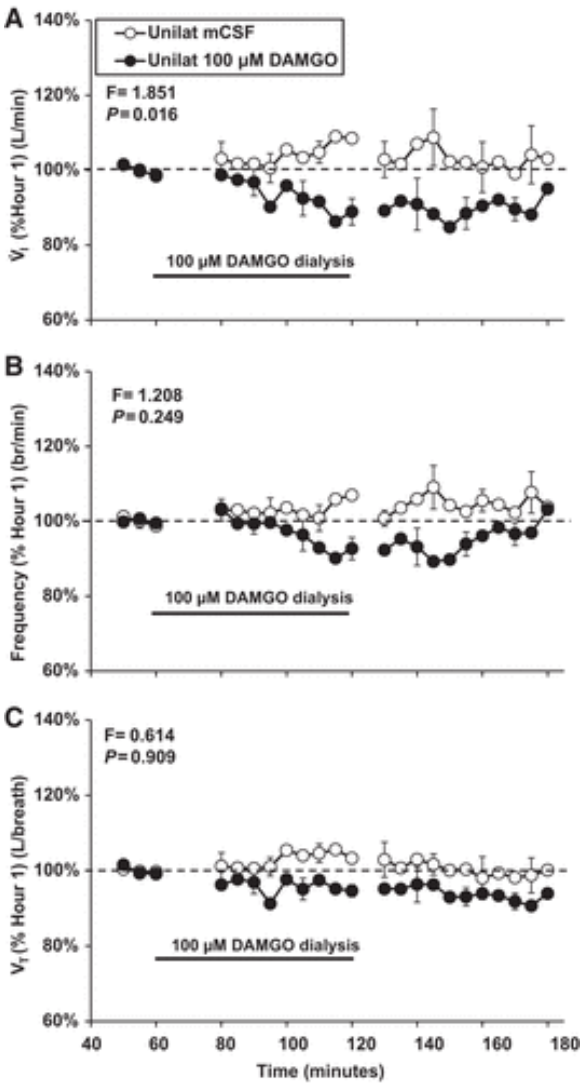

Fig. 2. When data during and after unilateral (Unilat) 100 µM [d-Ala^2, *N*-MePhe^4, Gly-ol]enkephalin (DAMGO) dialysis are expressed as a percentage of the final 15 min of the preceding hour of mock cerebrospinal fluid (mCSF) dialysis, ventilation ($\dot{V}$i, $P = 0.016$; *A*) was lower but breathing frequency ($P \geq 0.249$; *B*) and tidal volume (V_T, $P = 0.909$; *C*) were not different from dialysis of mCSF for 3 h ($n = 8$). ○, Studies during which mCSF was dialyzed for all 3 h; ●, studies during which 100 µM DAMGO was dialyzed between 60 and 120 min (DAMGO dialysis period indicated by the horizontal solid line). The *x*-axis values are time from the start of 180 min of dialysis. Data are means ± SE; br/min, breaths/min. *F* and *P* values were obtained from two-way repeated-measures ANOVA (time and dose as factors).

The EMG recordings suggested that unilateral dialysis of 100 µM DAMGO depressed GG activity compared with dialysis of mCSF alone (Fig. 3). Indeed, analysis of the integrated EMG (area under the curve) indicated that compared with mCSF dialysis, unilateral dialysis of 100 µM DAMGO significantly ($P< 0.001$) decreased GG muscle activity (Fig. 4*A*; 2-way RM ANOVA, dose and time as factors; $n = 8$). However, dialysis of 100 µM DAMGO did not significantly ($P = 0.809$) alter diaphragm activity compared with dialysis of mCSF alone (Fig. 4*B*). Unilateral 100 µM DAMGO dialysis also significantly ($P \leq 0.012$) decreased absolute values of $\dot{V}o_2$and HR, but did not significantly ($P \geq 0.05$) alter T_I, V_T/T_I, TR, BP, arterial partial pressure of CO_2 (PaCO2PaCO2), or PaO2PaO2 (2-way RM ANOVA, time and dose as factors). Neither 10 µM nor 100 µM DAMGO dialysis significantly ($P \geq 0.05$) increased the variability of any ventilatory parameter (calculated as the CV within each 5-min bin for each dose of DAMGO).

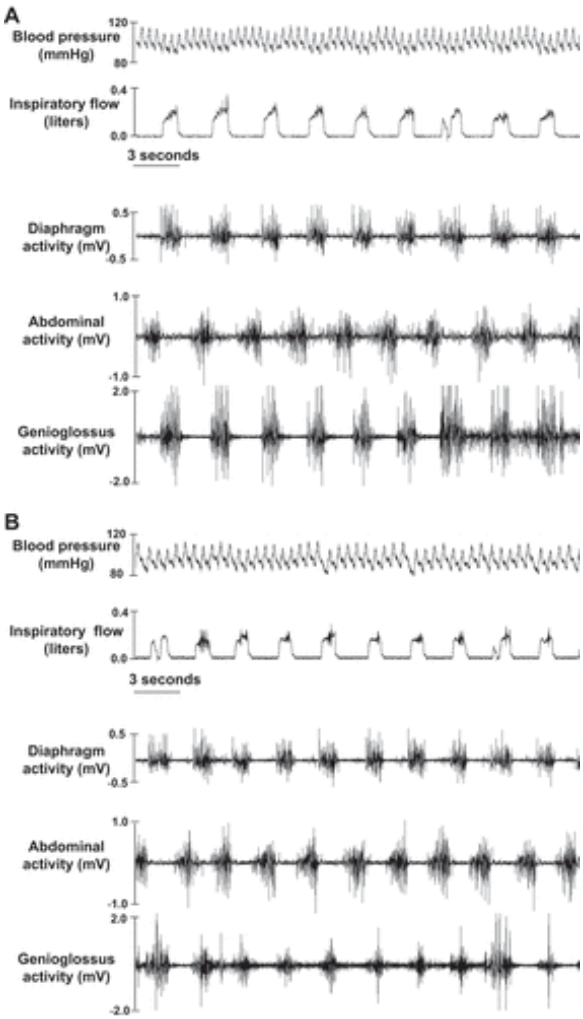

Fig. 3. Visual inspection of raw ventilatory and electromyographic data tracings before (*A*) and during (*B*) unilateral 100 μM [d-Ala^2, *N*-MePhe^4, Gly-ol]enkephalin (DAMGO) dialysis revealed that ventilation and genioglossus muscle activity were decreased by DAMGO dialysis. It is also evident that there were no major DAMGO-induced changes in heart rate, arterial blood pressure, or diaphragm and abdominal activity. Most of the goats chewed the abdominal electrodes, rendering them dysfunctional; thus insufficient data were obtained to determine whether any treatment significantly altered abdominal muscle activity. However, the data in this figure are from a goat in whom the abdominal electrodes remained functional, and it appears that DAMGO did not affect activity of this muscle.

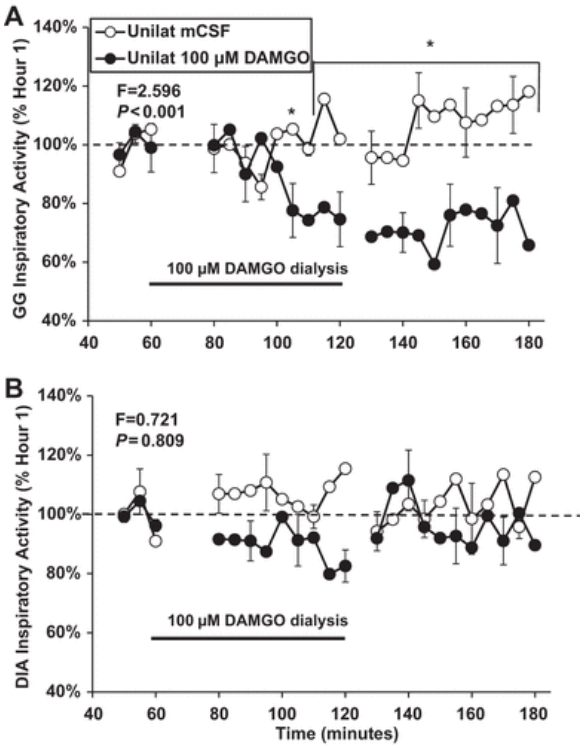

Fig. 4. Unilateral (Unilat) dialysis of 100 µM [d-Ala^2, *N*-MePhe^4, Gly-ol]enkephalin (DAMGO; *n* = 8) during daytime studies significantly (*P*< 0.001) decreased genioglossus (GG) muscle activity (*A*) but did not change (*P* = 0.809) diaphragm (DIA) muscle activity (*B*; *n* = 8). ●, Studies during which 100 µM DAMGO was dialyzed during *hour 2*; ○, studies during which mock cerebrospinal fluid (mCSF; *n* = 8) was dialyzed for 3 h. The *x*-axis indicates time from start of 180-min dialysis studies (horizontal solid line indicates time of 100 µM DAMGO dialysis). Data (means ± SE) are presented as a percentage of control using the last 15 min of *hour 1* mCSF dialysis as control values. *F* and *P* values were obtained from two-way repeated-measures ANOVA (time and dose as factors). *Time periods when a significant depression of GG activity occurred.

Absolute values for the awake state at night were generally significantly ($P < 0.05$) higher than during NREM sleep. However, the effect of unilateral DAMGO dialysis did not differ between awake and NREM sleep. This conclusion is based on finding that the percent change in $\dot{V}i$, *f,* and V_T did not differ between awake and NREM sleep when data during and after DAMGO dialysis were normalized to values over the last 15 min before dialysis of 10 µM (Fig. 5) or 100 µM DAMGO (2-way ANOVA, state and time as factors). Moreover, using one-way ANOVA (time as factor), $\dot{V}i$, *f,* and V_T did not change over time during dialysis of DAMGO in the awake state (Fig. 5). However, *f* ($P < 0.013$) and, to a lesser extent, $\dot{V}i$ and V_T ($P = 0.067$ and 0.084, respectively) decreased during and after dialysis of 10 µM DAMGO (Fig. 5). Dialysis of 100 µM DAMGO did not result in a similar time-dependent decrease in $\dot{V}i$, *f,* and V_T in the awake or NREM states ($P > 0.05$, data not shown).

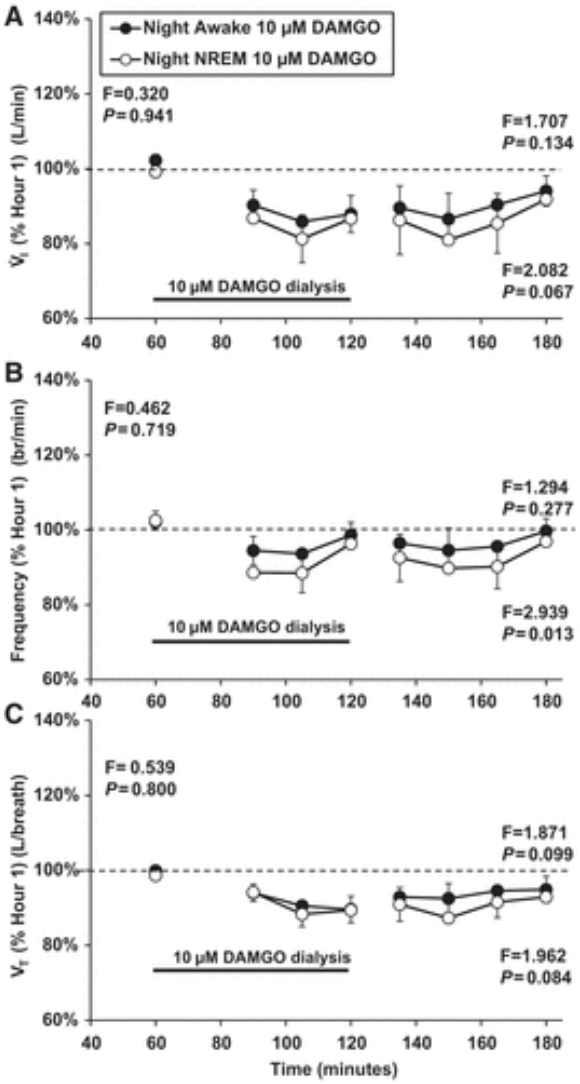

Fig. 5. When data during and after unilateral 10 µM [d-Ala^2, *N*-MePhe^4, Gly-ol]enkephalin (DAMGO; *n* = 8) dialysis are expressed as a percentage of the final 15 min of the preceding hour of mock cerebrospinal fluid dialysis (*n* = 8), there were no significant (*P* ≥ 0.771) interaction terms for ventilation (V̇i; *A*), breathing frequency (*f*; *B*), or tidal volume (V_T; *C*) as reported by two-way repeated-measures ANOVA comparing non-rapid eye movement (NREM) sleep (○) and night awake states (●; state and time as factors, *F* and *P* values at *top left* of each panel). Dialysis of 10 µM DAMGO did significantly (*P* = 0.009) depress *f*during the NREM state but did not alter V̇i or V_T or any ventilatory variable during the night awake state (*P* ≥ 0.171; 1-way repeated-measures ANOVA with time as factor; *F* and *P* values for the night awake state at *top right* and NREM state *bottom right* of each panel). The *x*-axis is time from start of dialysis in 180-min dialysis studies (horizontal solid line indicates period of dialysis); br/min, breaths/min.

There were no significant (*P* ≥ 0.992) interaction terms for V̇i, *f,* or V_T comparing bilateral dialysis of 100 µM DAMGO to bilateral dialysis of mCSF (2-way RM ANOVA, dose and time as factors; *n* = 5). However, bilateral dialysis of 100 µM DAMGO significantly (*P* ≤ 0.008) decreased *f* and V_T over time (*P* value of time factor from 2-way RM ANOVA), but V̇idid not differ significantly over time (*P* = 0.992) between bilateral DAMGO and mCSF dialysis (Fig. 6). During the hour of and the hour after unilateral and bilateral dialysis of 10 and 100 µM DAMGO during the day, 5-HT, SP, glutamate, norepinephrine, dopamine, glycine, and GABA in the effluent mCSF did not differ from studies in which only mCSF dialyzed.

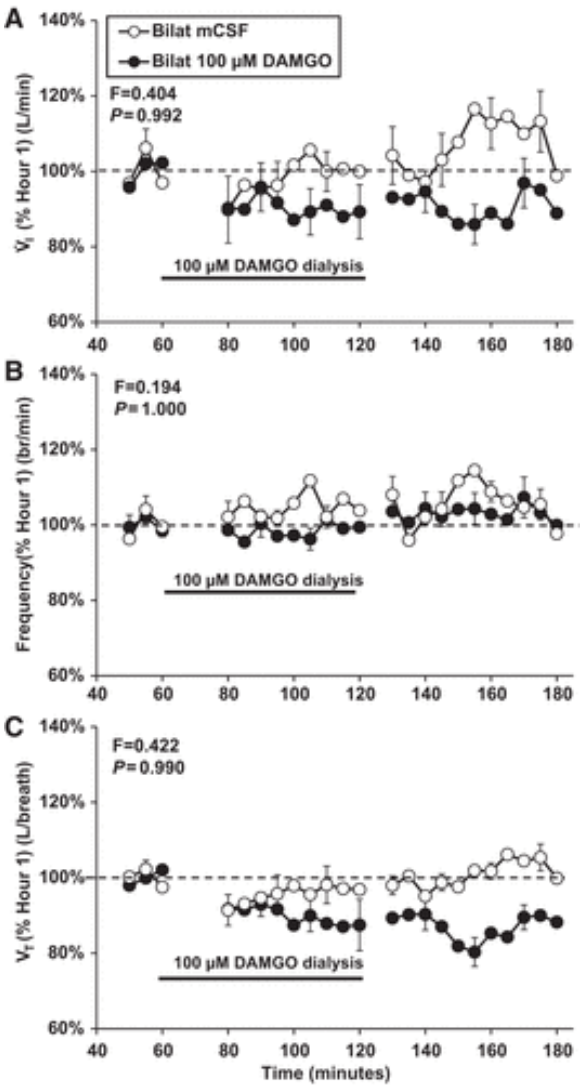

Fig. 6. When data during and after bilateral (Bilat) 100 µM [d-Ala^2, *N*-MePhe^4, Gly-ol]enkephalin (DAMGO) dialysis (*n* = 5) are expressed as a percentage of the final 15 min of the preceding hour of mock cerebrospinal fluid (mCSF) dialysis, there was no significant (*P* ≥ 0.990) interaction between dose and time (2-way repeated-measures ANOVA, dose and time as factors) for ventilation ($\dot{V}i$; *A*), breathing frequency (*f*; *B*), or tidal volume (V_T; *C*) from 3 h of mCSF dialysis (*n* = 4). However, bilateral 100 µM DAMGO dialysis significantly decreased *f* (*P* = 0.008) and V_T (*P* < 0.001) over time (*P* values from time factor of 2-way repeated-measures ANOVA). Studies during which 100 µM DAMGO was dialyzed from 60 to 120 min are indicated by ● (horizontal solid line indicates period of DAMGO dialysis). Data are means ± SE; br/min, breaths/min. *F* and *P* values were obtained from two-way repeated-measures ANOVA (time and dose as factors).

Effects of unilateral 50 mM atropine dialysis in the HMN.

When values during and after unilateral dialysis of 50 mM atropine into the HMN of awake goats were normalized to values before atropine dialysis, there were no significant (*P* ≥ 0.176) differences in $\dot{V}i$, *f,* or V_T (Fig. 7) compared with dialysis of mCSF alone (2-way RM ANOVA, time and dose as factors; *n* = 8). However, when a comparison was made of the absolute values before, during, and after atropine dialysis, *f* was significantly (*P* < 0.05) increased by atropine. Irrespective of comparisons of absolute or normalized data, there were no significant (*P* ≥ 0.225) effects of 50 mM atropine dialysis on GG and DIA muscle activity, T_E, T_I, V_T/T_I, $\dot{V}o_2$, HR, BP, TR, PaCO2PaCO2, or PaO2PaO2 (2-way RM ANOVA, dose and time as factors). Unilateral dialysis of 50 mM atropine significantly (*P* < 0.001) increased 5-HT (Fig. 8) but did not significantly (*P* ≥ 0.093) alter norepinephrine,

dopamine, glycine, GABA, or glutamate in effluent dialysate compared with dialysis of mCSF alone (2-way RM ANOVA, time and dose as factors).

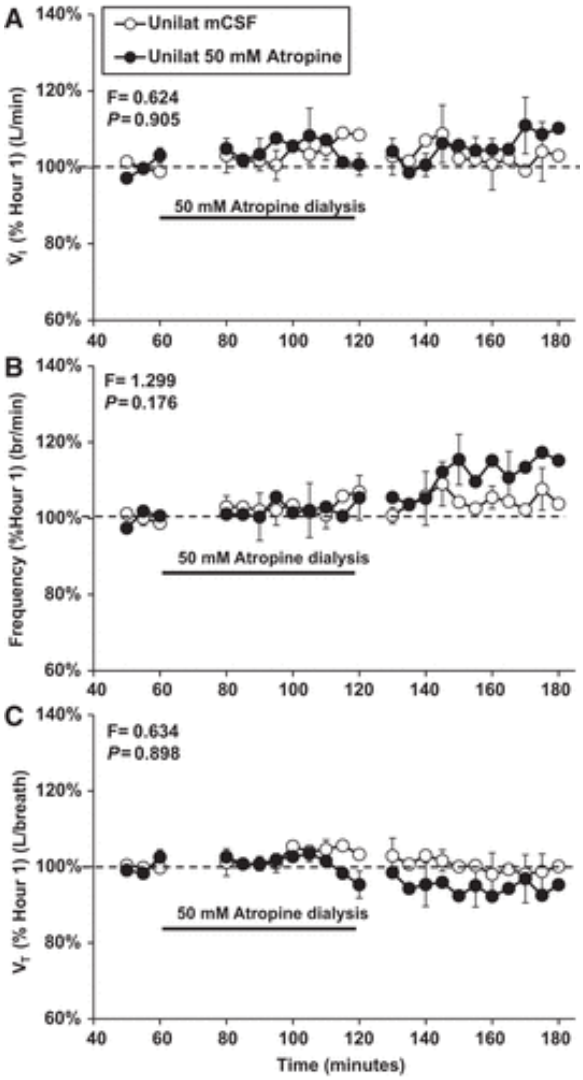

Fig. 7. Unilateral (Unilat) dialysis of 50 mM atropine during the day (n = 6) did not significantly ($P \geq 0.176$) alter ventilation ($\dot{V}i$; *A*), breathing frequency (*f*; *B*), or tidal volume (V_T; *C*) compared with dialysis of mock cerebrospinal fluid (mCSF) alone (n = 8). ○, Studies during which mCSF alone was dialyzed for 3 h; ●, studies during which 50 mM atropine was dialyzed from 60 to 180 min (horizontal solid line indicates period of atropine dialysis). *F* and *P* values were obtained from two-way repeated-measures ANOVA with drug and time as factors. Data are means ± SE expressed as percentage of control (using the last 15 min of *hour 1* mCSF dialysis as control values); br/min, breaths/min. The *x*-axis indicates time from start of 180 min of dialysis.

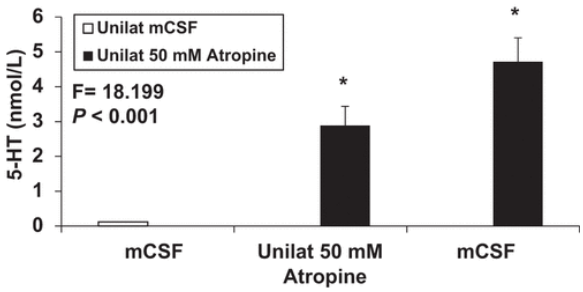

Fig. 8. Serotonin (5-HT) was significantly (*P < 0.001) increased in *hours 2*and *3* of 50 mM atropine dialysis studies (n = 6) compared with dialysis of mock cerebrospinal fluid (mCSF) alone. Unilat, unilateral. The *x*-axis indicates the period of

dialysis, and data are means ± SE. *F*and *P* values were obtained using two-way repeated-measures ANOVA analysis with time and drug as factors.

Effects of dialysis of a mixture of muscarinic, 5-HT, and NK_1 receptor antagonists in the HMN.

There were no significant ($P \geq 0.153$) changes in $\dot{V}i$, *f,* or V_T(Fig. 9) in response to unilateral daytime dialysis of a mixture of antagonists to muscarinic (5 mM atropine), 5-HT_{2A} (0.5 mM MDL 11,939), and NK_1 (500 μM spantide) receptor agonists (2-way RM ANOVA, dose and time as factors). Similarly, GG and DIA muscle activity, T_E, T_I, V_T/T_I, $\dot{V}o_2$, TR, HR, BP, TR, Pa_{CO2} (2-way RM ANOVA, dose and time as factors) were not altered by unilateral daytime dialysis of the excitatory antagonist mixture. This lack of a drug effect was apparent irrespective of comparing absolute or normalized data. We also found no significant ($P \geq 0.435$) interaction between state (awake vs. NREM) and time (2-way RM ANOVA) during antagonist mixture dialysis at night for $\dot{V}i$, *f,*or V_T (Fig. 9). Analysis by one-way ANOVA (time as factor) indicated that there were also no significant ($P \geq 0.300$) time-dependent effects of antagonist mixture dialysis on $\dot{V}i$ or *f*during the night awake or NREM states (Fig. 10). One-way ANOVA also indicated that V_T was not altered ($P = 0.715$) at night while in awake state but V_T was significantly ($P = 0.005$) decreased during NREM sleep. During the hour of and the hour after dialysis of the mixture of antagonists to excitatory receptors, 5-HT, SP, norepinephrine, dopamine, glutamate, GABA, and glycine levels did not differ significantly ($P \geq 0.116$) from the periods when mCSF alone was dialyzed (data not shown).

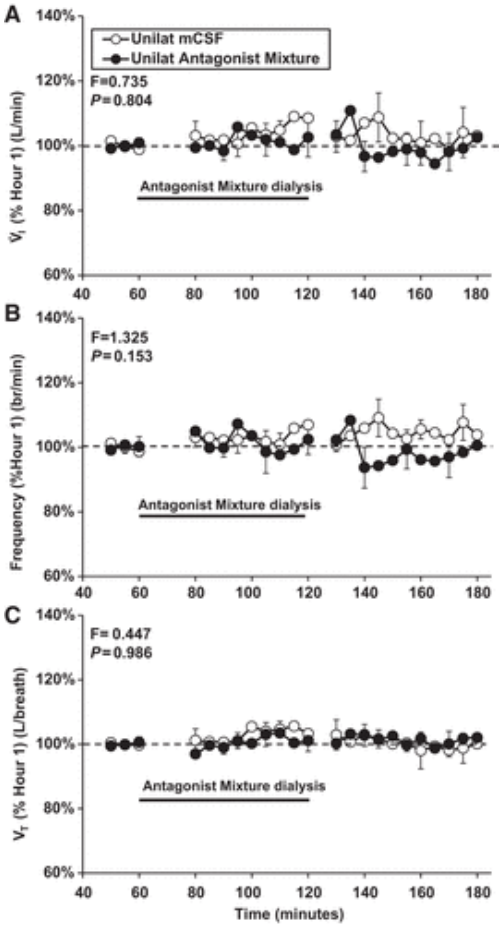

Fig. 9. Unilateral (Unilat) dialysis of a mixture of antagonists ($n = 7$) to muscarinic receptors (5.0 mM atropine), neurokinin-1 (NK_1) receptors (500 μM spantide), and serotonin 2A (5-HT_{2A}) receptors [0.5 mM α-phenyl-1-(2-phenylethyl)-4-piperidine methanol (MDL 11,939)] did not significantly ($P \geq 0.153$) alter ventilation ($\dot{V}i$; *A*), breathing frequency (*B*), or tidal volume

(V_T; *C*) compared with dialysis of mock cerebrospinal fluid (mCSF) alone (*n* = 8). ○, Studies during which mCSF alone was dialyzed for 3 h; ●, studies during which the antagonist cocktail was dialyzed during *hour 2* (60–120 min). *F* and *P*values were obtained using two-way repeated-measures ANOVA (dose and time as factors), and data are means ± SE (expressed as percentage of control with the last 15 min of *hour 1* mCSF dialysis used as control values); br/min, breaths/min. The *x*-axis indicates time from start of 180-min dialysis.

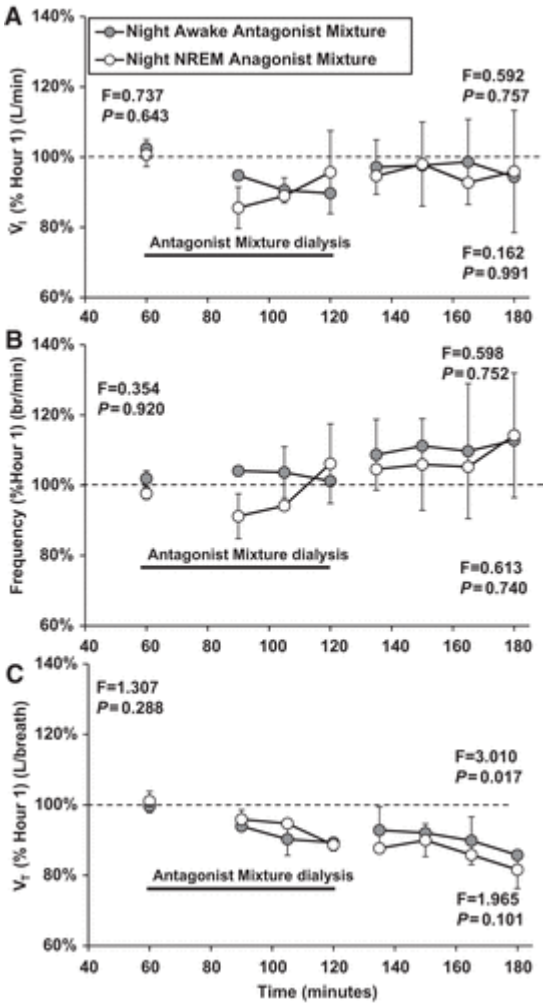

Fig. 10. Unilateral nighttime dialysis of a mixture of antagonists (*n* = 5) to muscarinic receptors, neurokinin-1 (NK_1) receptors, and serotonin 2A (5-HT_{2A}) receptors [5.0 mM atropine, 500 μM spantide, and 0.5 mM α-phenyl-1-(2-phenylethyl)-4-piperidine methanol (MDL 11,939)] did not result in any significant ($P \geq 0.435$) interactions between sleep state and time for ventilation (V̇i; *A*), breathing frequency (*f*; *B*), or tidal volume (V_T; *C*) using two-way repeated-measures ANOVA (state and time as factors). The antagonist mixture did not significantly ($P \geq 0.715$) alter V̇i or *f* in either the night awake (●) or non-rapid eye movement (NREM) sleep (○) state; however, V_T was significantly ($P = 0.005$) depressed in the NREM sleep state, but not in the night awake ($P = 0.197$) state. One-way repeated-measures ANOVA (with time as factor) was used to obtain *F* and *P* values for each state. The *x*-axis indicates time from start of 180-min dialysis studies, and the horizontal solid line indicates period of antagonist cocktail dialysis (60–120 min); br/min, breaths/min.

DISCUSSION

Our overall hypothesis was that mechanisms of compensatory neuromodulation are not uniform throughout the respiratory control network. Accordingly, since others (10, 13, 48) found that dialysis of various μ-opioid receptor agonists into the HMN depressed respiratory output and GG muscle activity, we hypothesized that unilateral and bilateral dialysis of DAMGO in the HMN would depress respiratory output and GG muscle activity in awake and sleeping goats and would not elicit local changes in neuromodulators. Our findings support this

hypothesis. In addition, since others (1, 48) have shown that acetylcholine modulation of the HMN is via the inhibitory M2 receptor and since there appears to be minimal stimulation of the HMN by 5-HT (15), we hypothesized that dialysis of antagonists to muscarinic, NK_1, and 5-HT receptors would not depress ventilation and there would be no compensatory increase in neuromodulators. Our findings support the ventilatory but not the neurochemical part of these hypotheses.

Concept of neuromodulator interdependence.

The neural circuitry controlling ventilation is modulated largely through G protein-coupled metabotropic receptors to determine the network excitability and stability (7, 8). Doi and Ramirez tested the concept of neuromodulator interdependence where changes in one or more neuromodulators are compensated for by changes in other modulators to maintain stability in the respiratory control network (7, 8). Subsequent studies support this concept as compensatory neuromodulation was elicited by dialysis of agonists/antagonists in the respiratory rhythm/pattern-generating VRC of awake and sleeping goats (20, 21, 32–35). The objective herein was to study this concept as it applies to the motor neuron-rich HMN, focusing on effects of activating μ-opioid receptors (DAMGO) or inhibiting muscarinic (atropine), neurokinin-1 (spantide) and select serotonergic receptors ($5\text{-}HT_{2A}$; MDL 11,939).

Compensatory neuromodulation in the HMN could occur through several different neuromodulators. For example, 5-HT (14–16, 50–52) has excitatory effects through $5\text{-}HT_{2A}$receptors and inhibitory effects through presynaptic $5\text{-}HT_{1B}$receptors (46, 47). Acetylcholine (ACh) in adult mammals has an inhibitory effect through the type 2 (M2) muscarinic receptor (1, 23), which is dominant over the nicotinic ACh receptors that excite the HMN (22). Norepinephrine modulates excitability of HMN through presynaptic α_1-adrenergic receptors (15), indirectly by potentiating the excitatory effects of glutamate when coreleased onto motor neurons (3, 15). Antagonists to GABA (23) and glycine (31) receptors administered to the HMN result in robust increases in GG activity indicating a significant role of inhibitory amino acids in regulating tonic GG activity (23, 31). In addition, opioids depress ventilation through G-protein inwardly rectifying potassium channels and voltage-dependent Ca^{2+}channels (30). However, opioids can also have excitatory effects by inhibition of nonopioid inhibitory interneurons or by receptor subtypes that cause opposite effects on voltage-sensitive K^+ and Ca^{2+} channels that cause hyperpolarization (4–6, 45). Indeed, conformational changes in opioid receptors cause coupling to inhibitory G proteins when exposed to high opioid concentration, whereas the same receptor subtype might switch to a stimulatory G protein during exposure to low opioid concentrations (11, 49).

Opioids modulate neural activity throughout the central nervous system, including the preBötC and HMN (10, 18, 20, 48). The effects of opioid administration in the preBötC, critical for the generation of respiratory rhythm and pattern (18, 21), have been equivocal, where some studies found breathing increases (20, 36), breathing decreases (28, 29), or no effect (18, 20). Similarly, there are differences between studies on respiratory effects of opioids delivered to the HMN. In rodents, the dominant effect of opioids is to reduce the amplitude of hypoglossal activity suggesting that there would be a reduction in tidal volume but not breathing frequency (10, 24). In awake goats, opioids delivered to the HMN also decreased hypoglossal muscle activity, which did not significantly decrease tidal volume, but there was an increase in expiratory time and decrease in breathing frequency. The cause of the decreased frequency is unclear, but it is possible that the DAMGO dialysis centered on the HMN may have diffused more widely than expected (see *Caveats and limitations*) and reached more lateral regions including but not limited to the nucleus of the solitary tract (NTS), a nucleus critical for integrating peripheral afferent information and cardiorespiratory control (12). Opioids injected directly into the NTS of anesthetized rats slowed respiratory frequency and significantly altered heart rate (12), which might help explain the decreases in respiratory frequency and heart rate observed herein.

Differences between sites in respiratory effects of opioids might be due to differences in compensatory neuromodulation. Dialysis of 100 μM DAMGO unilaterally into the VRC resulted in a mild and transient decrease in $\dot{V}i$, and bilateral DAMGO increased breathing (20). Concomitant with these effects was a decrease in local GABA (20), which may have compensated for the opioid-mediated depression. Herein we found that both unilateral and bilateral dialysis of DAMGO into the HMN decreased $\dot{V}i$ without concomitant changes in measured neuromodulators. These results are consistent with compensatory neuromodulation in the VRC but not in the HMN.

To our knowledge, the only other study finding increased release of neuromodulators during opioid dialysis into the HMN was by Skulsky et al., who found a dose-dependent increase in ACh in the effluent mCSF during dialysis of morphine into the HMN of anesthetized rats (48). Skulsky et al. speculated that the ACh increase was secondary to disinhibition by opioid receptors on GABAergic neurons controlling ACh release from hypoglossal motoneurons (48). This response would not be compensatory as the increased ACh would add to and/or could be the mechanism by which opioids depress neuronal activity. This ACh increase appears unique to medullary neurons as dialysis of opioids into nuclei rostral to the brain stem decreases ACh release (9, 25, 39).

Since ACh modulation of the HMN is via the inhibitory M2 receptor (22, 26), it could be expected that atropine dialysis into the HMN would increase breathing. However, there was an increase in 5-HT during atropine dialysis that may have offset the M2 atropine disinhibition through stimulation of 5-HT_{1B} receptors, which are inhibitory to breathing (15). It is likely that the increased 5-HT minimally stimulated breathing as others have shown that 5-HT stimulation through the 5-HT_{2A} receptor is minimal.

Significance of the present findings.

Since we used the same model and microdialysis protocol as in our previous VRC studies, we can compare physiological and neurochemical responses to neuromodulatory perturbations in the VRC and HMN. Significant differences noted particularly in the effects of dialysis of DAMGO included *1*) ventilatory instability only at the VRC, *2*) decreased $\dot{V}o_2$ and HR only at the HMN, *3*) marked hyperpnea during bilateral dialysis only at the VRC, and *4*) absence of measured neurochemical compensation to DAMGO only at the HMN. The site differences in physiologic responses could all be due to a site difference in compensation by an excitatory neuromodulator. Alternatively, the site differences in physiologic responses could be due to differences in effects of increased inhibition/decreased excitation in a rhythm-generating network (VRC) vs. a site of motoneurons (HMN; 19, 53, 54). Finally, the differences in effects between this and our previous studies may not reflect only VRC vs. HMN differences because there may have been diffusion of drugs to adjacent nuclei that, for example, decreased $\dot{V}o_2$ and HR in this but not in previous studies. Irrespective of the mechanism, site differences in physiologic and neuromodulator responses lead to the conclusion previously made by Skulsky et al. that the existence of these differences "emphasizes the need to characterize opioid effects throughout the widely distributed neural networks that regulate sleep and breathing" (48).

Also significant are findings that neuromodulator compensation is relatively less robust in the HMN. In other words, compensatory neuromodulation does not appear to be uniform across as least two critical sites in the ventilatory control network. It appears that mechanisms that maintain a patent airway are less well protected against opioid depression than the respiratory rhythm- and pattern-generating network, which would seem important to obstructive sleep apnea.

Also significant are findings that DAMGO and the mixture of antagonists to receptors of excitatory neuromodulators depressed components of the ventilatory control system during NREM sleep but not at night in the awake state (Figs. 5 and 10). These data are consistent with previous findings showing state-dependent difference in compensatory neuromodulation in the VRC (21, 32).

Finally, it is significant that dialysis of 50 mM atropine into the HMN increased 5-HT in the effluent mCSF virtually as much as dialysis into the VRC. This finding suggests that the composition of cells and the function of the nucleus are not strict determinants of changes in neuromodulators to compensate for presumed alterations in effects of endogenous neuromodulators. As explained earlier, the same change in 5-HT might result in compensation at both sites by activating two different 5-HT receptors (20, 21, 32–35). This compensatory 5-HT response is thus very robust, as also indicated by findings that dialysis of 50 mM atropine into the VRC was state independent, occurring to the same extent during awake, asleep, and anesthetized states (21, 32).

Caveats and limitations.

The limitations of our model and experimental protocol have been discussed in detail in previous articles (20, 21, 32–35). Nevertheless, we point out that even though the HMN was targeted for microtubule placement, the need to avoid blood vessels on the surface of the brain stem and/or surgical complications resulted in variation in placement of the microtubules (Fig. 1). However, the uniformity among goats in the decrease in GG muscle activity during dialysis of DAMGO provides evidence that the HMN was indeed perfused. The range of diffusion of drugs from the dialysis probe means that we may have affected other nuclei outside the HMN; thus it is possible that the effects on ventilation, $\dot{V}o_2$, and HR may have been caused in part by DAMGO effects outside the HMN. Another limitation is that we cannot measure all the potential neurochemicals in effluent dialysate; thus we could have missed changes in other neuromodulatory molecules. In addition, some neuromodulators might be metabolized rapidly, only released transiently, or degraded in the tubing and collection vials; thus the concentration in the effluent mCSF likely will not exactly represent the neuromodulator at the tissue level.

Conclusions.

We conclude that overall the mechanisms of compensatory neuromodulation are less robust in the HMN than in the VRC conceivably because of site differences in *1*) mechanisms through which neuromodulator perturbations alter neuronal activity and/or *2*) responsiveness of receptors to compensatory neuromodulators.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.M.L., S.E.N., M.R.H., L.P., and H.V.F. conceived and designed research; T.M.L., S.E.N., E.C., N.J.B., S.T., L.P., and H.V.F. performed experiments; T.M.L., S.E.N., E.C., N.J.B., S.T., and H.V.F. analyzed data; T.M.L., N.J.B., M.R.H., and H.V.F. interpreted results of experiments; T.M.L., S.E.N., and M.R.H. prepared figures; T.M.L. and H.V.F. drafted manuscript; T.M.L., S.E.N., M.R.H., and H.V.F. edited and revised manuscript; T.M.L., S.E.N., M.R.H., and H.V.F. approved final version of manuscript.

AUTHOR NOTES

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