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Effects on Breathing of Agonists to μ -opioid or GABA_A Receptors Dialyzed into the Ventral Respiratory Column of Awake and Sleeping Goats

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Contents

Keywords3

Highlights **Error! Bookmark not defined.**

Abstract3

1. Introduction.....3

2. Methods4

 2.1. Goats.....4

 2.2. Surgical procedures5

 2.3. Physiological studies.....6

 8

 2.4. Neurochemical analyses9

 2.5. Data and statistical analyses9

 2.6. Neurochemical analysis 10

3. Results 10

 3.1. Placement of microtubules (MTs) 10

 3.2. DAMGO microdialysis..... 11

 3.3. Muscimol microdialysis 23

4. Discussion	26
4.1. Ventilatory and neurochemical effects of opioids dialyzed into the VRC.....	26
4.2. Ventilatory and neurochemical effects of unilateral muscimol dialysis in the VRC.....	28
4.3. μ -Opioid and GABA receptor-mediated breathing instabilities	28
5. Caveats and limitations	28
6. Summary and conclusions.....	29
Author contributions.....	29
Disclosures.....	29
References.....	30

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Breathing, Neuromodulation, μ -Opioids, GABA_A, Ventral respiratory column, Sleep

Abstract

Pulmonary ventilation (\dot{V}_i) in awake and sleeping goats does not change when [antagonists](#) to several excitatory [G protein-coupled receptors](#) are dialyzed unilaterally into the ventral respiratory column (VRC). Concomitant changes in excitatory neuromodulators in the effluent mock cerebral spinal fluid (mCSF) suggest neuromodulatory compensation. Herein, we studied neuromodulatory compensation during [dialysis](#) of [agonists](#) to inhibitory [G protein-coupled](#) or [ionotropic receptors](#) into the VRC. [Microtubules](#) were implanted into the VRC of goats for dialysis of mCSF mixed with agonists to either μ -opioid (DAMGO) or [GABA_A](#) (muscimol) receptors. We found: (1) \dot{V}_i decreased during unilateral but increased during bilateral dialysis of [DAMGO](#), (2) dialyses of DAMGO destabilized breathing, (3) unilateral dialysis of [muscimol](#) increased \dot{V}_i , and (4) dialysis of DAMGO decreased GABA in the effluent mCSF. We conclude: (1) neuromodulatory compensation can occur during altered inhibitory neuromodulator receptor activity, and (2) the mechanism of compensation differs between G protein-coupled excitatory and inhibitory receptors and between G protein-coupled and [ionotropic](#) inhibitory receptors.

1. Introduction

From invertebrates to mammals, the excitability of neural circuits that govern motor outputs depends largely upon the local levels of excitatory and inhibitory [neuromodulators](#). However, the mechanisms that regulate the local release of neuromodulators, or compensatory shifts in [second messengers](#) downstream of neuromodulatory receptors remain unclear. Gaining insight into regulation of neuromodulation is important to improve treatment of disorders such as [epilepsy](#), depression, and Parkinson's disease, as well as [opioid](#) induced respiratory depression; thus, there is a need for studies on control of neuromodulators.

Data from rodent models suggests there is "neuromodulator interdependence", whereby changes in the activity of one or more neuromodulatory receptors are compensated by changes in the release of other neuromodulators to maintain stable ventilation ([Doi and Ramirez, 2008](#), [Doi and Ramirez, 2010](#)). This concept has also been studied in awake and sleeping goats by dialyzing [atropine](#) (50 mM) unilaterally into the ventral respiratory column (VRC) to reduce or block excitatory [muscarinic receptor](#) activity ([Muere et al., 2013](#)). Rather than a hypothesized decrease in pulmonary ventilation (\dot{V}_i) and breathing frequency (f), atropine [dialysis](#) increased f and \dot{V}_i ([Muere et al., 2013](#)) which could have been due to increases in the excitatory neuromodulators [substance P](#) (SP) and [serotonin](#) (5-HT) as measured in the effluent dialysate. Subsequent studies in this goat model found that a lower dose of atropine (5 mM), or selective M2 (methoctramine) or M3 (4-DAMP) [receptor antagonists](#) had no effect on f and \dot{V}_i , but increased SP in the effluent dialysate to a lesser extent than 50 mM atropine ([Muere et al., 2015a](#)). Moreover, dialysis into the VRC of antagonists to excitatory SP [receptors](#) ([neurokinin-1](#); NK-1) or 5-HT receptors (5-HT_{2A}) alone or in combination with 5 mM atropine did not affect f and \dot{V}_i , but increased SP and decreased [GABA](#) in effluent dialysate ([Muere et al., 2015b](#)). These findings suggest that presumptive decreases in activity of one or more excitatory receptors are accompanied by changes in other neuromodulators to maintain f and \dot{V}_i in awake and [NREM sleep](#) states which supports the concept of neuromodulator interdependence ([Doi and Ramirez, 2008](#)). In other words, there is "compensation" defined as

no change in ventilation with dialysis of excitatory antagonists and that this may be due to the observed concomitant increase in excitatory (SP) and decrease in [inhibitory neurotransmitters](#) (GABA).

The studies above examined the effects of blocking excitatory, [G protein-coupled](#) neuromodulatory receptors. It is unknown whether increased activation of inhibitory receptors in the VRC will also elicit compensatory changes in neuromodulators and/or affect f and \dot{V}_i . Widely expressed in the VRC are μ -opioid inhibitory receptors ([Krause et al., 2009a](#), [Pattinson, 2008](#)), which are also [G protein-coupled receptors](#) (GPCRs). When activated, μ -opioid receptors act through second messenger cascades to reduce [intracellular calcium](#) and increase potassium [efflux](#) to decrease cellular activity ([Pattinson, 2008](#)). Administration of μ -opioid [receptor agonists](#) to the preBötzing Complex (preBötC) region of the VRC has, in some studies, caused respiratory depression ([Montandon and Horner, 2014](#), [Montandon et al., 2011](#), [Pattinson, 2008](#)). However, other studies found that eupneic breathing is unchanged ([Krause et al., 2009a](#)) or increased with μ -opioid receptor agonists administration to the preBötC/VRC ([Mustapic et al., 2010](#)). The absence of a depression of breathing with increased μ -opioid receptor-mediated inhibition could be due to neuromodulatory compensation.

Another endogenous inhibitory neuromodulator controlling breathing is γ -aminobutyric acid (GABA) acting at GABA_B [metabotropic receptors](#) to regulate neuronal [membrane potential](#). However, acting through GABA_A [ionotropic receptors](#), GABA also directly affects [neuronal activity](#) by altering intracellular [chloride](#) ([Tillakaratne et al., 1995](#)). It is unknown whether neuromodulator interdependence occurs when the activity of ionotropic receptors is altered, and whether compensation occurs by similar or different mechanisms to increases in inhibitory signaling through GPCRs.

Herein we studied whether neuromodulatory compensation occurs in response to increased inhibitory receptor activity by unilaterally dialyzing the μ -opioid receptor agonist [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (DAMGO) or the GABA_A receptor agonist [muscimol](#) into the VRC while measuring breathing and [neurochemicals](#) in the effluent dialysate of awake and sleeping goats. We chose these inhibitory agonists due to lack of agreement among several previous studies on effects on respiratory control of these agonists ([Curran et al., 2000](#), [Gatti et al., 1987](#), [Krause et al., 2009a](#), [Lalley, 2003](#), [Lalley, 2008](#), [Montandon et al., 2011](#), [Mustapic et al., 2010](#), [Nattie and Li, 2000](#), [Taylor et al., 2006](#), [Yamada et al., 1981](#), [Yamada et al., 1982](#)). We hypothesized that f and \dot{V}_i would not change during [DAMGO](#) or muscimol dialysis, but that there would be changes in neurochemicals in the effluent dialysate. We also hypothesized that the mechanism of compensation would differ between antagonism of GPCR and ionotropic receptors.

Through bilateral dialysis of DAMGO, we also tested the hypothesis that increased activity of the contralateral VRC may contribute to the stable f and \dot{V}_i during unilateral DAMGO dialysis. We did not test this same hypothesis for muscimol because of the large ventilatory and behavioral responses observed in some goats with unilateral muscimol dialysis (see Results).

2. Methods

2.1. Goats

Fifteen adult female, non-pregnant goats with an average body weight of 51.0 \pm 8.5 kg were housed and studied in an environmental chamber with a fixed ambient temperature and alternating 12-h light-dark cycles set between 7am and 7pm. There was free access to food and water except for study periods and the 24 h preceding surgeries. All protocols and procedures utilized in this study were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

2.2. Surgical procedures

The goats were anesthetized with [ketamine](#) (IV), [intubated](#), and mechanically ventilated with [isoflurane](#) in 100% oxygen for the duration of the surgery. Flunixin [meglumine](#) (2 mg/kg, IM) was administered once pre-operatively and for two days post-operatively for pain. To minimize infection, [ceftriaxone](#) (25 mg/kg, IV) in 100 mL saline was administered over 30 min twice daily at 8–12 h intervals for three days starting the morning of a [craniotomy](#) surgery. Subsequently, ceftiofur sodium (4 mg/kg, IM) and [gentamycin](#) (6 mg/kg, IM) were administered once daily for the remainder of the study period. To minimize intracranial [edema](#) following the craniotomy, [dexamethasone](#) (4 mg/mL, IV) was administered three times daily and tapered for a week following surgery. [Surgical sites](#) were treated daily for 7 days with [triple antibiotic ointment](#). Rectal temperature (TR), heart rate (HR), and [oxygen saturation](#) were continuously monitored during surgery. Over the following 24 post-operative hours these variables were monitored at predetermined intervals.

Under sterile conditions, two surgeries were performed separated by two weeks. During one surgery, 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 overlying skin before closing the incision. Also, [electroencephalographic](#) (EEG) and electrooculographic (EOG) electrodes were inserted into the midline cranium and the superior orbital ridge for recordings to score wakefulness/sleep during night studies. The electrodes were attached to screws, secured with dental acrylic, and the wires were pulled through the skin prior to closure of the incision.

In a second surgery two weeks later, stainless steel [microtubules](#) (70.0 mm length, 1.27 mm outer diameter, 0.84 mm inner diameter) were chronically implanted unilaterally (n = 3) or bilaterally (n = 12) into or near the preBötC of the VRC ([Fig. 1A & B](#)). An incision was made that bisected the nuchal ligament along the dorsal midline of the skull and neck. A rotary drill was used to create an [occipital](#) craniotomy for visualization of the dura which, when opened, exposed the dorsal [medulla](#). [Obex](#) served as an anatomical reference for micromanipulator controlled microtubule placement into the VRC. The target for placement was the preBötC which in goats is approximately 2.5–3.5 mm rostral from obex, 4.0–5.0 mm lateral from the midline and 4.0–6.0 mm from the dorsal surface of the medulla ([Krause et al., 2009a](#), [Wenninger et al., 2004](#)). However, in some cases adjustments in microtubule placement had to be made to avoid blood vessels. Following microtubule placement, screws were inserted into the skull to which the microtubules were chronically secured using dental acrylic. Stainless steel stylets, slightly shorter than the microtubules, were inserted into the microtubules and the incisions sutured closed. Following the procedure, post-operative monitoring and medications were administered as described above.

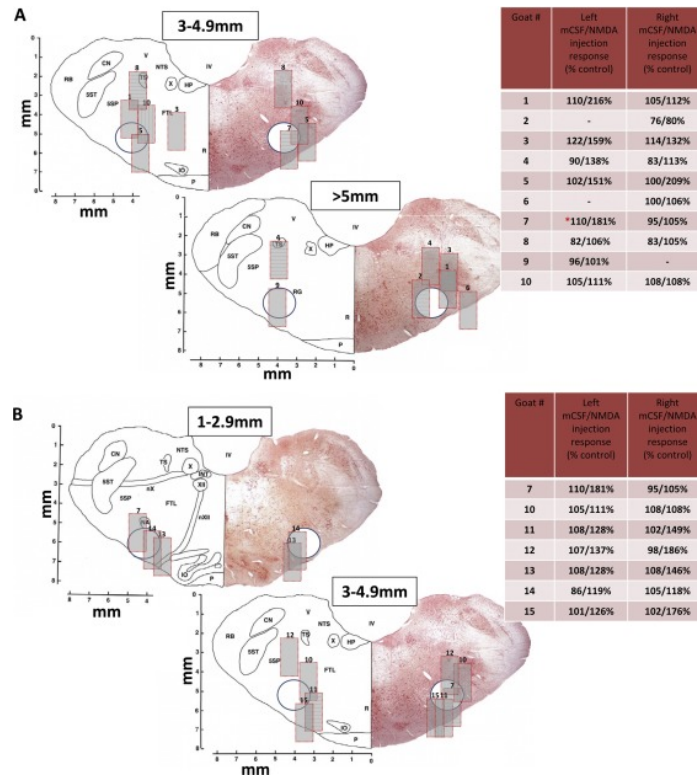


Fig 1. A and B show sections of the goat [medulla](#) at two rostral to caudal distances from [obex](#) depicting the [microtubule](#) location in which [DAMGO](#) or [muscimol](#) were dialyzed unilaterally (1A) and bilaterally (1B). Each goat was numbered for unilateral and bilateral studies, and the numbers for the left and right microtubule are located in the images where the distal end of the microtubule was identified by post mortem histological analysis. The 0.5 mm wide porous membrane of the [dialysis](#) probe extended 2 mm beyond the distal end of the microtubule; thus, we inserted a 2 mm long, 1 mm wide gray filled rectangle for each number to represent a conservative estimate of the region of diffusion of the dialyzed substances (see text for basis of minimal diffusion). The circle in each image is the approximate location of the VRC in goats. The inset in 1A and 1B is the quantitation of the breathing frequency responses to mCSF and [NMDA](#) (mCSF/NMDA) injections displayed as percent of control. Goats number 7 and 10 were included in both groups of studies.

2.3. Physiological studies

The goats were acclimated to study protocols and equipment during the weeks of recovery from surgery. To obtain ventilatory data, a custom fitted mask was secured to the goat's muzzle. A two-way breathing valve was inserted into the front of the mask with hoses attached to the inlet and outlet of the valve. For all studies, normal room air was inhaled though the inlet hose which was attached to a pneumotachograph connected to a computer. Windaq data acquisition software was utilized for breath-by-breath recording of inspiratory flow for computation of inspiratory (T_I) and [expiratory](#) (T_E) time, breathing frequency (f), [tidal volume](#) (V_T), mean inspiratory flow rate (V_T/T_I) and inspiratory [minute ventilation](#) (\dot{V}_I). The outlet hose was attached to a Tissot [spirometer](#) for collection of expired air (the volume of which was measured at 5 min intervals). Expired air was analyzed for O_2 and CO_2 concentration and those values were used to calculate O_2 consumption and CO_2 excretion. In several goats, heart rate (HR) and blood pressure (BP) were recorded via an indwelling catheter secured in the elevated carotid artery (which also facilitated blood sampling). [Arterial blood](#) samples were analyzed for pH, $paCO_2$, paO_2 , and O_2 saturation using a Siemens RapidLab248 [blood gas](#) analyzer. For night/sleep studies, the only change was the addition of [EEG](#) and [EOG](#) wires attached to the implanted electrodes. Rectal temperature was routinely recorded.

[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 is a semi-permeable membrane (membrane diameter 0.5 mm, 20kda cut-off, 3 μ L internal volume). Only the 2 mm membrane penetrated the brain tissue. During studies, the perfusate was either mock cerebral spinal fluid (mCSF: 124 mM NaCl, 2.0 mM KCL, 2.0 mM MgCl₂, 1.3 mM K₂PO₄, 2.0 mM CaCl₂, 11 mM [glucose](#), 26 mM NaHCO₃⁻ and pH 7.32 in sterile distilled H₂O) alone or with the μ -opioid [agonist DAMGO](#) (10, 50 or 100 μ M, Sigma-Aldrich: E7384) or the [GABA_A](#) agonist [muscimol](#) (0.5, 1.0, or 10.0 mM, Abcam: ab120094). The solutions were prepared in a [tonometer](#) flask, warmed to 39 °C, and equilibrated with 6.4% CO₂ and 12% O₂ balance N₂. A [syringe pump](#) (Harvard Apparatus) delivered the dialysate to the dialysis probe at a flow rate of 25 μ L/min. To minimize distraction of the goat, the pump was outside of the chamber; thus, a 150 cm length of polypropylene tubing (PE50) was needed to connect the syringe to the probe. The length of the tubing caused a delay of about 20 min between the start of dialysis and the arrival of perfusate at the probe tip. Moreover, during the initial 15–20 min of hour three, 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 temporal pattern of ventilatory responses (shown in Figures and Tables) likely reflect these drug delivery and washout characteristic. 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 analysis by department biochemical core lab facilities.

We performed uni- and bilateral dialysis of DAMGO, but dialysis of muscimol was only unilateral for reasons stated in the introduction. Provided in [Table 1](#) is a list of studies which were completed on each of the 15 goats. Thirty minutes after probe insertion, we collected data over a fifteen-minute pre-dialysis control period which was followed by three continuous hours of dialysis. Hour 1 was mCSF alone, hour 2 was mCSF alone or mCSF mixed with a drug, and hour 3 was again mCSF alone. DAMGO dialysis was at concentrations of 10, 50, or 100 μ M which is over the range and duration others used in dialyses studies on rats or dogs ([Montandon et al., 2011](#), [Mustapic et al., 2010](#)). Muscimol dialysis was at concentrations of 0.5, 1.0, or 10.0 mM which is over the range and duration others used in studies on awake and sleeping rats and piglets ([Curran et al., 2001](#), [Nattie and Li, 2008](#), [Taylor et al., 2006](#)). Day studies were completed between 9 AM and 2 PM. Night studies were completed between 7 P.M. and 2 A.M. A minimum of 36 h was allowed between consecutive studies on individual goats.

Table 1. This table summarizes which studies each of the fifteen goats completed. Abbreviations are as follow: u denotes unilateral [dialysis](#), b denotes bilateral dialysis, L and R denote dialysis on the left and right side respectively, and mCSF denotes mock cerebral spinal fluid.

DRUG	Goat	Goat	Goat	Goat	Goat	Goat	Goat	Goat	Goat	Goat	Goat	Goat	Goat	Goat	Goat
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
mCSF		u (R)	u (L)	u (L)	u (L)	u (R)	u (L)		u	b	b	b	b	b	b
									(L x 2)						
10µM DAMGO	u (L)	u (R)	u (L)	u (L)	u (L)	u (R)	u (L)	u (L)							
50µM DAMGO	u (L)	u (R)	u (L)	u (L)	u (L)	u (R)	u (L)	u (L)							
100µM DAMGO	u	u (R)	u	u (L)	u	u (R)	u (L)	u (R)	u (L)	b	b	b	b	b	b
0.5 mM MUSCIMOL		u (R)	u (L)	u (R)	u (R)	u (R)	u (L)		u (L)						
1.0 mM MUSCIMOL		u (R)	u (L)	u (L)		u (R)	u (R)		u (L)	u (L)					
10.0 mM MUSCIMOL		u (R)	u	u (L)	u (R)	u (R)	u (L)		u (L)	u (L)					
NIGHT DAMGO	u	u (R)	u (L)	u (L)	u	u (R)	u (L)		u (L)	b	b	b	b	b x 2	b
	(L&R)								& b						

2.4. Neurochemical analyses

To measure the [amino acids glutamine](#) (GLN), [glycine](#) (GLY) and GABA present in effluent dialysate [reverse phase high performance liquid chromatography](#) was used as published by Muere et al. with fluorescent detection using: Waters Resolve C18 column (150 × 3.9) and a fluorescent detector with excitation at 229 nm and emission at 470 nm, [β-alanine](#) internal standard was used with *O*-phthaldialdehyde derivatization ([Muere et al., 2013](#)). To measure [5-HT](#) and its metabolite HIAA, an identical column was used but the potential was set at 0.6 V vs. an Ag/AgCl reference electrode and a N-methylserotonin internal standard was used. A commercially available assay (Enzo Life Sciences, Assay Designs 900-018, range 9.76–10,000 pg/mL) and a microplate reader at 405 nm were used to determine SP levels. Measurement of [norepinephrine](#), [dopamine](#) and their metabolites (DOPAC and HVA) was accomplished by using a Waters μBondapak C18 column (300 × 3.9), potential setting of 0.65 V vs Ag/AgCl reference electrode, and a 2, 5-dihydroxybenzylamine hydrobromide internal standard.

Microtubule location was determined both functionally and histologically. Functional determination was accomplished via injections of *N-methyl-d-aspartic acid* (NMDA). The rationale for [NMDA](#) injections was based on previous findings that glutamate receptor agonist injections into the preBötC elicits a [tachypneic](#) ventilatory response ([Krause et al., 2009b](#), [McCrimmon et al., 2000](#), [Solomon et al., 1999](#), [Wenninger et al., 2004](#)) thus, to approximate microtubule placement relative to the preBötC, 500 nL injections were made of mCSF alone or mixed with NMDA (100 mM). These injections were made in the awake state at least two weeks after microtubule implantation when the goat had recovered from the surgery. Injection tubes were inserted only to the distal end of the microtubule to avoid tissue damage. Breathing was monitored continuously during a 30-min control period and over two hours during which, at 30 min intervals, either mCSF or NMDA was injected unilaterally.

A post-mortem histological determination of microtubule location was also performed in each animal. The [cerebral circulation](#) was isolated under deep [anesthesia](#) before flushing the [brainstem](#) with [phosphate buffered saline](#), followed by 4% paraformaldehyde in PBS following euthanasia. The brainstems were then extracted, dehydrated in successive 20 and 30% sucrose solutions at 4 °C and then frozen (–80 °C). The brainstems were serially sectioned at 25 μM in the transverse plane. One series was used for Nissl (cresyl violet) staining to determine microtubule (MT) placement. Nissl-stained images (scanned resolution of 4000 dots/inch) 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 and the ventral medullary surface) near the middle of the rostral-caudal MT damage range.

2.5. 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. Individual breath ventilatory data were averaged into 5 and 15 min bins. For all ventilatory variables, metabolic rate ($\dot{V}O_2$), arterial blood gases, arterial blood pressure (BP), heart rate (HR), and rectal temperature (TR), a two-way repeated (RM) measures ANOVA compared all drug (DAMGO or muscimol) doses to mCSF alone (one factor repetition with dose and time as factors). Statistical analyses were over different portions of the predialysis period and 3-h dialysis protocol. 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 (two factor repetition, state and time as factors) were performed to determine the effect of sleep state on ventilatory variables. Statistical analyses were on absolute values for each variable and again for each

variable during hours 2 and 3 of the protocol expressed as a percent of the variable over the last 15 min of hour 1. Variability in 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 if significant interactions occurred resulting from DAMGO or muscimol dialysis. Holm-Sidak [post hoc tests](#) were used where appropriate after each ANOVA.

2.6. Neurochemical analysis

For statistical analyses of [neurochemical](#) data, two-way RM ANOVAs (one 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, GLY, GABA, and norepinephrine for both muscimol and DAMGO studies. Also, we computed the change in each neurochemical between pre and post dialysis of DAMGO and muscimol, and then used a one-way ANOVA (drug as factor) to determine whether the neurochemicals significantly changed.

3. Results

3.1. Placement of microtubules (MTs)

Shown in [Fig. 1A](#) and B are representative transverse sections of the goat [medulla](#) in which the location of the distal end of the histologically identified [microtubule](#) tract is identified for each animal (represented by number). The [dialysis](#) membrane extended 2 mm beyond (ventral) the dorsal-most aspect of the microtubule implantation site. We did not measure drug diffusion in this study, but conservatively estimated an affected region ([Fig. 1](#)) based on our previous dialysis studies in which we measured brain pH changes at two distances that resulted from dialysis of CO₂-enriched mCSF ([Hodges et al., 2004](#)). In that study, we measured a dose-dependent decrease in extracellular fluid pH ~220 μm from the dialysis probe when 25% or 80% CO₂ was dialyzed, but simultaneous measurements of pH at a distal [brainstem](#) site (~1.4 mm) from the site of focal [acidosis](#) indicated no detectable change in pH. Accordingly, we conservatively estimate a minimal diffusion of [DAMGO](#) to be ~0.25 mm from the dialysis probe recognizing that others in rats have found greater DAMGO diffusion distance (1.5–2.0 mm) from the dialysis probe ([Montandon et al., 2011](#)). We included a 2 × 1 mm gray box which represents a minimal estimate of the affected brainstem region relative to the estimated location of the VRC (circle). However, our model of pH changes with dialysis of acidified mCSF may underestimate the diffusion area as the [in vivo](#) brainstem has great capacity to buffer protons but there is no comparable uptake of DAMGO.

As indicated in [Table 1](#), we unilaterally dialyzed on both sides of the VRC in six goats and in four other goats, unilateral dialysis was only on one side; thus, there were 16 sites of unilateral dialysis. Of these sixteen, eleven were in the estimated affected regions. ([Fig. 1A](#)). Bilateral dialysis was in seven goats. Thirteen of the fourteen estimated affected regions for the bilateral studies ([Fig. 1B](#)) reached the VRC.

As in past studies, we used the breathing frequency response to the glutamate [receptor agonist NMDA](#) as a functional marker of proximity to the preBötC. We found breathing frequency increased at least 10% after twenty-two of thirty-one NMDA injections made in the fifteen goats (insets [Fig. 1A](#) and B). In contrast, when only mCSF was injected, breathing frequency increased more than 10% in only two of the thirty-one injections. The variation in the responses to NMDA and the variation in histologically identified MT placement suggests that the dialyzed receptor agonists likely were not restricted to the targeted preBötC or the VRC in all goats. This possibility is a limitation of our study and will subsequently be discussed.

3.2. DAMGO microdialysis

There were no effects of time or dose (no significant interaction term; $P > 0.05$) in the two-way repeated (RM) measures ANOVA comparing absolute values measured during unilateral dialyses of mCSF alone or three doses of DAMGO (Table 2). Moreover, when the physiologic responses during and after unilateral DAMGO dialysis were normalized to Hour 1 control values (immediately prior to DAMGO dialysis), there also was no significant effects of time and dose for \dot{V}_I , f , V_T , (Fig. 2) or any other measured physiologic parameter. However, we noted a consistent trend for a transient reduction in \dot{V}_I during the final 30 min of 50 μM and 100 μM DAMGO dialyses. The individual goat data suggested there were differences between goats in the time within this period when \dot{V}_I was reduced; thus, a moving time average was computed for 3 successive 5 min intervals, which is shown in Fig. 3. Using this approach, there was a significant difference in the \dot{V}_I responses among mCSF dialysis and the 3 doses of DAMGO ($P = 0.042$) (one-way ANOVA, dose as factor). The [post hoc test](#) indicated that the response to mCSF dialysis differed significantly from 50 μM DAMGO dialysis. Finally, dialysis of DAMGO destabilized breathing (Fig. 4), where the coefficient of variation in f during and after the last 30 min of DAMGO dialysis was significantly ($P < 0.042$) increased compared to mCSF dialysis.

Table 2. Average of raw values for physiologic variables obtained during studies testing the effect of unilateral [dialysis](#) of [DAMGO](#) into the ventral respiratory column of awake goats. Presented are data for four, fifteen minute time periods for each of four studies (A–D). The first period of each study was predialysis control. During the subsequent periods of all studies, mock cerebral spinal fluid (mCSF) was dialyzed. During period 3 of studies B-D, DAMGO was added to the mCSF to provide concentration of 10 μM , 50 μM or 100 μM . Abbreviation of physiologic variables are defined in the text. For all variables, there was no statistically significant ($P > 0.05$) interaction value from the two-way repeated measures ANOVA (time and study as factors) comparisons of all 4 studies. For Study A $n = 7$, Study B & C $n = 8$, and Study D $n = 9$.

Study A														
Period	Data	V _I (L/min)	f(hr/min)	T _I (sec)	T _E (sec)	V _E (L/hr)	V _I /T _I	VO ₂ (ml/min)	TR(°C)	HR(bpm)	BP(mmHg)	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH
1 Predialysis	Average	7.73	25.03	0.94	1.47	0.32	0.33	287.71	39.04	-	-	-	-	-
	Std. Error	0.57	1.86	0.06	0.09	0.04	0.03	36.34	0.21	-	-	-	-	-
	Average	7.39	26.84	0.88	1.44	0.29	0.34	273.81	39.07	-	-	-	-	-
	Std. Error	0.71	1.89	0.07	0.11	0.03	0.04	28.49	0.16	-	-	-	-	-
3 mCSF	Average	7.25	27.14	0.81	1.41	0.28	0.34	265.38	39.12	-	-	-	-	-
	Std. Error	0.59	2.48	0.06	0.10	0.03	0.04	26.72	0.16	-	-	-	-	-
	Average	7.65	29.47	0.79	1.28	0.28	0.35	256.14	39.25	-	-	-	-	-
	Std. Error	0.61	3.03	0.07	0.10	0.02	0.04	28.52	0.21	-	-	-	-	-

Study B														
Period	Data	V _I (L/min)	f(hr/min)	T _I (sec)	T _E (sec)	V _E (L/hr)	V _I /T _I	VO ₂ (ml/min)	TR(°C)	HR(bpm)	BP(mmHg)	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH
1 Predialysis	Average	7.90	24.76	0.93	1.78	0.34	0.38	264.52	39.17	-	-	-	-	-
	Std. Error	0.53	2.19	0.07	0.23	0.03	0.02	31.50	0.15	-	-	-	-	-
	Average	7.03	22.67	0.96	1.92	0.33	0.36	250.86	39.17	-	-	-	-	-
	Std. Error	0.63	1.97	0.09	0.25	0.03	0.02	29.79	0.15	-	-	-	-	-
3 10 μM	Average	7.10	23.69	0.85	1.79	0.31	0.37	233.62	39.17	-	-	-	-	-
	Std. Error	0.63	2.13	0.05	0.25	0.02	0.02	28.72	0.15	-	-	-	-	-
	Average	7.48	24.98	0.79	1.75	0.32	0.39	240.76	39.17	-	-	-	-	-
	Std. Error	0.62	2.62	0.07	0.26	0.03	0.02	29.58	0.15	-	-	-	-	-

Study C														
Period	Data	V _I (L/min)	f(hr/min)	T _I (sec)	T _E (sec)	V _E (L/hr)	V _I /T _I	VO ₂ (ml/min)	TR(°C)	HR(bpm)	BP(mmHg)	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH
1 Predialysis	Average	7.28	23.43	0.95	1.79	0.33	0.34	263.50	38.88	-	-	-	-	-
	Std. Error	0.45	1.86	0.07	0.23	0.03	0.02	21.83	0.10	-	-	-	-	-
	Average	7.49	23.82	0.99	1.73	0.34	0.37	280.33	38.88	-	-	-	-	-
	Std. Error	0.55	2.45	0.13	0.26	0.02	0.01	33.64	0.10	-	-	-	-	-
3 50 μM	Average	6.76	23.17	0.84	1.86	0.30	0.36	231.42	38.88	-	-	-	-	-
	Std. Error	0.50	2.10	0.07	0.18	0.02	0.02	24.12	0.10	-	-	-	-	-
	Average	7.19	24.05	0.64	1.60	0.28	0.38	220.38	38.88	-	-	-	-	-
	Std. Error	1.07	3.85	0.08	0.32	0.03	0.06	38.58	0.10	-	-	-	-	-

Study D														
Period	Data	V _I (L/min)	f(hr/min)	T _I (sec)	T _E (sec)	V _E (L/hr)	V _I /T _I	VO ₂ (ml/min)	TR(°C)	HR(bpm)	BP(mmHg)	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH
1 Predialysis	Average	8.05	29.19	0.85	1.41	0.29	0.35	284.19	39.52	110.93	70.36	41.64	96.70	7.46
	Std. Error	0.57	3.09	0.08	0.14	0.01	0.03	30.48	0.23	8.98	2.79	1.13	5.60	0.01
	Average	7.75	27.19	0.90	1.58	0.31	0.36	278.85	39.40	87.20	71.52	41.42	101.33	7.45
	Std. Error	0.64	3.01	0.08	0.23	0.02	0.02	28.61	0.20	9.22	3.97	0.99	5.38	0.01
3 100 μM	Average	7.38	26.96	0.79	1.67	0.29	0.38	263.00	39.25	86.55	71.25	41.30	101.03	7.46
	Std. Error	0.68	3.12	0.07	0.25	0.02	0.03	33.68	0.17	7.90	4.22	1.59	3.57	0.01
	Average	7.47	28.19	0.74	1.63	0.28	0.40	244.26	39.21	85.67	71.08	40.12	100.92	7.46
	Std. Error	0.60	3.14	0.07	0.23	0.02	0.02	27.63	0.18	6.39	3.43	1.07	5.18	0.01

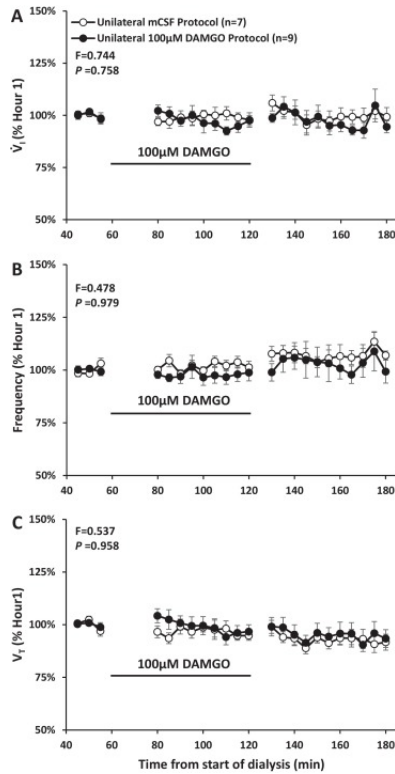


Fig. 2. Unilateral dialysis during the day of 100 μM DAMGO ($n = 9$, filled circles) did not significantly ($P \geq 0.758$) alter ventilation (\dot{V}_I , panel A), breathing frequency (f , panel B) or tidal volume (V_T , panel C) compared to dialysis of mCSF alone ($n = 7$, open circles). Data are means \pm SE. The x Axis is time from start of 180 min of dialysis. As indicated by the black line, 100 μM DAMGO was dialyzed between 60 and 120 min. The values shown during and after DAMGO dialyses are the percent of the last 15 min prior to DAMGO dialyses. F and P values are the interaction term obtained from two-way repeated measures ANOVA using time and dose as factors. Note the trend of a transient reduction in \dot{V}_I and f over the last 30 min of DAMGO dialysis.

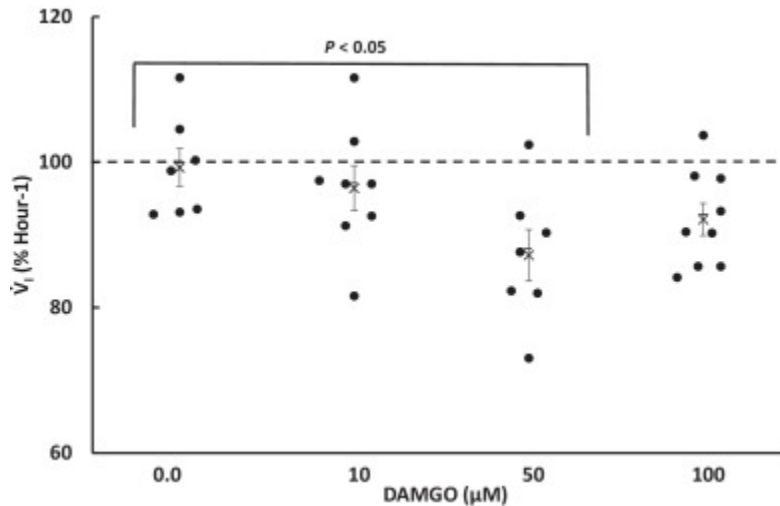


Fig. 3. Because of the trend of a transient reduction in \dot{V}_I over the last 30 min of DAMGO dialysis (Fig. 2), a moving time average was computed using 3 successive 5 min intervals and the lowest value for each goat over the last 30 min of DAMGO dialysis and mCSF are plotted in this figure (filled circles). Average values for each dose of DAMGO are plotted using mean symbols, data are means \pm SE. One-way ANOVA indicated \dot{V}_I as a percent of control differed significantly

($P < 0.042$) over the 4 conditions shown. The [post hoc test](#) indicated the responses to 0 and 50 μM differed significantly ($P < 0.05$).

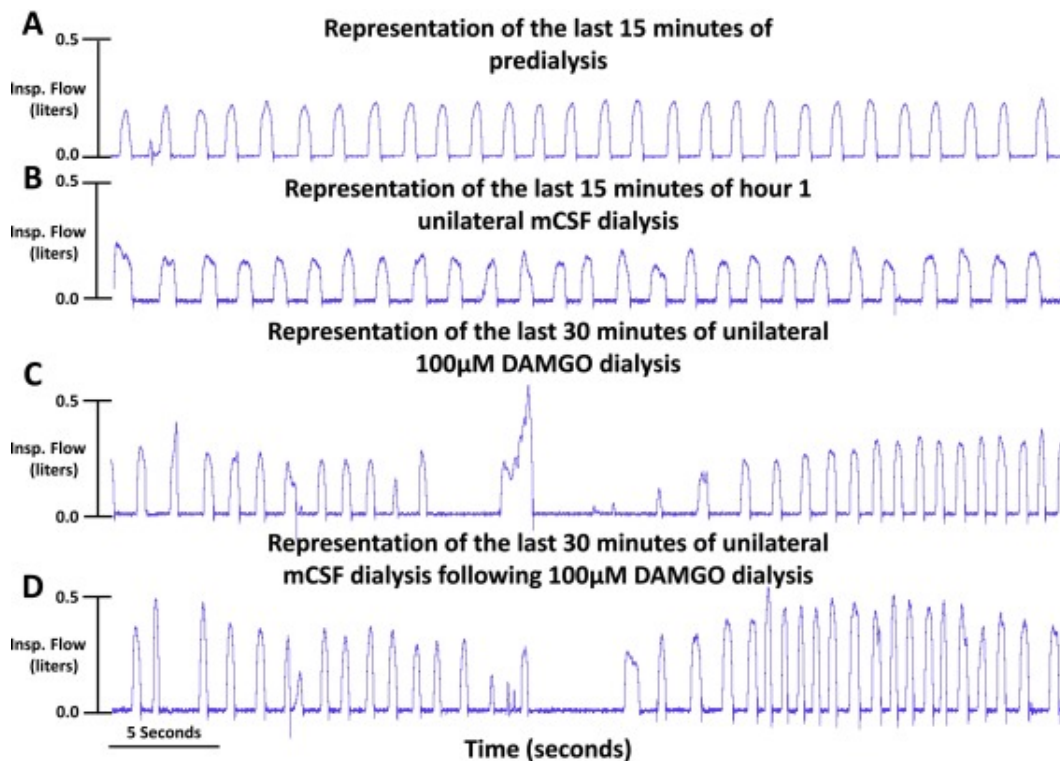


Fig. 4. Visual inspection of ventilatory tracings indicates variability of breathing increased during and after unilateral [dialysis](#) of 100 μM [DAMGO](#). The four tracings were from a single goat for a portion of the last 15 min of the predialysis control period (panel A) and the last 15 min of each of three hours of mCSF dialysis (panels B–D) with DAMGO increased during the second of the three hours (Panel C).

There were significant ($P < 0.05$) effects of time and drug for \dot{V}_I , f , T_I , T_E , and V_T/T_I when comparing bilateral dialysis of mCSF with bilateral dialysis of 100 μM DAMGO during the day. Expressing the data as absolute values over the entire study protocol ([Table 3](#)), or when expressed as percent of control values immediately prior to DAMGO dialysis ([Fig. 5](#), [Fig. 6](#)), we found that bilateral dialysis of 100 μM DAMGO during the day significantly ($P < 0.05$) increased \dot{V}_I , f , T_E , and V_T/T_I and decreased T_I . Bilateral dialysis of DAMGO also destabilized breathing ([Fig. 7](#)), where the coefficient of variation of \dot{V}_I and f ([Fig. 8](#)) were significantly ($P < 0.001$) greater during the hour after bilateral DAMGO dialysis compared to the hour after mCSF dialysis. There were no significant ($P \geq 0.516$) changes in any other measured variable during bilateral dialysis of DAMGO during the day ([Table 3](#)).

Table 3. Averages of raw values for physiologic variables obtained during studies testing the effect of bilateral [dialysis](#) of [DAMGO](#) into the ventral respiratory column of awake goats. Presented are data for four, fifteen minute time periods for each of two studies (A and B). The first period of each study was predialysis control. During the subsequent three periods of both A and B studies, mock cerebral spinal fluid (mCSF) was dialyzed. The only difference between A and B was in study B when DAMGO was added to the mCSF in period 3 to obtain a concentration of 100 μM . Abbreviation of physiologic variables are defined in the text. Results of statistical analyses using two-way repeated measures ANOVA (time and study as factors) indicated there was a significant interaction term for \dot{V}_I , ($P = 0.025$), f ($P = 0.019$), and T_I ($P = 0.007$). There were significant ($P < 0.05$) effects of time indicated as follows: * 4 differed from 1, ** 4 differed from 1 and 2, and ***1 differed from 2, 3, & 4. For Studies A & B $n = 7$.

Study A															
Period	Data	V _i (L/min)	f (br/min)	T _i (sec)	T _e (sec)	V _i (L/hr)	V _i /T _i	VO ₂ (ml/min)	TR (°C)	HR (bpm)	BP (mmHg)	Paco ₂ (mmHg)	PaO ₂ (mmHg)	pH	
1 Predialysis	Average	8.14	30.49	0.90	1.26	0.28	0.38	264.62	39.13	-	-	-	-	-	
	Std. Error	0.74	2.76	0.10	0.10	0.01	0.04	21.99	0.14	-	-	-	-	-	
	Average	8.15	33.50	0.80	1.17	0.25	0.34	275.29	39.15	-	-	-	-	-	
	Std. Error	0.79	2.93	0.08	0.11	0.02	0.03	25.65	0.12	-	-	-	-	-	
3 mCSF	Average	8.49	35.79	0.75	1.12	0.25	0.36	288.43	39.19	-	-	-	-	-	
	Std. Error	0.90	3.50	0.07	0.10	0.02	0.04	23.92	0.11	-	-	-	-	-	
	Average	8.93	38.63*	0.70*	1.04	0.24	0.37	308.33	39.21	-	-	-	-	-	
	Std. Error	0.92	4.08	0.08	0.10	0.02	0.04	32.00	0.10	-	-	-	-	-	
Study B															
Period	Data	V _i (L/min)	f (br/min)	T _i (sec)	T _e (sec)	V _i (L/hr)	V _i /T _i	VO ₂ (ml/min)	TR (°C)	HR (bpm)	BP (mmHg)	Paco ₂ (mmHg)	PaO ₂ (mmHg)	pH	
1 Predialysis	Average	7.24***	32.74**	0.80**	1.20**	0.23***	0.29***	290.14	38.88	72.51	79.72	41.75	88.75	7.45	
	Std. Error	0.53	2.29	0.07	0.08	0.02	0.02	24.20	0.12	3.44	1.64	0.95	2.45	0.00	
	Average	7.72	36.38	0.77	1.12	0.22	0.33	302.29	38.91	73.23	80.49	41.95	91.25	7.45	
	Std. Error	0.95	4.08	0.12	0.12	0.02	0.04	40.71	0.11	2.61	3.92	0.15	13.15	0.01	
3 100 µM	Average	8.84	45.95	0.51	1.09	0.21	0.41	283.62	39.05	70.07	76.95	43.40	89.70	7.43	
	Std. Error	0.82	5.16	0.06	0.16	0.02	0.05	32.99	0.10	1.92	3.05	1.30	13.10	0.02	
	Average	9.09	47.34	0.47	1.34	0.21	0.45	272.43	39.12	68.94	82.78	41.60	83.65	7.43	
	Std. Error	0.88	6.73	0.07	0.18	0.02	0.06	37.04	0.11	1.47	2.16	0.90	7.55	0.03	

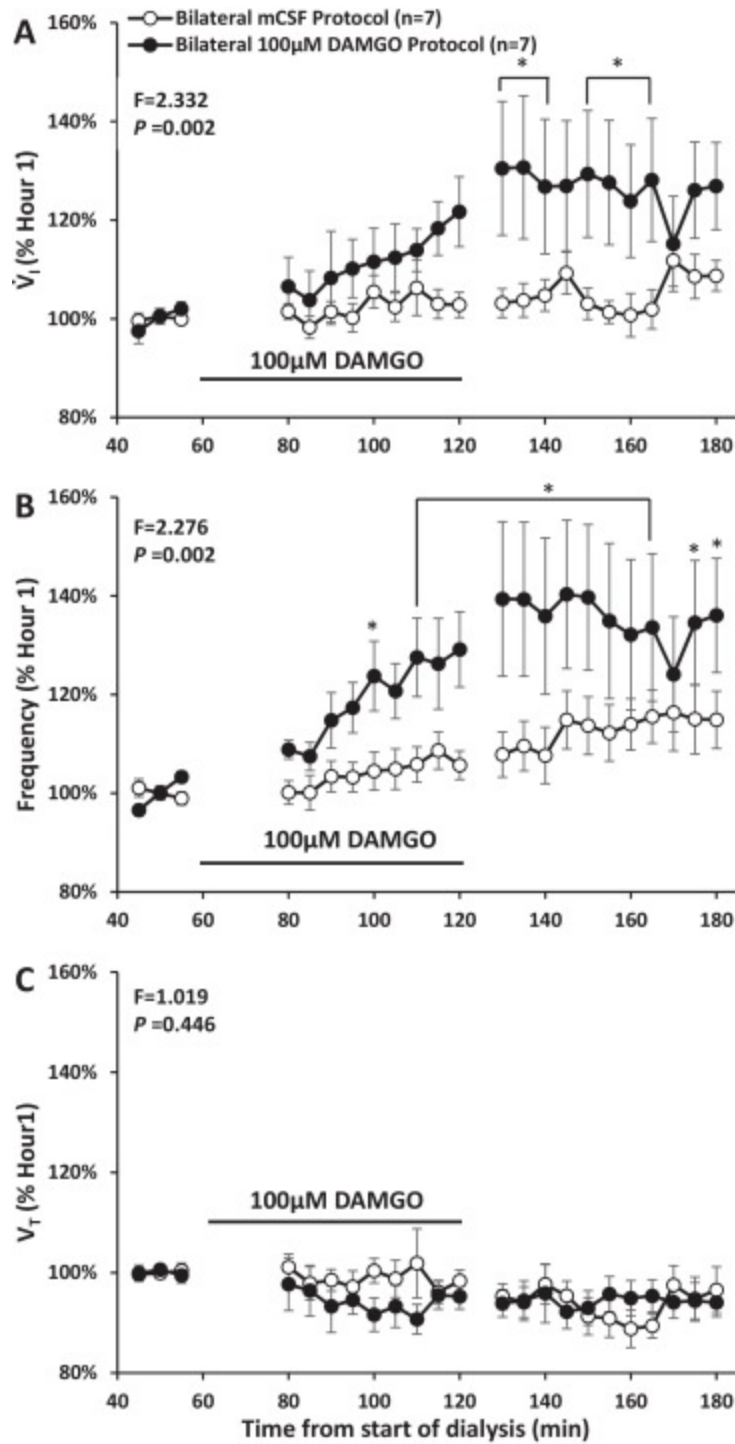


Fig. 5. Bilateral dialysis of 100 μ M DAMGO during the day (filled circles, n = 7) significantly ($P < 0.002$) increased ventilation (\dot{V}_T , panel A) and breathing frequency (f, panel B) but did not alter ($P < 0.446$) tidal volume (V_T , panel C) compared to dialysis of mCSF alone (open circles, n = 7). Data are means \pm SE. Dialysis of 100 μ M DAMGO occurred between 60 and 120 min (indicated by solid line). The values shown during and after DAMGO dialyses are the percent of the last 15 min prior to DAMGO dialyses. The F and P values are interaction terms from two-way repeated measures ANOVA using dose and time as factors. Asterisks denote values different between open and closed symbols ($P < 0.05$), post hoc test).

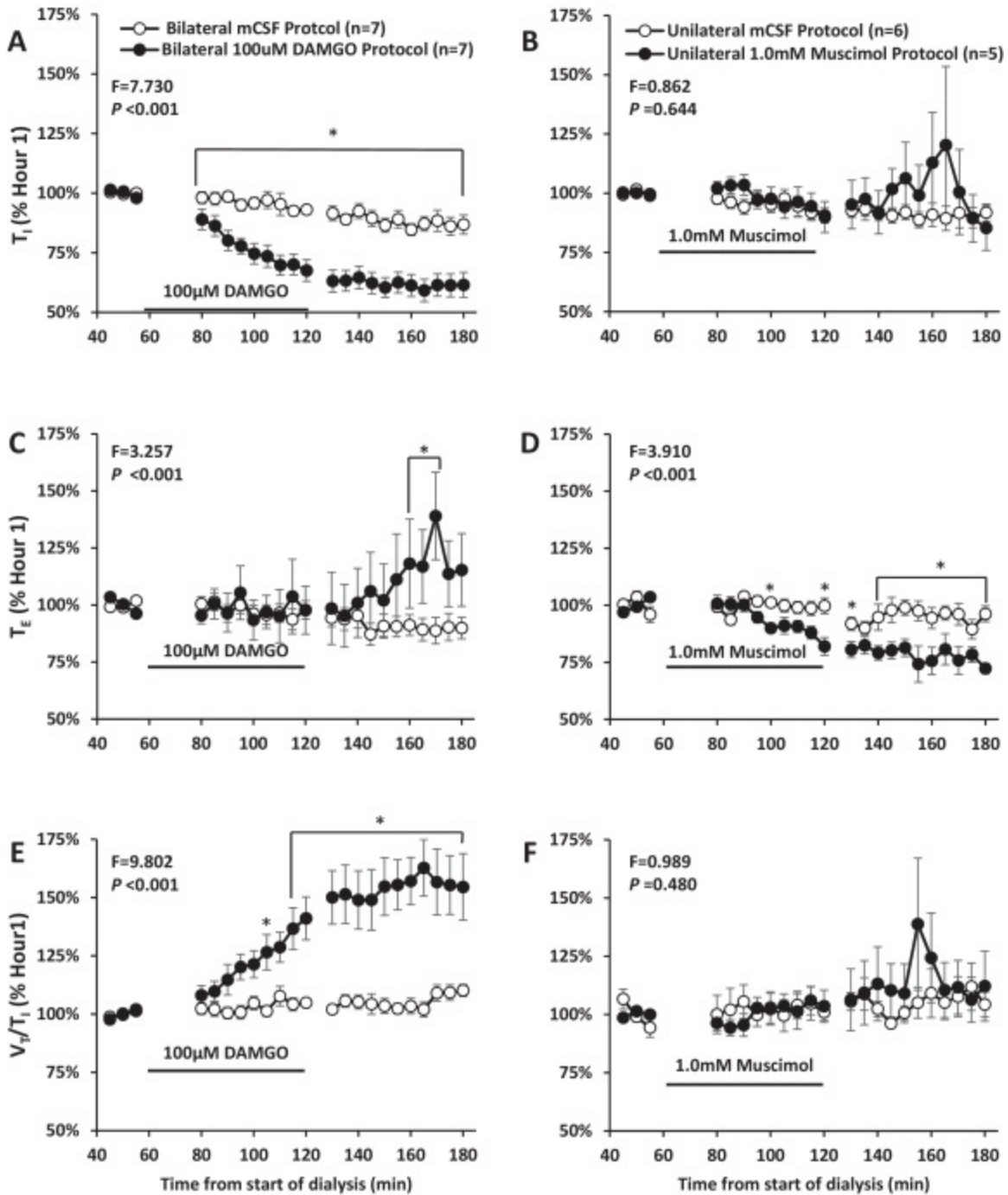


Fig. 6. Inspiratory time (T_I), [expiratory](#) time (T_E) and tidal volume/inspiratory time changed differently between bilateral [dialysis](#) of [DAMGO](#) (left panels) and unilateral dialysis of [muscimol](#) (right panel). Data are means \pm SE. Bilateral dialysis of 100 μ M DAMGO ($n = 7$) during the day significantly ($P < 0.001$) decreased T_I , increased T_E during the post DAMGO dialyses period, and increased V_T/T_I during and after DAMGO dialysis. T_I and V_T/T_I increased after muscimol dialysis while T_E was significantly ($P < 0.001$) decreased during and after muscimol dialysis. Filled circles indicate studies during which 100 μ M DAMGO was dialyzed during 60–120 min (solid line indicates period of DAMGO dialysis). Two-way repeated measures ANOVA was used to obtain F and P values and values shown are the interaction term of the ANOVA. Asterisks denote values different between open and closed symbols ($P < 0.05$, post hoc test).

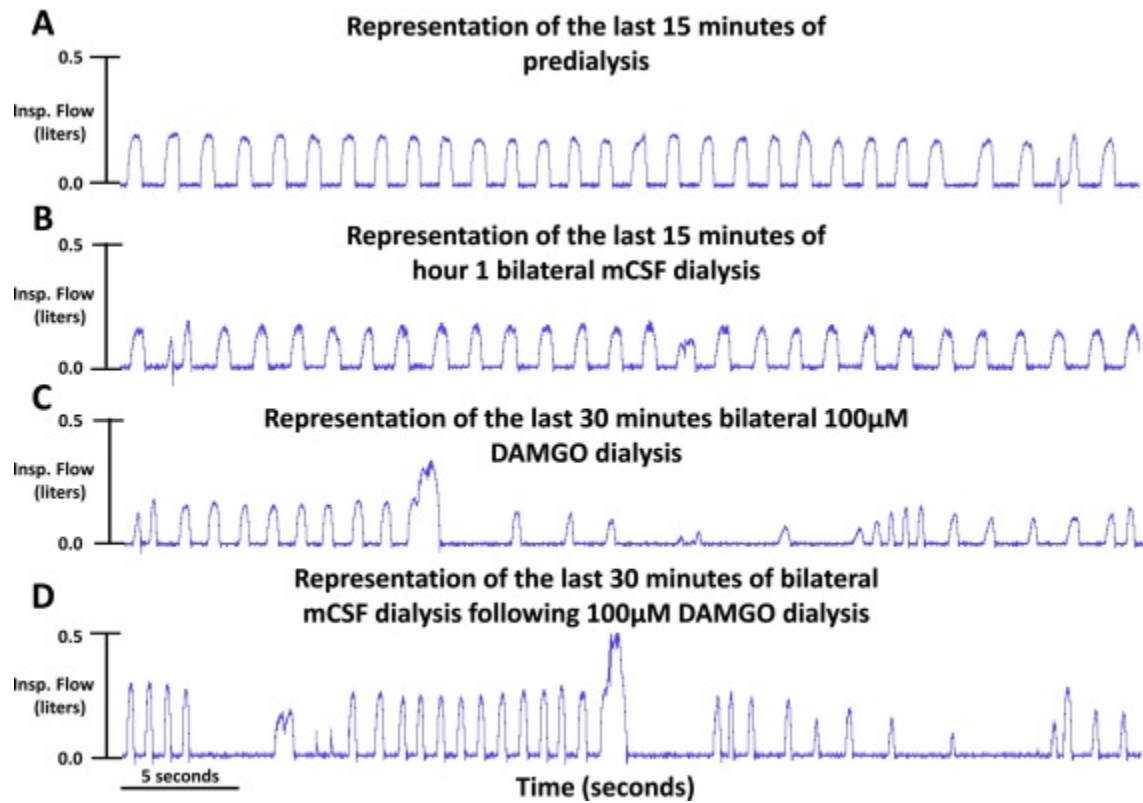


Fig. 7. Visual inspection of ventilatory tracings indicates variability of breathing increased and inspiratory time decreased during and after bilateral [dialysis](#) of 100 μ M [DAMGO](#). The four tracings were from a single goat for a portion of the last 15 min of the predialysis control period (panel A) and the last 15 min of each of three hours of mCSF dialysis (panels B–D) with DAMGO increased during the second of the three hours (Panel C).

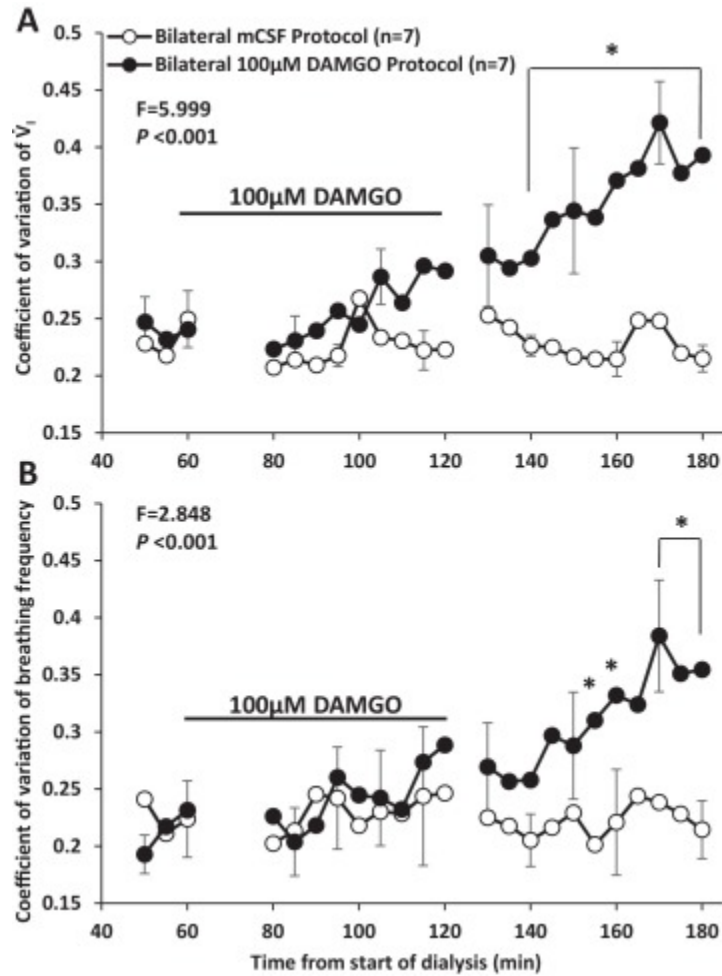


Fig. 8. The coefficient of variation of ventilation (\dot{V}_I , panel A) and breathing frequency (f , panel B) within 5 min periods was significantly ($P < 0.001$) greater during the period after bilateral 100 μM DAMGO dialysis than after bilateral mCSF dialysis. Data are means \pm SE. The x Axis is time from start of 180 min of dialysis. As indicated by the black line, DAMGO was dialyzed between 60 and 120 min. The F and P values are interaction terms from two-way repeated measures ANOVA using dose and time as factors. Asterisks denote values different between open and closed symbols ($P < 0.05$, post hoc test).

At night, for unilateral and bilateral (Fig. 9) dialysis of 100 μM DAMGO, there were no significant interaction terms in two-way ANOVA analysis (time and state as factors) when comparing responses during awake and NREM sleep (Table 4, Table 5).

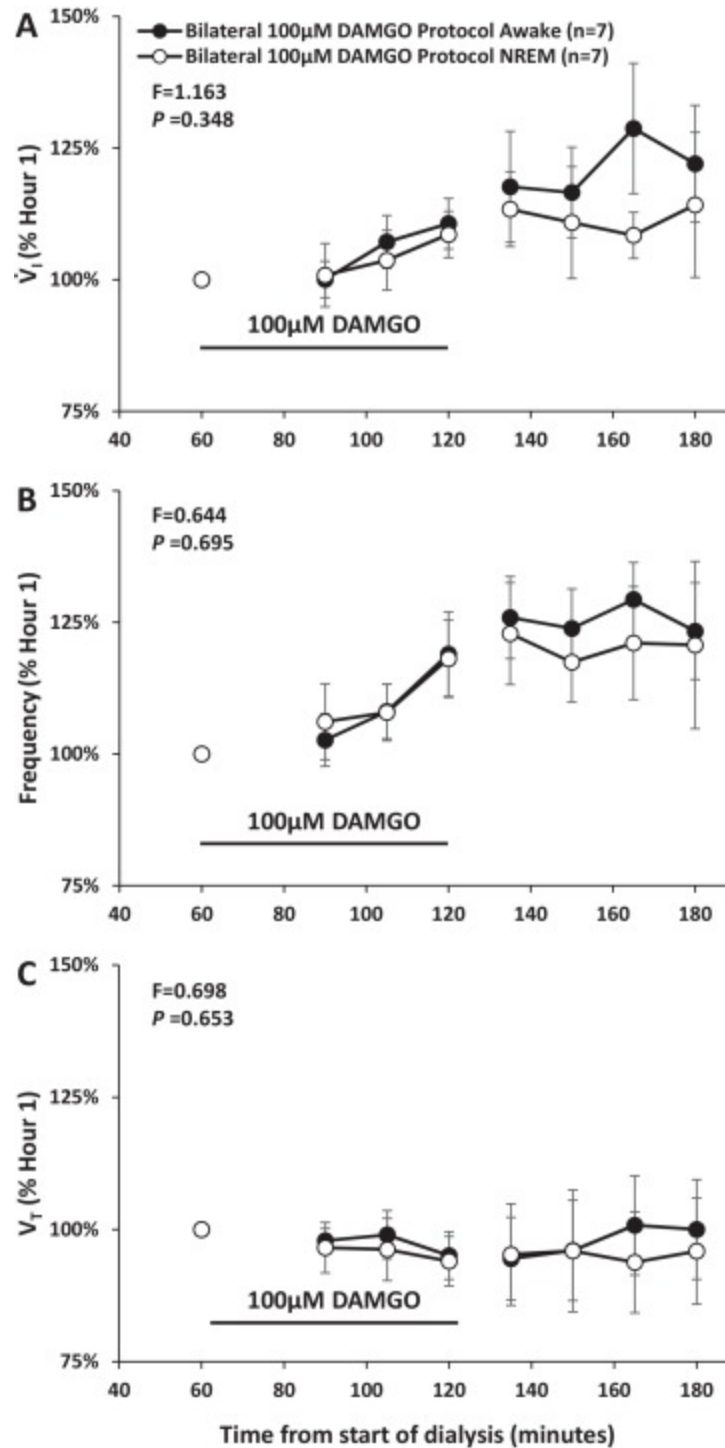


Fig. 9. During bilateral dialysis of 100 μ M DAMGO ($n = 7$) at night, there were no significant ($P \geq 0.348$) interaction terms for any ventilatory variable of the two-way ANOVA comparing awake and NREM sleep (state and time). Data are means \pm SE. Data from each state were averaged in 15 min bins, the filled circles indicate the awake state and open circles indicate the NREM sleep state. Dialysis of 100 μ M DAMGO occurred during minutes 60–120 of the study and is indicated by the solid line.

Table 4. Average of raw values for physiologic variables obtained during studies testing the effect of unilateral [dialysis](#) of [DAMGO](#) into the ventral respiratory column of goats during awake and [NREM sleep](#) states. Presented are data for the last fifteen minutes of three hours of mCSF dialysis. During the second hour, DAMGO was added to the mCSF to provide a concentration of 100 μ M. Abbreviation of physiologic variables are defined in the text. Ventilatory data were obtained on seven goats, but HR, BP, [blood gases](#), and pH were obtained on only four goats. For all variables, there was no statistically significant ($P > 0.05$) interaction value from the two-way repeated measures ANOVA (time and study as factors) comparing awake and NREM sleep.

Period	Data	\dot{V}_I (L/min)	f (br/min)	T_I (sec)	T_E (sec)	V_T (L/br)	HR (bpm)	BP (mmHg)	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH
1 (awake) mCSF	Average	7.46	24.65	1.14	1.84	0.32	81.57	74.49	37.50	87.54	7.47
	Std. Error	0.77	2.73	0.09	0.19	0.02	10.21	7.90	1.88	4.86	0.01
2 (awake) 100 μ M	Average	7.99	27.03	0.95	1.64	0.31	83.98	73.37	35.64	85.66	7.46
	Std. Error	0.78	2.88	0.10	0.17	0.02	10.45	7.45	1.11	4.01	0.01
3 (awake) mCSF	Average	7.67	27.39	0.95	1.71	0.29	83.47	71.56	36.73	82.78	7.46
	Std. Error	0.89	3.19	0.11	0.17	0.02	11.54	5.81	0.74	4.60	0.00
Period	Data	\dot{V}_I (L/min)	f (br/min)	T_I (sec)	T_E (sec)	V_T (L/br)	HR (bpm)	BP (mmHg)	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH
1 (NREM) mCSF	Average	6.79	23.09	1.14	1.81	0.30	77.90	80.06	-	-	-
	Std. Error	0.56	1.97	0.08	0.14	0.02	11.27	5.54	-	-	-
2 (NREM) 100 μ M	Average	7.54	25.87	0.97	1.61	0.29	78.95	72.97	-	-	-
	Std. Error	0.68	2.34	0.11	0.15	0.02	10.14	8.35	-	-	-
3 (NREM) mCSF	Average	6.17	24.82	1.01	1.79	0.26	90.82	65.95	-	-	-
	Std. Error	0.45	2.25	0.11	0.13	0.02	13.57	6.47	-	-	-

Table 5. Average of raw values for physiologic variables obtained during studies testing the effect of bilateral [dialysis](#) of [DAMGO](#) into the ventral respiratory column of goats during awake and [NREM sleep](#) states. Presented are data for the last fifteen minutes of three hours of mCSF dialysis. During the second hour, DAMGO was added to the mCSF to provide a concentration of 100 μ M. Abbreviation of physiologic variables are defined in the text. For all variables, there was no statistically significant ($P > 0.05$) interaction value from the two-way repeated measures ANOVA (time and study as factors) comparisons of all 4 studies. Ventilatory data were obtained on seven goats, but HR, BP, [blood gases](#), and pH were only obtained on four goats.

Period	Data	\dot{V}_I (L/min)	f (br/min)	T_I (sec)	T_E (sec)	V_T (L/br)	HR (bpm)	BP (mmHg)	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH
1 (awake) mCSF	Average	9.20	35.33	0.85	1.16	0.27	91.55	61.83	42.95	90.58	7.45
	Std. Error	1.30	3.78	0.14	0.16	0.02	15.69	3.95	1.80	4.72	0.02
	Average	10.16	42.82	0.67	1.20	0.25	89.38	62.52	42.30	93.63	7.45
	Std. Error	1.45	6.01	0.12	0.26	0.02	14.61	4.56	1.19	5.71	0.02
	Average	10.87	44.59	0.60	1.29	0.26	92.03	63.70	42.80	89.58	7.44
Std. Error	1.50	6.62	0.10	0.25	0.02	13.01	5.25	1.62	4.55	0.01	
1 (NREM) mCSF	Data	\dot{V}_I (L/min)	f (br/min)	T_I (sec)	T_E (sec)	V_T (L/br)	HR (bpm)	BP (mmHg)	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH
	Average	7.80	33.05	0.89	1.16	0.24	88.97	61.30	-	-	-
	Std. Error	1.00	3.57	0.16	0.12	0.02	9.72	2.22	-	-	-
	Average	8.42	39.38	0.67	1.22	0.23	90.36	63.71	-	-	-
	Std. Error	1.05	5.08	0.10	0.25	0.02	8.89	4.17	-	-	-
3 (NREM) mCSF	Average	8.57	40.89	0.60	1.46	0.23	89.55	64.14	-	-	-
	Std. Error	1.24	7.44	0.09	0.30	0.03	9.20	2.21	-	-	-

During daytime unilateral DAMGO dialysis, a one-way ANOVA analysis of the change in [GABA](#) between pre and post dialysis of DAMGO indicated there was a significant ($P < 0.020$) decrease in GABA during dialysis of 50 μM and 100 μM DAMGO ([Fig. 10](#)). In addition, during both unilateral and bilateral dialysis of 100 μM DAMGO at night, there were significant ($P < 0.001$) decreases in GABA in the effluent mCSF. However, bilateral dialysis of 100 μM DAMGO during the day did not significantly ($P > 0.05$) alter GABA. Lastly, there were no significant changes ($P > 0.05$) in [5-HT](#), [SP](#), [glutamine](#), [glycine](#), [dopamine](#), [norepinephrine](#) and the metabolites [DOPAC](#), [HIAA](#), and [HVA](#) in the effluent dialysate during DAMGO dialyses studies (data not shown).

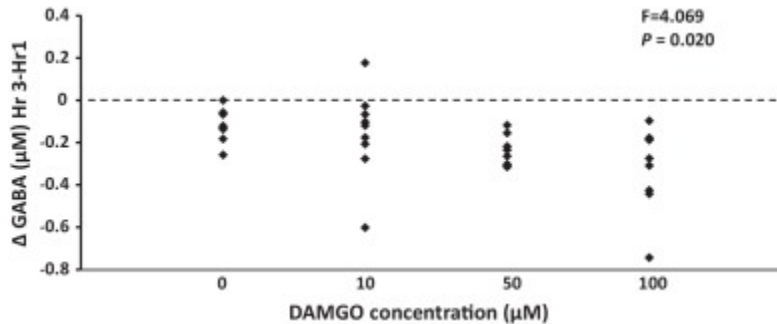


Fig. 10. Unilateral [dialysis](#) of 50 μM and 100 μM [DAMGO](#) during the day significantly ($P < 0.02$) decreased [GABA](#) in the effluent dialyzed mCSF. Data are means \pm SE. On the x-axis are the DAMGO doses and y axis indicates the change in GABA between hours 1 and 3 of dialysis. Statistical analysis was by one-way ANOVA (drug as factor).

3.3. Muscimol microdialysis

A two-way ANOVA (time and dose as factors) indicated there were no significant ($P > 0.05$) differences in absolute values of physiologic variables between mCSF dialysis and dialysis of three [muscimol](#) concentrations (0.5, 1.0, or 10 mM) ([Table 6](#)). However, when variables during and after muscimol dialysis were expressed as a percent of the periods just prior to muscimol dialysis, there were significant effects of time and dose for \dot{V}_i ($P < 0.025$) and f ($P < 0.044$) when all muscimol doses were compared and when each elevated muscimol concentration was individually compared to the mCSF study ([Fig. 11](#), 1.0 mM shown). Expressed as percent change, only 0.5 mM muscimol significantly ($P < 0.005$) altered V_T . All doses of muscimol significantly ($P < 0.023$) decreased T_E but did not significantly ($P \geq 0.577$) alter T_I or V_T/T_I ([Fig. 6](#)).

Table 6. Average of raw values for physiologic variables obtained during studies testing the effect of unilateral [dialysis](#) of [muscimol](#) into the ventral respiratory column of awake goats. Presented are data for four, fifteen minute time periods for each of four studies (A–D). The first period of each study was predialysis control. During the subsequent periods of all studies, mock cerebral spinal fluid (mCSF) was dialyzed. During period 3 of studies B–D, muscimol was added to the mCSF to provide concentrations of 0.5 μM , 1.0 μM or 10 μM respectively. Abbreviation of physiologic variables are defined in the text. For all variables, there was no statistically significant ($P > 0.05$) interaction value from the two-way repeated measures ANOVA (time and study as factors) comparisons of all 4 studies. For studies A–C $n = 7$, study D $n = 6$.

Study A														
Period	Data	V _I (L/min)	f (br/min)	T _I (sec)	T _E (sec)	V _E (L/hr)	V _I /T _I	VO ₂ (ml/min)	TR (°C)	HR (bpm)	BP (mmHg)	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH
1 Predialysis	Average	7.73	25.03	0.94	1.47	0.32	0.33	287.71	39.04	-	-	-	-	-
	Std. Error	0.57	1.86	0.06	0.09	0.04	0.03	36.34	0.21	-	-	-	-	-
	Average	7.39	26.84	0.88	1.44	0.29	0.34	273.81	39.07	-	-	-	-	-
	Std. Error	0.71	1.89	0.07	0.11	0.03	0.04	28.49	0.16	-	-	-	-	-
3 mCSF	Average	7.25	27.14	0.81	1.41	0.28	0.34	265.38	39.12	-	-	-	-	-
	Std. Error	0.59	2.48	0.06	0.10	0.03	0.04	26.72	0.16	-	-	-	-	-
	Average	7.65	29.47	0.79	1.28	0.28	0.35	256.14	39.25	-	-	-	-	-
	Std. Error	0.61	3.03	0.07	0.10	0.02	0.04	28.52	0.21	-	-	-	-	-
Study B														
Period	Data	V _I (L/min)	f (br/min)	T _I (sec)	T _E (sec)	V _E (L/hr)	V _I /T _I	VO ₂ (ml/min)	TR (°C)	HR (bpm)	BP (mmHg)	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH
1 Predialysis	Average	8.24	27.90	0.80	1.21	0.31	0.37	301.96	38.87	-	-	40.54	97.99	7.45
	Std. Error	0.57	3.32	0.04	0.13	0.02	0.01	25.00	0.2	-	5.23	0.70	4.82	0.01
	Average	8.22	29.03	0.77	1.13	0.30	0.40	285.33	39.0	-	-	40.58	98.87	7.44
	Std. Error	0.85	4.07	0.05	0.11	0.02	0.01	28.32	0.18	-	-	1.14	5.22	0.01
3 0.5 mM	Average	9.36	31.62	0.72	1.03	0.31	0.44	304.95	39.0	86.53	71.96	40.38	102.74	7.47
	Std. Error	1.06	4.47	0.05	0.10	0.02	0.02	29.36	0.19	9.92	4.04	1.56	5.49	0.01
	Average	9.06	30.22	0.75	1.08	0.30	0.41	276.67	39.0	82.00	62.76	40.75	96.80	7.43
	Std. Error	1.01	4.26	0.05	0.12	0.02	0.02	27.21	0.19	11.34	3.75	0.75	4.38	0.01
Study C														
Period	Data	V _I (L/min)	f (br/min)	T _I (sec)	T _E (sec)	V _E (L/hr)	V _I /T _I	VO ₂ (ml/min)	TR (°C)	HR (bpm)	BP (mmHg)	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH
1 Predialysis	Average	7.83	27.80	1.02	1.24	0.31	0.32	289.75	39.22	89.63	69.36	42.09	88.17	7.44
	Std. Error	0.65	3.30	0.11	0.15	0.02	0.03	35.94	0.13	7.59	5.63	1.17	4.76	0.01
	Average	7.75	30.63	0.80	1.18	0.28	0.32	292.92	39.19	88.96	66.42	41.67	88.98	7.44
	Std. Error	0.47	4.03	0.08	0.17	0.02	0.02	31.42	0.10	6.37	5.23	0.80	4.61	0.01
3 1.0 mM	Average	8.21	34.54	0.77	1.06	0.27	0.32	288.63	39.24	91.47	63.67	42.20	87.42	7.42
	Std. Error	0.69	5.50	0.08	0.16	0.02	0.01	30.31	0.12	6.72	3.07	1.15	4.00	0.01
	Average	8.90	39.98	0.68	0.87	0.25	0.34	311.67	39.29	90.66	68.59	42.53	87.80	7.43
	Std. Error	0.97	6.28	0.08	0.10	0.02	0.03	36.91	0.14	9.40	3.58	1.07	4.24	0.01
Study D														
Period	Data	V _I (L/min)	f (br/min)	T _I (sec)	T _E (sec)	V _E (L/hr)	V _I /T _I	VO ₂ (ml/min)	TR (°C)	HR (bpm)	BP (mmHg)	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH
1 Predialysis	Average	8.58	30.46	0.53	0.72	0.29	0.39	326.78	39.22	-	-	-	-	-
	Std. Error	0.98	3.96	0.18	0.24	0.01	0.04	47.34	0.29	-	-	-	-	-
	Average	8.27	30.03	0.48	0.76	0.29	0.40	298.33	39.11	-	-	-	-	-
	Std. Error	0.84	4.16	0.16	0.26	0.01	0.03	45.33	0.22	-	-	-	-	-
3 10.0 mM	Average	9.40	32.66	0.44	0.74	0.30	0.49	306.00	39.23	-	-	-	-	-
	Std. Error	1.27	5.54	0.15	0.25	0.01	0.06	49.11	0.22	-	-	-	-	-
	Average	10.61	36.17	0.45	0.61	0.30	0.51	303.56	39.18	-	-	-	-	-
	Std. Error	2.16	7.10	0.16	0.21	0.05	0.13	59.56	0.2	-	-	-	-	-

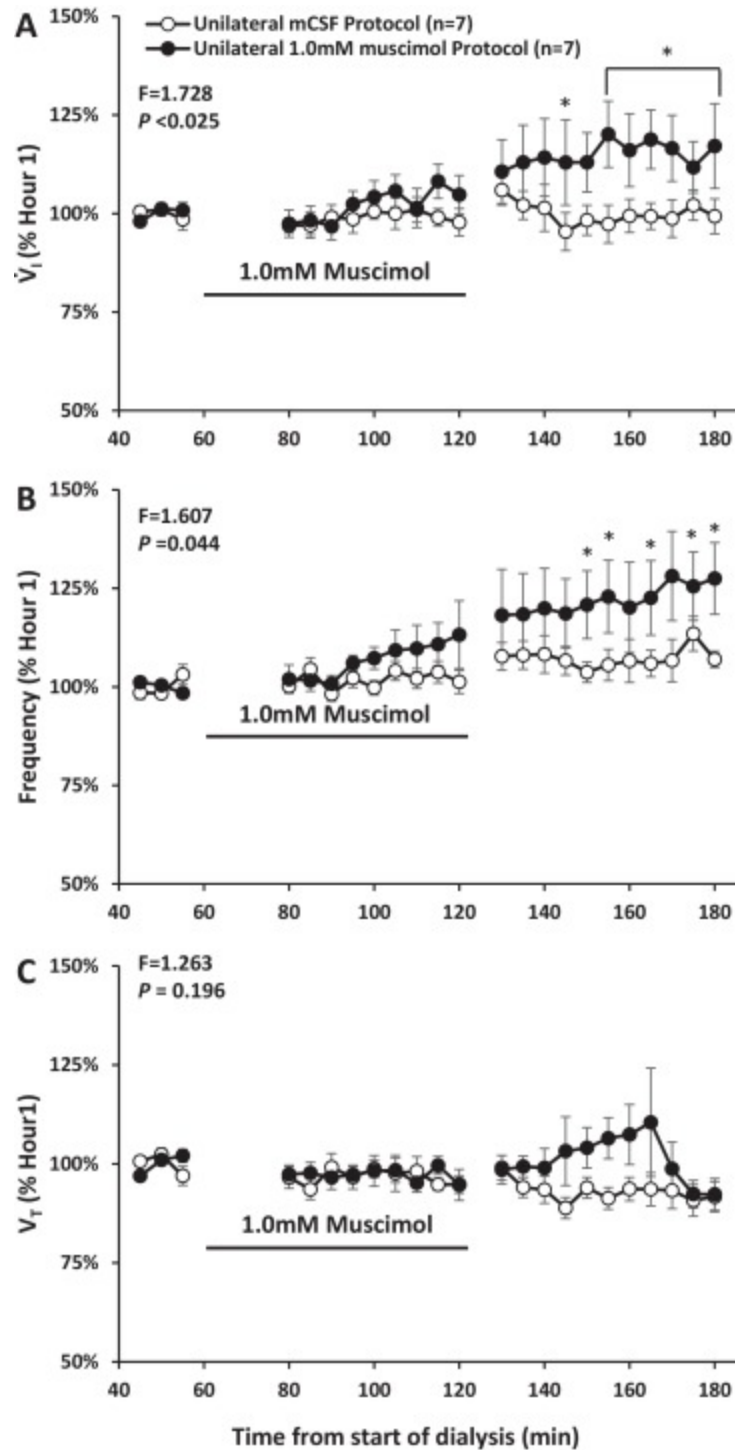


Fig. 11. Unilateral dialysis of 1.0 mM (n = 7) muscimol (filled circles) during the day significantly increased ventilation (\dot{V}_I , panel A, $P = 0.025$) and breathing frequency (f , panel B, $P = 0.044$) but did not alter tidal volume (V_T , panel C, $P = 0.196$) compared to dialysis of mCSF alone (open circles) (n = 7). Data are means \pm SE. The solid lines indicate the period during which muscimol was dialyzed (60–120 min). Two-way repeated measures ANOVA was used (dose and time as factors) to obtain F and P values. Asterisks denote values significantly different between open and closed symbols ($P < 0.05$, post hoc test).

The highest concentration of muscimol (10.0 mM) induced varied degrees of behavioral excitability, [nystagmus](#), spastic limb movements, and [postural instability](#) lasting up to 4 h following the completion of studies. In 3 of 9 studies, these [behavioral effects](#) were too severe to warrant continuation of the study beyond the second hour. Two of the three animals had a dramatic increase in f accompanied by severe behavioral effects, while the third animal's breathing frequency decreased eventually leading to terminal [apnea](#). Despite these major behavioral changes, there were no significant changes during or after muscimol dialysis in all [neurochemicals](#) analyzed in the effluent mCSF, although norepinephrine increased in 4 of 6 goats during dialysis of 10.0 mM muscimol (data not shown). For all doses of muscimol, there were no significant ($P \geq 0.094$) changes in PaO₂, PaCO₂, BP, HR, TR, or $\dot{V}O_2$ ([Table 6](#)).

4. Discussion

[Microdialysis](#) of [antagonists](#) to excitatory, neuromodulatory, [G protein-coupled receptors](#) into the VRC of awake and sleeping goats ([Langer et al., 2016](#), [Muere et al., 2013](#), [Muere et al., 2015a](#), [Muere et al., 2015b](#), [Muere et al., 2015c](#)) support the concept of [neuromodulator](#) compensation, whereby decreases in excitatory receptor activity are compensated by increases in other (predominantly excitatory) neuromodulators to prevent a decrease in ventilation and breathing frequency ([Doi and Ramirez, 2008](#), [Doi and Ramirez, 2010](#)). In other words, there is "compensation" defined as *no change* in ventilation with [dialysis](#) of excitatory antagonists and that this may be due to the observed concomitant increase in excitatory (SP) and decrease in [inhibitory neurotransmitters](#) (GABA).

The objective herein was to determine whether compensation would also occur during VRC dialyses of [agonists](#) to inhibitory, [G protein-coupled](#) (μ -opioid) or inhibitory, [ionotropic](#) (GABA_A) receptors in awake and sleeping goats. Ventilatory data indicated that what appears to be compensation occurred during dialysis of both types of inhibitory receptor agonists. Furthermore, it appears the compensation in neuromodulators differed between agonists to inhibitory G protein-coupled (μ -opioid) receptors and agonists of inhibitory ionotropic (muscimol) receptors.

4.1. Ventilatory and neurochemical effects of opioids dialyzed into the VRC

μ -opioid receptors are widely and abundantly expressed throughout respiratory control regions in the [brainstem](#), including the preBötC region of the VRC ([Gray et al., 1999](#), [Haji et al., 2003](#), [Krause et al., 2009a](#), [Pattinson, 2008](#), [Stornetta et al., 2003](#)). These inhibitory, neuromodulatory receptors may contribute to the regulation of normal eupneic ventilation through the activity of [endogenous opioids](#). The activation of these receptors also contributes to unwanted depression of ventilation with [opiate](#) use clinically, or [cardiorespiratory](#) failure in cases of opiate abuse/overdose. Previous studies have addressed the effects of exogenously applied opioid receptor agonists within the [neural networks](#) that control breathing, but these studies show inconsistent effects on ventilatory variables ([Krause et al., 2009a](#), [Lalley, 2008](#), [Montandon et al., 2011](#), [Mustapic et al., 2010](#), [Pattinson, 2008](#)). Montandon et al. dialyzed [DAMGO](#) into the preBötC of awake and anesthetized rats and found that breathing frequency was depressed at low concentrations, and found complete [apnea](#) at higher concentrations ([Montandon et al., 2011](#)). In contrast, Mustapic et al. reported that the injection of DAMGO into the preBötC of [decerebrate](#) (unanesthetized) dogs increased breathing frequency and [phrenic nerve](#) output ([Mustapic et al., 2010](#)). Different still were studies by Krause et al., in which DAMGO bilaterally injected into the preBötC of awake goats did not affect respiratory rhythm or pattern while breathing room air ([Krause et al., 2009a](#)). Our current results differ from each of the above as unilateral DAMGO dialysis decreased ventilation, but bilateral dialysis increased ventilation, breathing frequency and ventilatory drive while it decreased inspiratory time. In addition, both unilateral and bilateral dialysis of DAMGO destabilized breathing frequency,

which is similar to previous reports that opioids can cause increased variability or destabilization of breathing ([Mitsis et al., 2009](#), [Pattinson, 2008](#), [van den Aardweg, 2009](#), [Walker et al., 2007](#)). Collectively, these data suggest that the effect of opioids on ventilation might differ between species, state of [wakefulness](#), and the nature of the experimental preparation.

During unilateral DAMGO dialysis, a local decrease in [GABA](#) (as indicated in the collected effluent mCSF, [Fig. 10](#)) may have provided neuromodulatory compensation for a presumed DAMGO-induced depression of ventilation ([Fig. 3](#)). In other words, an initial direct depression of ventilation ([Fig. 3](#)) by DAMGO may have been offset within minutes by a secondary/compensatory decrease in GABA or by a change in another [neurochemical](#) to return ventilation to normal. This secondary/compensatory response may have been relatively greater with 100 μM compared to 50 μM which is why a significant depression in ventilation was found with 50 μM but not 100 μM DAMGO. A secondary/compensatory response is sufficiently rapid to prevent a detectable decrease in ventilation during dialysis of antagonists to excitatory neuromodulator receptors ([Muere et al., 2013](#), [Muere et al., 2015a](#), [Muere et al., 2015b](#), [Muere et al., 2015c](#)). Compensation during DAMGO dialysis may also have been within the contralateral VRC. However, findings herein ([Fig. 5](#)) do not support this possibility as bilateral DAMGO dialysis *increased* ventilation. This finding is similar to bilateral dialysis of antagonists to excitatory receptors which also increased ventilation even though ventilation did not change during unilateral dialysis of these antagonists ([Langer et al., 2016](#)). Accordingly, during unilateral and bilateral dialysis, there likely are multiple factors affecting ventilation, which makes it difficult to measure a steady-state, maximal effect, or recovery effect. The cause of the increased ventilation with bilateral DAMGO was not associated with decreased GABA; thus, we speculate it is due to other mechanisms such as increases in excitatory neuromodulators not yet measured and/or changes in [second messengers](#) downstream of G protein-coupled neuromodulator receptors. We have no explanation for why the increased ventilation with bilateral DAMGO dialysis did not result in arterial [hypocapnia](#) even though metabolic rate did not change. Nevertheless, whatever the exact mechanism of the neurochemical response to perturbation of neuromodulator receptors, it does not appear unique to awake and [NREM sleep](#) states as Langer et al. found increases in [5-HT](#) and SP equal in awake and anesthetized goats during dialysis of 50 mM [atropine](#) ([Langer et al., 2016](#)).

Opioids administered clinically in humans for pain management clearly cause respiratory depression, and when given intravenously likely simultaneously depress multiple respiratory nuclei ([Pattinson, 2008](#)). Why does neuromodulator compensation not prevent this respiratory depression? It is not known whether neuromodulator compensation is widespread throughout the brain. Thus, the specific neural sites providing a tonic drive to breathe (chemoreceptors) and/or sites of respiratory-associated [motor neurons](#) (such as the [hypoglossal](#) nuclei) might not display neuromodulator compensation during opiate-induced depression, and thus could be the major substrate for respiratory depression during clinical intravenous opioid administration. Indeed opioid respiratory depression has been linked to increased incidence of obstructive sleep apnea and [sleep disordered breathing](#) ([Randerath and George, 2012](#), [Zutler and Holty, 2011](#)), which as reported by Skulsky et al. ([Skulsky et al., 2007](#)) and Hajiha et al. ([Hajiha et al., 2009](#)) could be due to opioid depression of hypoglossal muscle activity.

In reduced preparations opioids added to a perfusate of the [medulla](#) induced a quantal or step-wise, rather than progressive, slowing of respiratory frequency ([Mellen et al., 2003](#)). In awake goats quantal slowing does not occur during opioid administration to the VRC (which includes the preBötC). These findings suggest a fundamental difference in the effects of opioids between reduced and intact preparations. Indeed, an index of the drive to breathe (V_T/T_i) was increased during bilateral μ -opioid receptor stimulation, which likely represents the effects of compensation (or overcompensation) by neuromodulators. Moreover, the present findings demonstrate a fundamental difference between the effects of DAMGO and [muscimol](#) on respiratory drive and timing mechanisms.

4.2. Ventilatory and neurochemical effects of unilateral muscimol dialysis in the VRC

The current and past dialysis studies in unanesthetized goats found neuromodulator compensation during perturbation of G protein-coupled receptors ([Muere et al., 2013](#), [Muere et al., 2015a](#), [Muere et al., 2015b](#), [Muere et al., 2015c](#)). Herein we found what could be interpreted as overcompensation for increases in inhibitory ionotropic [GABA receptor](#) activity during muscimol dialysis. Several previous studies ([Akilesh et al., 1997](#), [Curran et al., 2000](#), [Curran et al., 2001](#), [Darnall et al., 2001](#), [Gatti et al., 1987](#), [Yamada et al., 1981](#), [Yamada et al., 1982](#)) found that administering muscimol to various brainstem regions depressed breathing and/or arousal from sleep. On the other hand, other studies ([Nattie and Li, 2008](#), [Taylor et al., 2006](#)) found that administration of muscimol increased breathing and/or increased the ventilatory response to CO₂ and [hypoxia](#) ([Nattie and Li, 2000](#), [Taylor et al., 2006](#)). Our findings are similar to these latter studies as we found that muscimol dialysis increased ventilation and breathing frequency.

We found no significant change in the levels of any measured neurochemicals in the effluent mCSF that could counter a [depressant](#) effect of muscimol on [neuronal activity](#). However, 10.0 mM muscimol dialysis increased [norepinephrine](#) in effluent dialysate in 4 of 6 goats, which could have contributed to the increased \dot{V}_I in these goats. A mechanism of GABA receptor-mediated increase in norepinephrine release has been proposed by [Scatton and Bartholini, 1981](#) who found that administration of a GABA [receptor agonist](#) (Progabide) in the rat [hypothalamus](#) activated noradrenergic neurons and increased norepinephrine turnover ([Scatton and Bartholini, 1981](#)). Another possible explanation is that muscimol acts on [presynaptic](#) GABA receptors to increase [glutamate](#) release and postsynaptic neuronal activity ([Jang et al., 2006](#), [Koga et al., 2005](#)); thus, presynaptic, GABA-mediated glutamate responses may contribute to the increased \dot{V}_I and f for all doses of muscimol. The [behavioral effects](#) observed with muscimol dialysis could also be due to increased glutamate release, as these effects were similar to those observed by Wenninger et al. after injection of [ibotenic acid](#) (an excitatory [neurotoxin](#) which acts glutamate receptors) into the preBötC of goats ([Wenninger et al., 2004](#)).

Irrespective of the mechanism and/or pathway, the ventilatory responses to muscimol indicate that some type of compensation and/or secondary effect exists to prevent respiratory depression during perturbations of inhibitory ionotropic receptors, which instead increased ventilation in the behaving goat.

4.3. μ -Opioid and GABA receptor-mediated breathing instabilities

There was significant ventilatory instability with dialyses of the μ -opioid receptor agonist ([Figs. 4, 7, 8](#)) and to some degree during muscimol dialysis. This instability contrasts to studies ([Langer et al., 2016](#), [Muere et al., 2013](#), [Muere et al., 2015a](#), [Muere et al., 2015b](#), [Muere et al., 2015c](#)) in which dialyses of antagonists to multiple excitatory receptors in the VRC did not destabilize breathing. The destabilization with DAMGO could be due to time-dependent changes in the direct effect of opioid-induced depression and the indirect effect of potential compensatory mechanisms. Or a compensatory increase in an unknown excitatory neuromodulator may have created a “high-gain controller”, which destabilizes breathing ([Asyali et al., 2002](#), [Khoo, 2000](#)). Finally, destabilization in the muscimol studies could be due to increased norepinephrine which during pathological states induced increased variability in breathing ([Viemari et al., 2013](#), [Zanella et al., 2014](#)).

5. Caveats and limitations

We previously summarized ([Muere et al., 2013](#), [Muere et al., 2015a](#), [Muere et al., 2015b](#), [Muere et al., 2015c](#)) the limitations and caveats of [dialyses](#) studies in the [medulla](#) of awake and sleeping goats. **One** limitation is that

the [microtubules](#) were not all implanted at the same site ([Fig. 1](#)). The variation in placement was due primarily to the need for avoiding blood vessels on the dorsal surface during surgical implantation. A **second** limitation is the uncertainty of the exact boundaries of the targeted preBötC. To assess the proximity of the microtubule to the preBötC, we utilize the ventilatory response to the glutamate [receptor agonist NMDA](#). Herein, there was a positive response to NMDA even in some goats (see # 11 and 15) whose post mortem histologic measurements indicated the microtubule was outside the estimated boundaries of the preBötC; thus, it is possible our anatomic estimate of the preBötC may be too restrictive. A **third** limitation is the study may have been under powered which could be why we did not detect a significant depression of ventilation with 100 μ M unilateral dialysis of [DAMGO](#) even though there was a significant depression with 50 μ M unilateral dialysis of DAMGO. A **fourth** limitation is uncertainty regarding the diffusion of the drugs dialyzed. A final limitation is that we currently only have the capability of measuring the selected [neurochemicals](#) in the effluent mCSF, and as a result we likely missed potential changes in other local neuromodulatory molecules.

6. Summary and conclusions

We found that: (1) \dot{V}_I decreased during unilateral but increased during bilateral [dialysis](#) of [DAMGO](#), (2) dialyses of DAMGO destabilized breathing, (3) unilateral dialysis of [muscimol](#) increased breathing and (4) dialysis of DAMGO decreased [GABA](#) in the effluent mCSF. We conclude: (1) neuromodulatory compensation can occur during altered inhibitory [neuromodulator](#) receptor activity, and (2) the mechanism of compensation differs between [G protein-coupled](#) excitatory and inhibitory receptors and between G protein-coupled and [ionotropic](#) inhibitory receptors, and (3) the effects of presumed perturbations of inhibitory receptor activity within the respiratory control network shown herein likely have general applicability to neuronal networks throughout the brain.

Author contributions

T.L. performed surgeries and experiments, analyzed data and wrote the MS, S.E.N. performed surgeries and experiments and histological analyses, contributed to data analysis and figures, and MS editing, N.J.B. performed experiments and contributed to intellectual discussions, S.T. performed experiments and contributed to data analysis and histological analyses, L.P. performed all surgeries, M.R.H. contributed to intellectual discussions, MS writing and editing, H.V.F. contributed to intellectual discussions and MS writing and editing. Funding for this study was provided by NIH HL 112996 and HL-007852 and the Veterans Administration. All experiments were performed at Medical College of Wisconsin, Milwaukee, Wisconsin, U.S.A.

Disclosures

There are no conflicts of interest to disclose.

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