Clinically relevant infusion rates of μ-opioid agonist remifentanil causes bradypnea in decerebrate dogs but not via direct effects in the pre-Bötzinger Complex Region.

Mustapic, Sanda1,2, Radocaj, Tomislav1,2, Sanchez, Antonio1,2, Dogas, Zoran4, Stucke, Astrid G.1,3, Hopp, Francis A.1,2, and Stuth, Eckehard A.E.1,3, Zuperku, Edward J.1,2

1Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, WI, 2Clement J. Zablocki VA Medical Center, Milwaukee, WI, and 3Children’s Hospital of Wisconsin, Pediatric Anesthesia, Milwaukee, WI

Pre-Bötzinger Complex and μ-opioid effects in vivo

Corresponding author: Edward J. Zuperku, Ph.D.
Research Service 151
Clement J. Zablocki VA Medical Center
5000 West National Avenue
Milwaukee, WI 53295
(414) 384-2000 ext. 41579
ezuperku@mcw.edu
ABSTRACT:

Systemic administration of μ-opioids at clinical doses for analgesia typically slows respiratory rate. Mu-opioid receptors (MOR) on pre-Bötzinger Complex (preBötC) respiratory neurons, the putative kernel of respiratory rhythmogenesis, are potential targets. The purpose of this study was to determine the contribution of preBötC MORs to the bradypnea produced in vivo by intravenous administration of clinically relevant infusion rates of remifentanil (remi), a short-acting, potent μ-opioid analgesic. In decerebrate dogs, multibarrel micropipettes were used to record preBötC neuronal activity and to eject the opioid antagonist naloxone (NAL, 0.5 mM), the glutamate agonist DL-homocysteic acid (DLH, 20 mM) or the MOR agonist [D-Ala², N-Me-Phe⁴, gly-ol⁵]-enkephalin (DAMGO, 100µM). Inspiratory and expiratory durations (T_I and T_E) and peak phrenic nerve activity (PPA) were measured from the phrenic neurogram. The preBötC was functionally identified by its rate altering response (typically tachypnea) to DLH microinjection. During intravenous remi-induced bradypnea (~ 60% decrease in central breathing frequency, f_B), bilateral injections of NAL in the preBötC did not change T_I, T_E, f_B and PPA. Also, NAL picoejected onto single preBötC neurons depressed by intravenous remi had no effect on their discharge. In contrast, ~60 µg/kg of intravenous NAL rapidly reversed all remi-induced effects. In a separate group of dogs, microinjections of DAMGO in the preBötC increased f_B by 44%, while subsequent intravenous remi infusion more than offset this DAMGO induced tachypnea. These results indicate that μ-opioids at plasma concentrations that cause profound analgesia produce their bradypneic effect via MORs located outside the preBötC region.
INTRODUCTION

Systemic administration of µ-opioids at clinical doses for analgesia typically produces bradypnea during sedation and sleep (26, 35). Mu-opioid receptors (MORs) on pre-Bötzing Complex (preBötC) neurons, the putative kernel of respiratory rhythmogenesis, are potential targets (15). Studies in brain slices that contain the preBötC show that µ-opioids markedly slow the burst rate of respiratory-related output (15, 20), and produce slowing that has been characterized as quantal in neonatal rat brainstem-spinal cord preparations (29). Perturbations of neuronal function within the preBötC of adult animals severely disrupt breathing (14, 22, 32, 36, 41). In vivo studies using multibarrel micropipettes and microiontophoresis have shown that localized application of µ-opioids to medullary respiratory neurons causes a decrease in neuronal discharge and membrane hyperpolarization demonstrating the presence of functional MORs on these neurons (16, 17, 25).

Further evidence in support of the preBötC region as the site of the opioid-induced depression of breathing rate is suggested by the coexpression of 5-HT4a and MOR receptors in the preBötC and the ability of a 5-HT4a agonist to reverse most of the opioid effect on breathing rate without reversing analgesia (27). The underlying mechanism for this functional antagonism was hypothesized to act by counterbalancing the opioid-induced decrease in intracellular cyclic adenosine monophosphate (cAMP) via an increase in cAMP levels produced by activation of 5-HT4a receptors. The functional antagonism did not affect the antinociceptive action of opioids, presumably because 5-HT4a receptors are absent in the regions of the spinal cord involved in the processing of pain stimuli. Similarly, a study by Lalley (24) demonstrated that selective D1-dopamine receptor agonists, which are known to activate the cAMP-PKA signaling pathway in a
variety of neurons, restored phrenic nerve activity after it had been abolished by the selective MOR agonist fentanyl in anesthetized and unanesthetized decerebrate cats. Again it was suggested that the potential site of action could be on preBöTc respiratory neurons.

In a recent study using the picoejection technique combined with extracellular single unit recording (42), we found that respiratory bulbospinal premotor neurons in decerebrate dogs are depressed by μ- and δ-opioids when applied directly to neurons in millimolar concentrations. However, a depression of these neurons of similar magnitude (50%) by intravenous remifentanil (remi), a short-acting, potent, selective MOR agonist used for clinical analgesia, which leads to effect site (brain) agonist concentrations in the nanomolar range, could not be reversed by picoejection of the opioid antagonist naloxone directly onto the neurons. Thus, we concluded that the μ-opioid effects occurred at sites upstream (presynaptic) from the respiratory premotor neurons. Since it has been shown that preBöTc respiratory neurons supply synaptic inputs to the premotor neurons, we hypothesized that clinically relevant opioid-induced effects may be mediated via MORs on preBöTc neurons. Accordingly, the purpose of our study was to determine the contribution of preBöTc MORs to the bradypnea produced in vivo by intravenous remifentanil.

METHODS

This research was approved by the subcommittee on animal studies of the Zablocki VA Medical Center, Milwaukee, WI, in accordance with provisions of the Animal Welfare Act, the PHS Guide for the Care and Use of Laboratory Animals and VA policy. Experiments were performed on mongrel dogs of either sex, weighing from 8 to 16 kg. Inhalational anesthesia was induced by mask and maintained with isoflurane at 1.5-2.5% end-tidal concentration. The
animals were monitored for signs of inadequate anesthesia such as salivation, lacrimation, and increases in blood pressure and heart rate. If required, anesthetic depth was increased immediately.

**Surgical procedures**

The trachea of dogs was intubated with a cuffed endotracheal tube and their lungs mechanically ventilated with an air-O\textsubscript{2}-isoflurane mixture. The surgical procedures, monitoring and maintenance of body homeostasis have been previously described in detail (Dogas et al. 1998). Briefly, after cannulating the femoral artery for blood pressure recording and blood gas sampling and femoral vein for continuous infusion of maintenance fluids and administration of drugs, a bilateral pneumothorax was performed to reduce motion artifacts. The animal was then decerebrated by midcollicular transection (45) and isoflurane was discontinued. After decerebration the animal was ventilated with an air-O\textsubscript{2} mixture and maintained in hyperoxic normocapnia (FiO\textsubscript{2} >0.6). The dorsal surface of the medulla oblongata was exposed by an occipital craniotomy for neuronal recording and microinjections. Phrenic nerve activity was recorded from the desheathed right C5 rootlet. The phrenic neurogram (PNG) was obtained from the moving-time average (100 ms) of the amplified phrenic nerve activity and was used to produce timing pulses corresponding to the beginning and end of the inspiratory phase for the measurement of inspiratory duration (T\textsubscript{I}) and expiratory duration (T\textsubscript{E}). Peak phrenic activity (PPA) was also obtained from the PNG. Continuous neuromuscular block was achieved with pancuronium (0.1 mg/kg/h) to reduce motion artifacts during neuronal recordings.

**Microinjection Technique**

A minimum of one hour was allowed for preparation stabilization before data collection. Extracellular neuronal recordings were obtained using multibarrel micropipettes (10-30 μm
composite tip diameter) consisting of a recording barrel containing a 7 μm thick carbon filament and three drug barrels. The micropipettes were used to locate preBötC neurons within the ventral respiratory column (VRC) prior to pressure microinjections of the glutamate agonist DL-homocysteic acid (DLH, 1 mM) and the MOR antagonist naloxone (NAL, 500 μM and 5 mM) or the MOR agonist [D-Ala\(^2\), N-Me-Phe\(^4\), Gly-ol\(^5\)]-Enkephalin (DAMGO, 100 μM), which were dissolved in artificial cerebrospinal fluid (aCSF). Microinjections were made using an in-house, custom-built, 4-channel microinjection system, which allowed independent control of ejection duration, repetition rate, and pressure 0-80 psi. The microinjected drug volumes were measured via height changes of the meniscus in the pipette barrel with a 100x magnification microscope equipped with a reticule (resolution ~ 2 nl).

**Method for Locating the preBötC Region**

We used three criteria to locate the preBötC region: 1) predetermined stereotaxic coordinates, 2) presence of a mixture of respiratory neuron subtypes within the VRC, and 3) PNG tachypneic response to DLH microinjections (30-40 nl; 20 mM). Our previous studies showed that the canine preBötC can be found within a region in the VRC extending from ~3 to ~6 mm rostral to the obex. The region where the largest DLH-induced rate altering (typically tachypneic but see below) responses were observed consisted of a heterogeneous mixture of propriobulbar I and E neuron subpopulations (e.g. ref. 22), which is consistent with observations in other species (8, 11, 39, 44). The tachypneic response produced by DLH microinjection into the VRC has been accepted by many investigators as a functional marker of the preBötC region (8, 22, 32, 41, 46), and is the reason we used it in this study as one of the main criteria. For the present study, starting at 3 mm rostral to obex and typically 4.5 mm lateral to the midline the micropipette was advanced into the VRC and the subtypes of neurons noted from the dorsal to
the ventral aspects of the VRC. The pipette was withdrawn to the midpoint and DLH was then microinjected while monitoring the PNG response. The micropipette was then removed, the tip cleaned, and then reinserted 1 mm more rostral, and the procedure was repeated, continuing to 7 mm rostral. The site within this region of the VRC with the maximum tachypneic response to DLH microinjection (~30 nl) was considered to represent the preBötC region (22). If no tachypneic response was found within the VRC, then coordinates and neuron subtype composition were used to locate the preBötC region.

**Remifentanil Infusion**

The distinct advantages of using remifentanil (remi) is its rapid onset and short latency to peak effect, as well as its rapid recovery, which is independent of dose rate and length of infusion. These properties of remi are due to its rapid metabolism by nonspecific esterases in the blood and tissues. Because it is short acting, remi must be continuously infused, and the infusion rate can be adjusted to produce steady-state responses of various degrees. The context-sensitive half-life, the time to a 50% decrease of an effective site concentration after infusion is stopped has been estimated to be ~ 4 min for remi, and is independent of infusion duration (3). The 50% effective dose to surgical stimuli in humans was found to be ~0.5 µg/kg/min, and appears to be similar in dogs (30, 31).

**Protocols**

1. **Bilateral microinjection of naloxone into the preBötC during intravenous remifentanil-induced bradypnea**

   After establishing a stable PNG baseline pattern, the preBötC region was located as previously described. Subsequently, remi was infused at an intravenous rate (~0.1-1.0 µg/kg/min) that resulted in a marked steady-state bradypnea (~60% decrease in central breathing
During continued steady-state remi infusion a series of bilateral injections of NAL (500 µM solution, ~120 nl each), 3 on each side, centered in the preBötC and 1 mm rostral and caudal, were performed to locally block MORs. T_i, T_E, and PPA were continuously monitored online with a PowerLab system (ADInstruments, Castle Hill, Australia, 16SP and Chart v5.5.6). Average values of these variables and f_B were obtained for offline analysis before remi infusion and from data 1 minute before and after each NAL microinjection during remi infusion. The same concentration (500 µM) NAL solution was finally given intravenously (total IV dose ~60 µg/kg) following completion of all direct preBötC NAL microinjections to verify its continued effectiveness in reversing the intravenous remi effects.

2. Picoejection of naloxone onto single preBötC neurons during remifentanil-induced bradypnea

After locating the preBötC region in a second set of animals, control recordings from single preBötC respiratory-related neurons were made before and during remi-induced bradypnea. During steady-state bradypnea, which typically depressed preBötC neuronal discharge frequencies by 25-50%, NAL (500 µM) was continuously picoejected onto the depressed neuron at increasing picoejection rates up to a level, which was estimated to produce a NAL concentration at 200 µm from the pipette tip that approached the barrel concentration. Subsequently, the intravenous remi infusion was stopped and neuronal activity was followed during the recovery phase from the effects of remi. The micropipette was then repositioned in a new location at least 500 µm distant from the previous track to avoid contamination from residual effects of intramedullary NAL, and the protocol was then repeated. In addition, at least once per animal, picoejection of the aCSF vehicle was used to verify that the aCSF constituents and ejected volumes were without effect on single neurons. A time-amplitude window
discriminator was used to generate a standard logic pulse for each spike. Neuronal discharge frequency, \( F_n \), was continuously determined online by the number of discharges in each 100 msec time increment. Neuronal and phrenic activity and the picoejection marker signals were recorded on a digital tape system (Model 3000A, A.R. Vetter, Rebersburg, PA) for further offline analyses. Cycle-triggered histograms (CTHs; 50 ms bins) of the tape-recorded neuronal discharge were generated from template-discriminated spikes (Spike2, v6.04, Cambridge Electronic Design, Cambridge, England). Peak and average discharge frequency were determined from the CTHs (15-20 cycles/CTH) and used to measure the remi and NAL effects.

3. Microinjection of DAMGO into the preBötC region: assessment of the direct MOR agonist effect on the phrenic nerve activity and breathing pattern

After locating the preBötC region in the VRC in a separate third set of animals, DAMGO (100µM) was microinjected into the preBötC region and the effects on the PNG were measured. After completion of the DAMGO injections, once maximal DAMGO effects on the PNG were observed, remi was infused intravenously to measure additional changes in the PNG that would have resulted from systemic opioid effects on areas other than the preBötC. Then the remi infusion was stopped and recovery from the remi-induced changes in \( f_B \) was awaited. Since the effects of preBötC DAMGO microinjections are long lasting, the phrenic tachypnea recurred. This persistent DAMGO effect was then reversed by microinjection of a concentrated solution of NAL (5 mM) into the same site (preBötC). To confirm that all DAMGO effects were limited to the preBötC area, this was followed, 10-15 minutes later, by intravenous NAL (~60 µg/kg), which would have antagonized any additional opioid effects on the brainstem. In a subset of 4 dogs, DAMGO was injected bilaterally into the preBötC to measure any additional direct effects.

Data analysis
Statistical procedures were carried out using SigmaStat 3.5 (Systat Software, Inc., Richmond, CA 94804). A One-Way Repeated Measures ANOVA was used on data that were normalized relative to control values. A general linear model was automatically used to provide least squares estimates of the means for cells with missing data and unbalanced data sets. The Holm-Sidak method was used for pairwise multiple comparisons with a familywise error rate of 0.05. For all data sets, tests for normality of the normalized data (Kolmogorov-Smirnov test) were performed before parametric procedures were used. For data sets that failed the normality test, a Kruskal-Wallis One-Way ANOVA on ranks was used with Dunn’s method for pairwise multiple comparisons. Differences were considered significant for p<0.05. Values are expressed as means ± SE.
RESULTS

We found that the tachypneic region associated with the preBötC region was located within the VRC at 4.3-6.8 mm rostral to obex, 4-5 mm lateral to the midline and 6-8 mm below the dorsal medullary surface. The area also contained a mixture of inspiratory and expiratory neuron subpopulations as has been previously shown by Krolo et al. (22).

Protocol 1. Bilateral microinjections of naloxone into the preBötC during intravenous remifentanil-induced bradypnea

An example of this protocol is shown in figure 1, which shows traces of the PNG, injection markers, and breathing rate $f_B$. DLH microinjection produced a strong tachypnea (from 12 to 30 breaths/min; BPM), indicative of the preBötC region. Intravenous infusion of remi (0.22 µg/kg/min) decreased $f_B$ to ~ 5 BPM. During steady-state remifentanil induced bradypnea, a series of NAL microinjections (120 nl each) were made on the right side and then the left side of the medulla centered in the preBötC region. Microinjections were placed at the center of the maximum tachypneic region and 1 mm rostral and 1 mm caudal to it. At each site two microinjections were made separated by ~0.5 mm in depth. Note that none of the local NAL microinjections had any effects on $f_B$. In figure 1,D augmented PNGs or sigh-like activity is apparent. Such sighs are seen at baseline and can become more frequent during IV remi-induced bradypnea in some but not all dogs, with and without NAL microinjections. In contrast to preBötC NAL microinjections, intravenous NAL rapidly reversed the remi-induced bradypnea and depression of phrenic amplitude. This reversal tended to transiently overshoot beyond the control baseline levels to varying degrees (Fig. 1 E, and Fig. 2, NAL IV bars). A transient IV NAL-induced overshoot was also regularly seen in systemic blood pressure (data not shown). Note that due to drift in the central respiratory rate over time despite steady-state
intravenous remi, $f_B$ had increased prior to NAL microinjections and was much higher after the final intravenous NAL bolus compared to the initial baseline rate. A slow, spontaneous increase in breathing frequency can be often seen in decerebrate dogs without as well as during steady state remifentanil infusions, but such slow increases occur over many hours so that they do not influence or negate assessment of the effects of interventions over shorter time spans (e.g. < 30 min).

In 15 dogs, remi (0.50±0.51 µg/kg/min) decreased $f_B$ by 41±7% from 27.5±4.6 to 14.5±3.6 BPM, decreased PPA (peak phrenic activity) by 32±3%, decreased neural minute ventilation by 60±6% (Fig. 2, left), increased $T_I$ by 17±4% and increased $T_E$ by 143±14% (Fig. 2. right). An average protocol took 2.6 hours to complete. The time from the start of the remi infusion to the first NAL injection was 55±10 minutes and the average time between injection sites was 16±2 minutes, which included cleaning the micropipette tip, insertion in the new location, locating neuronal activity in the VRC, placing two injections of ~125 nl each and ~0.5 mm apart in the dorsal and ventral regions of the VRC and micropipette withdrawal. Based on a brain extracellular volume fraction of 0.21 (fraction of the total brain tissue volume (34)) each 125 nl injection volume would have a spherical diameter of ~ 1 mm and with subsequent diffusion would have an even larger effective volume diameter. Thus, 3 injections that are 1 mm apart would allow the effects of NAL to extend over 3 mm in the rostral-caudal direction of VRC-preBötC region and well over 1 mm in the other directions. Despite the relative large projected volume of spread, figure 2 shows that the NAL injections had no effect on any of the phrenic respiratory timing and drive variables. To correct for the effects of time drift on respiratory rate (see above) from the onset of the protocol, changes in the variables were always analyzed relative to their immediate local control values, that is the value preceding each
injection. As shown in figure 3 with such corrections, there were no changes in PPA, T_I and T_E following the NAL injections. In stark contrast, intravenous NAL (60.7±19 µg/kg) during the remi infusion promptly and fully reversed the remi-induced effects (Fig. 2, NAL IV).

Protocol 2. Picoejection of naloxone onto single neurons in or near the preBötC during remifentanil-induced bradypnea

Figure 4 shows an example of protocol 2 for an inspiratory decrementing neuron in or near the preBötC region. Intravenous infusion of remi (0.16 µg/kg/min) decreased the peak discharge frequency (F_n) from 32.2 to 20.2 Hz (~37%) and decreased the decrementing rate (Fig. 4A). Picoejection of NAL (500 µM at 2.9 nl/min or 1.45 pmol/min) onto this neuron had no effect on reversing the remi-induced depression (Fig. 4B), but the neuron’s discharge pattern completely recovered following termination of the remi infusion (Fig. 4C). Pooled data from 3 inspiratory and 3 expiratory neurons show that highly localized application of NAL did not reverse the remi-induced depression of neuronal activity. Remi intravenous infusion (0.20±0.03 µg/kg/min) produced a 37.2±9.8% decrease in peak F_n and a 29.8±11.2% decrease in the time-averaged F_n (Fig. 5, F_peak and F_ave). Picoejection of NAL (4.4±1.2 pmol/min) had no effect but neuronal activity completely recovered following termination of remi infusion. Even though we found that none of 6 neurons (0/6) respond to NAL, with this small sample it is possible to miss neurons depressed by remi that would have been reversed by local NAL. Without prior knowledge of the proportion of the neuron population that is unresponsive, a 95% confidence interval analysis suggests that the proportion of neurons that did not respond to NAL lies between 0% to 46% (33).

Protocol 3. Microinjection of DAMGO into the preBötC region: assessment of the direct MOR agonist effect on the phrenic nerve activity and breathing pattern
Figure 6 shows an example of the protocol used to study the effects of microinjection of the MOR agonist DAMGO into the preBötC region on the breathing pattern. The preBötC region was functionally identified in the VRC, where I and E neurons were found, by the typical tachypneic response produced by DLH microinjection into the left VRC. Following recovery of the response to DLH, 109 nl of 100 µM DAMGO was microinjected and \( f_B \) increased from ~12 to ~20 BPM (67% increase) over a 15 minute period. In contrast, intravenous infusion of 0.8 µg/kg/min remi after maximal DAMGO effects were reached produced a pronounced decrease in \( f_B \) to ~5 BPM. Following recovery from remi-induced bradypneic effects (~15 min) the prolonged residual DAMGO effect caused a return to relative tachypnea. At this point microinjection of NAL (360 nl; 5 mM) into the same site where DAMGO was microinjected gradually reversed the DAMGO-induced tachypnea with return of respiratory rate to pre DAMGO baseline. Subsequent intravenous naloxone (NAL IV; 79 µg/kg) produced no additional effect in this animal. Typically DAMGO-induced effects lasted >1 hour before spontaneous resolution. The overall protocol took ~90 minutes to complete.

The pooled data (n=16, Fig. 7) show that unilateral microinjection of 100 µM DAMGO (143±16 nl) into the right preBötC region increased \( f_B \) by 44.4±5.2% from the baseline \( f_B \) of 17.7±2.9 BPM. This was due to a 25±3% decrease in \( T_I \) and a 35±4% decrease in \( T_E \) (Fig. 7B), where baseline \( T_I \) and \( T_E \) values were 1.64±0.20 and 3.35±0.64 seconds, respectively. Peak phrenic activity was decreased by 12±4%. Subsequent microinjection of NAL (211±39 nl, 5 mM) at the same location as the DAMGO microinjections could only partially antagonize the DAMGO-induced tachypnea, reducing \( f_B \) to 25.7±7.3 % above control, but restored PPA to control levels (Fig. 7A, NAL pBC). However, intravenous administration of NAL (76±5 µg/kg intravenous) produced additional and nearly complete antagonism of the DAMGO effects in the
preBötC region. In 4 of the 16 dogs, bilateral DAMGO microinjections in the preBötC regions were studied to determine the magnitude of the additional DAMGO injection. The pooled data (Fig. 7C, top) show that the right-sided microinjections increased $f_B$ from $14.5\pm2.3$ to $21.4\pm4.3$ BPM or $\sim48\%$. Additional left-sided DAMGO microinjections increased $f_B$ to 24.6 BPM or a further $22\%$. Subsequent intravenous NAL returned $f_B$ to control levels.

In 7 of the 16 dogs, remi ($1.0\pm0.1 \, \mu g/kg/min$) was infused intravenously after the DAMGO microinjections, i.e., while the DAMGO effects persisted, to compare systemic versus local $\mu$-opioid effects on $f_B$. In these studies, unilateral microinjection of DAMGO increased $f_B$ by $51\pm16\%$ whereas intravenous remi decreased $f_B$ by $55\pm8\%$ relative to control (Fig. 7C, bottom). Only after complete recovery from the remi effects were local NAL microinjections given, followed by intravenous NAL injections.

The anatomical sites of the NAL and DAMGO microinjections and studied neurons as determined by the stereotaxic coordinates relative to the obex, midline, and dorsal surface are shown in figure 8. The microinjection sites were always in a region of respiratory neuronal activity, presumably the VRC.
DISCUSSION

The main finding of this study is that the bradypnea produced by systemically administered µ-opioids at clinically relevant doses for analgesia is not caused by direct MOR activation in the pre-Bötzinger Complex. Rather, direct activation of preBötC MORs with the selective agonist DAMGO consistently produced tachypnea. This conclusion is also supported by the lack of a reversing effect of NAL on single preBötC neurons that were depressed during intravenous remifentanil infusion, within the limits imposed by the small sample size (n=6). These findings suggest that MORs on neurons presynaptic to the preBötC neurons must be responsible for the bradypneic response. In addition, although there are functional MORs in the preBötC region, their in vivo role in the control of breathing is not clear. It is clear that high local opioid agonist concentrations in the micro- to millimolar range are required to directly depress respiratory neurons in the preBötC, similar to our findings for VRC premotor neurons (42). Such high concentrations may be reached by synaptic release of endomorphins, i.e., preBötC MORs may be synaptically activated by endogenous peptides (28, 47). It is known from other neurotransmitters that their transient synaptic concentrations can reach the millimolar range (2, 9).

In a minority of animals, mostly those with low baseline respiratory rates, we observed an increase in respiratory rate with remifentanil rather than a slowing. These animals were not used for the study protocols. This discrepancy to the clinical experience with patients is likely due to our particular experimental setup. Vagotomy may eliminate a direct opioid effect on vagal inputs and thus reduce slowing. Also, the increase in arterial CO2 that results from opioid application in patients can cause slowing
of the respiratory rate, while in our animals ventilation was controlled and CO2 remained unchanged.

*Methodological Considerations*

We used three criteria to locate the preBötC region: 1) predetermined stereotaxic coordinates, 2) presence of a mixture of respiratory neuron subtypes within the VRC, and 3) PNG tachypneic response to DLH microinjections (30-40 nl; 20 mM). Our previous studies showed that the canine preBötC can be found within a region in the VRC extending from ~3 to ~6 mm rostral to the obex. The region where the largest DLH-induced rate altering (typically tachypneic) responses were observed consisted of a heterogeneous mixture of propriobulbar I and E neuron subpopulations (e.g. (22)), which is consistent with observations in other species (8, 11, 39, 44). The tachypneic response produced by DLH microinjection into the VRC has been accepted by many investigators as a functional marker of the preBötC region (8, 22, 32, 41, 46), and is the reason we used it in this study as one of the main criteria. The preBötC region in the dog consists of a heterogeneous mixture of propriobulbar inspiratory and expiratory neuron subpopulations and is located within the VRC at 4.3-6.8 mm rostral to obex (22). We are fairly certain that our local microinjections were sufficient to achieve maximal drug effects on all neurons of the preBötC. The size and spread of the multiple NAL injections (~125nl, >3 mm in the rostral-caudal direction, centered at the point of maximum tachypneic response and >1 mm in the medial-lateral and dorsal-ventral aspects of the VRC) probably exceeded the boundaries of the preBötC region. Microinjections were only made in the ventrolateral medulla where respiratory neuronal activity was found. In addition, the concentration of microinjected NAL
(500 µM) was >1000 times greater than the plasma concentration that was required for reversal of remi-induced effects by intravenous NAL. Furthermore, since diffusion occurs with NAL microinjections, the effective volume of MOR antagonism by local NAL would be much greater than the injected volume. Finally, microinjections of DAMGO into the same region produced a reproducible tachypneic response consistent with direct activation of MORs on neurons in the preBöCtC region. On the other hand, the fact that NAL microinjections did not have any effects on the PNG suggests that diffusion of the drug was limited to the VRC and did not affect other areas of respiratory control.

Our conclusions are based on whole phrenic nerve recordings during microinjections into a network of neurons; however, these conclusions are also supported at the cellular level. Picoejection of NAL on single preBöCtC neurons (n=6) failed to reverse any of the neuronal depression induced by intravenous remi. Similarly, a previous study found that none of the 18 (95% confidence interval: 0-18.5%) bulbospinal respiratory premotor neurons studied were affected by NAL picoejection during IV remi-induced depression, even though these neurons have been shown to have µ- and ð-opioid receptors using selective agonists (42).

A possible explanation for the lack of a direct effect on preBöCtC MORs by systemic administration of remi is the low plasma and thus brain concentration levels that are attained. The typical remi infusion rates we used (~0.5 µg/kg/min) are expected to result in plasma concentrations of ~10 ng/ml or 24 nanomolar in dogs (31), which is ~4,000 times less than the concentration of microinjected DAMGO (100 µM), which produced a tachypneic response. An IC$_{50}$ of 35±9 nM for DAMGO inhibition of cAMP production was found in cells expressing cloned MORs (13). In mice, the unbound brain EC$_{50}$ concentrations for remi related opioids, fentanyl, alfentanil, and sufentanil, have been found to be the very similar to the unbound EC$_{50}$
serum concentrations and to the \textit{in vitro} affinity ($K_i$) (21). Since remi is about half as potent as fentanyl (31), the estimated affinity $K_i$ for remi would be between 5-10 nM. This may explain why the targeted ~24 nM remi plasma concentration we used in the current study induced the observed, marked bradypneic responses, assuming that highly opioid sensitive sites responsible for respiratory rate control were affected.

In our previous study on VRC bulbospinal premotor neurons, where intravenous remi had only upstream effects but no direct depressant effects at the premotor neuronal level, picoejection of remi at concentrations >20 times larger than reported peak plasma concentrations had no effect on the discharge of single neurons. A similar scenario may take place in the more rostral VRC preBötC region. Thus, it seems likely that the remi-induced depression of breathing rate occurs at respiratory-related neurons that are presynaptic to the preBötC neurons, which may express MORs that respond to low nanomolar opioid concentrations that are typical during clinical analgesia and respiratory depression. The sensitivity of neurons to low concentrations of opioids may not only be related to the number or density of MORs, but may also depend on the location of the MORs on the neuron. For example, if the receptors are strategically located on axon terminals, similar to the primary afferent fibers in the dorsal horn of the spinal cord that appear to inhibit the release of neurotransmitters via inhibition of voltage-dependent $Ca^{2+}$ channels, the opioid effect could be markedly enhanced compared to a location on or near the soma.

\textbf{DAMGO-induced tachypnea}

Microinjections of DAMGO into the preBötC region produced an increase in respiratory rate, which was opposite to the slowing response produced by intravenous remi and also in \textit{in vitro} studies. In brainstem slices containing the preBötC, rhythm generation appears to rely on
pacemaker neuron activity (15) (1, 4, 5). Opioid-induced membrane hyperpolarizations of pacemaker neurons produce slowing of the burst rate whereas depolarizations increase burst rate (15). Bath application of DAMGO in brainstem-spinal cord preparations (29) and systemic fentanyl in juvenile rats (P7-P13; 16-32 g)(19) produce quantal slowing of inspiratory burst activity, while the burst rate of expiratory related activity is unchanged. We have not observed quantal slowing in our in vivo dog model, but rather a gradual dose-dependent increase in \( T_I \) and \( T_E \) with IV remi. In addition, the discharge of canine expiratory neurons in the preBötC region is prolonged with remi-induced increases in \( T_E \) and remains coupled to inspiratory neuronal activity in a 1:1 manner (personal observation, E. J. Zuperku 7-15-09).

In developmentally mature in vivo preparations, rhythm generation appears to be the result of a network in which synaptic interactions among neurons play a key role (37). In such models that have been analyzed via computer simulation, the indispensable element is the reciprocal synaptic inhibition between I and E neurons with intrinsic adaptive properties that result in decrementing patterns (38). Computer simulation of such a reduced network model consisting only of I and E decrementing neurons with inhibitory reciprocal interconnections shows that a reduction in tonic excitatory drive produces an increase in oscillatory rate, whereas an increase in excitation produces slowing due to increased synaptic inhibition (43). Since many VRC neurons appear to express MORs (27, 42), a possible explanation for the tachypnea produced by microinjection of DAMGO into the preBötC region is that a simultaneous postsynaptic depression of preBötC region neurons by DAMGO may result in decreased excitation and thus an increase in the rate of the oscillator and thus of the respiratory rate. However, other mechanistic explanations are possible.

Possible sites for intravenous remifentanil-induced effects
Immunohistochemical studies, using antibodies to cloned MORs have been used to examine the distribution of MORs in the rat brain. Within respiratory-related areas, the most intense MOR-like immunoreactivity (LI) was seen in the pontine lateral parabrachial nuclei (PBN), the locus coeruleus, rostral ambiguous nucleus, and the medial and commissural subnuclei of the solitary tract (NTS) (12). The NTS contained nerve fibers with intense MOR-LI, including the primary afferent fibers of the vagus nerve. Endomorphin types 1 and 2 (EM-1 and EM-2) are endogenous peptides that have high affinity and selectivity for MORs and potent analgesic activity. Immunoreactivity to EM-1 and EM-2 was used to determine the location of fibers and cell bodies of endomorphin-containing neurons (28). In these studies dense EM-1 LI was seen in the PBN, locus coeruleus, and the NTS. Some of the endomorphin inputs to the PBN in rats arise from EM-1 and EM-2 LI neurons in the hypothalamus (7). Using dual labeling immunocytochemistry combined with electron microscopy, Silverman et al. (40) found EM-2 LI primarily in the unmyelinated axons and axon terminals in the NTS, including some vagal afferents, and MOR LI in many of the larger dendritic targets of the EM-2 LI terminals. In addition, there appear to be reciprocal connections of EM-1 and EM-2-containing neurons between the hypothalamus and the NTS in the rat (18).

It is well known that inputs to the preBötC region from the NTS and the PBN contribute to respiratory phase switching. Afferent inputs from pulmonary stretch receptors end in the NTS and mediate the Breuer-Hering inspiratory inhibitory and expiratory facilitatory reflexes (23). Outputs from the neurons in the parabrachial complex region, including the Kölliker-Fuse nucleus and intertrigeminal region, project to the VRC, and chemical or electrical activation of these neurons profoundly alter respiratory phase timing (6, 10). A recent network model, which is based on spike-train cross-correlation analysis and which assigns specific synaptic connections
between pontine neurons and preBötC/Bötzinger neurons, is able to simulate experimentally observed responses to various perturbations (38). Thus, it appears that the presence of dense MORs in the PBN and NTS could be potential sites where systemic opioids such as intravenous remi act to produce their effect on breathing rate. Another possible site may include the midline medullary raphe region where MOR LI has been seen (12). Zhang et al. (48) found that the systemic DAMGO-induced inhibition of ventilation and of the carbon dioxide response was significantly reduced after pretreatment of the caudal raphe region with a selective MOR antagonist.

Summary

Even though pre-Bötzinger Complex neurons contain µ opioid receptors, it does not appear that clinical doses of intravenous opioids act directly on them to produce bradypnea, in vivo. We speculate that the effect of intravenous opioids, such as remifentanil, may be on presynaptic neurons that project to the preBötzinger Complex region and are involved with the control of respiratory phase timing.
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REFERENCES


**Figure Legends**

**Figure 1:** Example of protocol 1: Bilateral microinjections of naloxone into the preBötzingerg Complex (preBötC) region fail to antagonize intravenous remifentanil-induced bradypnea: Top trace shows phrenic neurogram (PNG), middle trace microinjection marker and bottom trace displays phrenic breathing (burst) rate. Panel A: DLH microinjection induces tachypnea, which functionally identifies the preBötC location. Panels B through E: Intravenous remifentanil (IV REMI) infusion (upper horizontal bar) produces a gradual reduction in phrenic breathing rate during B. Panels C and D: During steady-state IV REMI induced bradypnea, multiple microinjections of naloxone (NAL) into area of preBötC (pBC) in the right and left ventral respiratory columns fail to antagonize IV REMI-induced bradypnea. Of note, remi can cause
occasional sighs as visible in D. Panel E: Intravenous NAL promptly reverses IV REMI bradypnea with overshoot tachypnea.

Figure 2: Summary data for Protocol 1 from 15 dogs confirm that right sided (Rt) and left sided (Lt) microinjections of naloxone (NAL) into preBötC fail to reverse the intravenous remifentanil (REMI IV) induced bradypnea, while intravenous naloxone (NAL IV) promptly reverses REMI IV effects with overshoot tachypnea. Top left panel shows phrenic (burst) breathing rate, middle left panel peak phrenic activity and bottom left panel neural minute ventilation. Right panel: intravenous remifentanil (REMI IV) induced a modest prolongation of inspiratory duration (T_i) and marked prolongation of expiratory duration (T_E), but bilateral microinjections of NAL into the preBötC failed to have any effect, while intravenous naloxone (NAL IV) promptly reversed REMI IV effects. All values are normalized to control values, see text for details. ***: p<0.001 for significant differences between pre-events (gray bars) and post-events (black bars).

Figure 3: Summary data for Protocol 1 from 15 dogs confirm that right and left sided microinjections of naloxone (NAL) into preBötzinger complex (pBC) region fail to reverse the intravenous remifentanil (REMI IV) induced depression of phrenic amplitude (PPA), prolongation of inspiratory duration (T_i) and expiratory duration (T_E) while intravenous naloxone (NAL IV) promptly reversed all REMI IV effects. To correct for the effects of time drift on phrenic respiratory rate (see text for details) from the onset of the protocol, changes in the variables were always analyzed relative to their immediate local control values, that is the value preceding each injection (e.g. pre-event in figure 2). **: p<0.01; ***: p<0.001 for significant differences from no change.
**Figure 4:** Example from an inspiratory preBötC neuron with a decrementing discharge pattern from protocol 2. Top trace of each panel shows effect on phrenic neurogram (PNG), bottom trace of each panel shows effect on neuronal discharge pattern as a cycle triggered histogram of the discharge frequency. Panel A: Phrenic and neuronal activities were depressed by intravenous remifentanil (REMI IV) from control (CON). Panel B: Picoejection of naloxone (picoejected NAL) onto the neuron during remifentanil-induced depression (REMI IV) did not reverse the decrease of the neuronal peak discharge. Panel C: Phrenic activity and neuronal discharge fully recovered after cessation of the intravenous remifentanil infusion (post-REMI IV CON).

**Figure 5:** Pooled data for protocol 2 from 3 inspiratory and 3 expiratory neurons. Second bars of each panel show that intravenous remifentanil infusion (REMI IV) decreased peak and time-averaged discharge frequency ($F_{peak}$ and $F_{ave}$). Picoejection of naloxone (NAL during REMI IV, 3$^{rd}$ bar) onto the neuron in the preBötC did not reverse the remifentanil-induced depression of neuronal activity. Neuronal activity completely recovered following termination of remifentanil infusion (END CON). ***: $p<0.001$ for significant differences from control (100%).

**Figure 6:** Example of a protocol 3 run shows that microinjections of the MOR agonist DAMGO into the preBötC region increased the phrenic burst rate. Top trace shows the phrenic neurogram (PNG) and bottom trace the fictive respiratory rate derived from the phrenic neurogram. From left to right: DLH microinjection (44 nl; 20 mM) induces tachypnea, which locates preBötC. Subsequent unilateral microinjection of DAMGO (109 nl; 100µM) at the same site increased respiratory rate with only minor effects on peak phrenic activity. Intravenous remifentanil
(REMI IV; 0.8 µg/kg/min) overcame the effects of DAMGO and, on the contrary, produced a profound bradypnea and depression of peak phrenic activity. Following recovery of respiratory rate and peak phrenic activity after cessation of intravenous remifentanil, microinjection of NAL (360 nl; 5 mM) into the same site where DAMGO was microinjected, gradually reversed the DAMGO-induced tachypnea. Subsequent intravenous naloxone (NAL IV; 79 µg/kg) produced little or no additional effect.

**Figure 7:** Summary of the normalized pooled data for protocol 3 (n=16) shows that unilateral microinjection of DAMGO into the right preBötC region increased breathing rate (panel A, top, second bar, DAM pBC). This was due to decrease in T₁ (panel B, top, second bar, DAM pBC) and in Tₑ (panel B, lower, second bar, DAM pBC). Peak phrenic activity (PPA) was only modestly decreased (panel A, lower, second bar, DAM pBC). Subsequent microinjection of naloxone (NAL pBC) at the same location as the DAMGO microinjections only partially antagonized the DAMGO-induced tachypnea (panel A, top, third bar), but restored PPA to control levels (panel A, lower, third bar). However, intravenous administration of naloxone (NAL IV) produced complete antagonism of the DAMGO effects in the preBötC region. Panel C, top: Summary of the pooled data from 4 of the 16 dogs with sequential bilateral DAMGO microinjections in the preBötC regions. Additional left-sided DAMGO (DAM Lt pBC) microinjections increased phrenic respiratory rate further, but the difference from the right-sided DAMGO (DAM Rt pBC) microinjections was not statistically significant. Subsequent intravenous naloxone (NAL IV) reversed the DAMGO effects and returned the rate to control levels. Panel C, lower: Summary data from 7 of 16 dogs, in which remifentanil (REMI IV) was infused intravenously after the DAMGO microinjections to compare systemic versus local µ-
opioid effects on phrenic respiratory rate. Unilateral microinjection of DAMGO (DAMGO pBC) increased respiratory rate whereas intravenous remifentanil (REMI IV) decreased the respiratory rate relative to control. The two effects acted in opposite directions. *: p<0.05; **: p<0.01; ***: p<0.001 for significant differences from control.

**Figure 8:** Coronal sections of the brainstem in the area of the preBötzinger complex show the anatomical sites of the naloxone (NAL, filled circles) and DAMGO (open circles) microinjections and neurons (x) relative to the obex, midline, and dorsal surface. For clarity NAL and DAMGO injection sites are shown on separate sides.
Remifentanil iv infusion (0.22 µg/kg/min)

A

PNG

B

Inj marker

C

120 nl NAL injections

D

120 nl NAL injections

E

200 nmoles

f_B

Rate (BPM)

2 min

5 min

2 min

5 min
REMI IV Continuous Infusion

NAL Microinjections into pBC

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**% change**

- PPA: Right -1 **,** Left +1 ***
- TI: Right -1 **,** Left +1
- TE: REMI IV **,** NAL IV ***