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Micro-Opioid Receptor Agonist Injections into The Presumed Pre- Botzinger Complex and The Surrounding Region of Awake Goats Do Not Alter Eupneic Breathing

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Abstract: Opioids are clinically important in the alleviation of pain. An undesirable side effect of opioids is depression of breathing. Data from isolated preparations suggest this effect is due to attenuation of discharge activity of neurons in the pre-Bötzinger complex (preBötzc), a medullary area with respiratory rhythmogenic properties. The purpose of this study was to examine how [d-Ala(2),N-Me-Phe(4),Gly(5)-ol]-enkephalin (DAMGO), a μ -opioid receptor agonist, affected breathing after injection into the presumed preBötzc of the adult awake goat. We hypothesized that DAMGO would cause breathing to decrease and become irregular when injected into the presumed preBötzc and the surrounding region of the conscious animal. We further hypothesized that ventilatory sensitivity to CO₂ and hypoxia would be blunted after the injection of DAMGO. Microtubules were bilaterally implanted into the presumed preBötzc of 10 adult female goats. After recovery from the surgery, DAMGO (0.5–10 μ l, 1 nM–10 μ M) was injected into the presumed preBötzc during the awake state. DAMGO had no effect on pulmonary ventilation [inspiratory minute ventilation (Vi)], respiratory rhythm and pattern, the activation pattern of inspiratory and expiratory muscles, or arterial blood gases during eupneic breathing conditions ($P > 0.10$). However, DAMGO attenuated ($P < 0.05$) the evoked increase in breathing frequency when inspired CO₂ was increased, and DAMGO attenuated the Vi response to reduction of inspired O₂ to 10.8% ($P < 0.05$). We conclude that our data do not provide support for the concept that in awake mammals opioid depression of breathing is due to a directed action of opioids on preBötzc neurons.

Keywords: chemosensitivity, [d-Ala(2),N-Me-Phe(4),Gly(5)-ol]-enkephalin

Opioids are routinely used in the treatment of pain, coughing, and smooth muscle spasticity. However, their therapeutic uses are limited by respiratory complications, including depression of respiratory frequency (f), tidal volume (Vt), and sensitivity to CO₂ and hypoxia.^{24,32} Accordingly, considerable research efforts have been focused on understanding the opioid influence on respiratory control.

The μ - and δ -opioid receptors are found throughout many of the respiration-related regions of the medulla;^{2,4,7,14,16} thus it is conceivable that the opioid effect on breathing is through the depression of neurons at multiple sites within the neural network that controls breathing. One apparent primary site that opioids affect is the pre-Bötzinger complex (preBötzc),^{1,3,5,20,22} an area thought to be critical in generating inspiratory muscle activity.^{9,10} Indeed, opioids cause quantal slowing of respiratory rhythm in a brain stem-spinal cord preparation containing both the preBötzc and the more rostrally located parafacial respiratory group (pFRG),^{3,10,18} which is hypothesized to generate expiratory muscle activity.^{10,11} However, when [d-Ala(2),N-Me-Phe(4),Gly(5)-ol]-enkephalin (DAMGO) is applied to a more isolated preparation that likely lacks a functional pFRG, there is a further pronounced slowing of respiratory rhythm.^{18,22} Thus it is apparent that in an in vitro preparation opioids exert an important influence on the preBötzc, and consequently the resultant respiratory rhythm.

The effect of opioids on preBötzc neurons in vivo is unclear. In one in vivo study on both anesthetized and unanesthetized decerebrate adult cats, intravenous injections of an opioid had no direct opioid-mediated membrane effects on inspiratory or expiratory bulbospinal neurons in the ventral respiratory group; thus the observed slowing of respiratory rhythm does not appear to be related to opioid postsynaptic actions on these types of neurons.¹⁵ Accordingly, it is possible that "other candidate sites of opiate-mediated rhythm slowing, in particular the preBötzc and the rostral pons, are implicated from the pattern of slowing".¹⁵ However, in studies on anesthetized rats and decerebrate dogs, injection of μ -opioids into the preBötzc increased respiratory rate.^{16,21}

We are not aware of any published reports on the effect of DAMGO injected into the preBötzc region of freely behaving, awake

mammals. Therefore, the purpose of the present study was to address this issue in awake adult goats that had been chronically implanted with microtubules within the preBötzC region. We hypothesized that because of the known respiratory depressant effects of opioids, together with the observed effects of the aforementioned in vitro preparations, DAMGO will cause breathing to decrease and become irregular when injected into the presumed preBötzC region of the conscious animal. We further hypothesized that because opioids are known to blunt breathing during hypercapnia and hypoxia,^{24,32} DAMGO injections into the preBötzC region of the conscious animal will cause the breathing responses to be blunted during these conditions as well.

Methods

Data were obtained on four groups (*group 1, n = 7; group 2, n = 3; group 3, n = 1, group 4, n = 7*) of adult female goats weighing 43.3 ± 2.4 kg. Goats in *groups 1–3* were utilized for physiological studies, and those in *group 4* were utilized solely for histology on the medulla. Goats were used for this study because this species has a regular pattern of inspiratory (diaphragm) and expiratory (abdominal oblique) muscle activity during eupnea.^{13,33,34} Goats were housed and studied in an environmental chamber with a fixed ambient temperature and photoperiod. All goats were allowed free access to hay and water, except for periods of study. The goats were trained to stand comfortably in a stanchion during periods of study. All aspects of the study were reviewed and approved by the Medical College of Wisconsin Animal Care Committee before the studies were initiated.

Surgical procedures.

Before all surgery, goats were anesthetized with a combination of ketamine and xylazine (15 mg/kg and 1.25 mg/kg, respectively), intubated, and mechanically ventilated with 1.5% halothane in 100% O₂. All surgeries were performed under sterile conditions. Each goat underwent an initial instrumentation surgery in which electromyogram (EMG) electrodes were implanted into the diaphragm (Dia) and abdominal internal and external oblique (Abd) muscles. The purpose of obtaining Dia and Abd muscle activity (inspiratory and expiratory muscles, respectively) was to determine whether a potential reduction

in Dia activity after the injection of DAMGO into the preBötzC would be compensated by an increase in expiratory activity to result in a partially passive inspiration.⁹ This type of change occurs after attenuation of preBötzC activity via injections of a neurotoxin into the preBötzC.³⁴ A 5-cm segment of each carotid artery was dissected from the vagus nerve and elevated subcutaneously for eventual insertion of a catheter. Ceftiofur sodium (Naxcel; 8 mg/kg im) was administered daily for 1 wk postoperatively to minimize infection.

After at least 2 wk of recovery, goats underwent another surgery for the purpose of bilaterally implanting 70-mm-long (OD = 1.27 mm, ID = 0.84 mm) stainless steel microtubules into the presumed preBötzC area. An occipital craniotomy was created, and the dura mater was excised to expose the posterior cerebellum and the dorsal aspect of the medulla for visualization of obex. In goats of *groups 1* and *2*, the microtubules were placed 2–3 mm rostral to obex, 4–5 mm lateral to the midline, and 4–6 mm from the dorsal surface. In the goat of *group 3*, the microtubules were 1 mm rostral to obex, 6 mm lateral to the midline, and 3 mm from the dorsal surface. The microtubules were chronically fixed with stainless steel screws and dental acrylic.

After brain surgery, the goats were visually monitored continuously by laboratory personnel for at least 6 h, or until they reached stable conditions, after which they were checked hourly for the next 24 h. Buprenorphine hydrochloride (Buprenex; 0.005 mg/kg) was administered for pain. After the brain surgery, the goats were placed on a regimen of medication to minimize infection (chloramphenicol 20 mg/kg iv, 3 times daily for 3 days) and swelling (dexamethasone iv, 3 times daily for 7 days, starting with 0.4 mg/kg and decreasing to 0.05 mg/kg). Everyday thereafter the goats were medicated with ceftiofur sodium (4 mg/kg im) and gentamicin (6 mg/kg im).

Experimental procedures and protocols.

Studies commenced after a minimum 2-wk period of recovery from the brain surgery. Before the present study, *group 1* animals were used to study respiratory chemosensitivity in the preBötzC by way of a microdialysis-induced focal acidosis.¹² For the present study,

all goats were monitored over a 30-min room air control period and arterial blood was sampled. DAMGO was then injected bilaterally into the preBötzC of awake goats over a period of 30 s. On separate days, *group 1* animals had 0.5 μ l of 1 nM DAMGO injected into the preBötzC, as well as 0.5 and 10 μ l of 100 nM DAMGO. *Group 2* animals had 0.5 and 10 μ l of 1 and 10 μ M DAMGO injected into the preBötzC. The volumes injected were chosen to affect either a small or a more expanded area of the preBötzC region. Physiological variables (see below) were monitored for 30 min after the injection during eupneic conditions, and arterial blood was sampled over the last 2 min of the study. The goat's sensitivity to either CO₂ or hypoxia was assessed immediately after the 30-min study period. Previous studies have found effects on physiological function over the time frame of our protocol.^{30,32} On separate days, CO₂ and hypoxia sensitivity were also assessed without prior DAMGO injection. On any single day, there was only one bilateral injection of DAMGO. Hypoxia studies were never completed on consecutive days. No more than two CO₂ studies were completed on a single day.

CO₂ sensitivity was assessed by sequentially increasing inspired CO₂ to 3%, 5%, and 7% at 5-min intervals. Arterial blood was sampled over the last 2 min of each level of inspired CO₂. Hypoxia sensitivity was assessed by decreasing inspired O₂ to 10.8% for 30 min, followed by a 15-min room air recovery. Arterial blood was sampled during *minutes 5* and 25 of hypoxia and over the last 2 min of the recovery period.

Glutamate receptor agonist injections.

A glutamate receptor agonist applied to the preBötzC elicits a unique tachypneic and dysrhythmic breathing response.^{13,17,25,31,34} Accordingly, after completion of the DAMGO injection studies reported here, the irreversible glutamate receptor agonist ibotenic acid (IA) was injected through the microtubules into the tissue of the first three groups of goats. IA was chosen for injection because in addition to providing evidence that the microtubules were correctly placed, the neurotoxic effect of IA also incrementally destroyed the presumed preBötzC. This destruction allowed for the subsequent study of the capability of the respiratory network to provide a normal respiratory

rhythm and pattern after near-total elimination of the preBötzC area.¹³ The experimental design consisted of injection of a series of incremental sequential volumes of IA (50 mM) that started with 0.5 μ l and increased to 10 μ l. Each injection was separated by 1 wk. After a 15-min eupneic control period, a unilateral injection of IA was made into the preBötzC through the microtubule. Physiological variables were monitored for 1 h, and then an identical injection was made into the contralateral microtubule, except that the contralateral injection of 10 μ l was made 1 wk later. The 10- μ l injections were separated by 1 wk because of the severe effects observed in a previous study when 10- μ l injections were made bilaterally on the same day.³⁴

Physiological variables.

During every study, inspiratory flow (V_i) was measured with a pneumotach by attaching a breathing valve to a custom-fitted mask secured to the goat's muzzle. Expired air was collected in a spirometer. The volume of expired air and the concentrations of CO₂ and O₂ in the air were measured, and from these measurements O₂ consumption and CO₂ excretion were computed. Mean arterial blood pressure (MABP) and heart rate (HR) were measured with a Statham transducer connected via tubing to a catheterized carotid artery. The proximal ends of the EMG electrodes were connected via microclips to a Grass recorder for signal filtering at a band pass of 3–500 Hz. The airflow and raw EMG signals were sent for display, digital recording, and analysis to a CODAS computer data acquisition system at a sampling rate of 250 Hz. Arterial blood was sampled in duplicate for blood gas and pH measurements. Rectal temperature was monitored at regular intervals.

Histological studies.

As stated above, after completion of the present study the animals were further studied to determine the effect of neurotoxin (IA)-induced destruction of the preBötzC area.¹³ Accordingly, it was impossible to histologically verify the presence of μ -opioid receptors within the preBötzC of these animals. Therefore, to confirm the presence of μ -opioid receptors within the presumed preBötzC area, an area known to have a high density of neurokinin-1 (NK1)-expressing

neurons,^{13,33} two unoperated control animals were euthanized (Beuthanasia), perfused with physiological buffer solution, and fixed with 4% paraformaldehyde. The medulla was excised and placed in a 4% paraformaldehyde solution for 24–48 h and then placed in a 30% sucrose solution. The medulla was then frozen and serial sectioned (10 or 25 μm) in a transverse plane, and the sections were adhered to chrome alum-coated slides. Immunohistochemistry for μ -opioid receptors was determined by complexing rabbit anti- μ -opioid receptor antibody (primary antibody, 1 μl antibody to 1,000 μl 0.2% Triton X-100 in physiological buffer solution; Chemicon International) with biotinylated anti-mouse antibody (secondary antibody, 1 μl antibody to 60 μl 0.2% Triton X-100 in physiological buffer solution).

Four additional unoperated control goats were euthanized, and the medulla was extracted, transversely sectioned (25 μm), and prepared for immunohistochemistry to determine the profile of NK1 receptor (NK1R)-expressing neurons in the ventral medulla. Rabbit anti-NK1R antibody (primary antibody, 1 μl antibody to 3,000 μl 0.2% Triton X-100 in physiological buffer solution, blocked with 5% normal horse serum and 0.2% Triton X-100 in physiological buffer solution) was complexed with biotinylated anti-mouse antibody (secondary antibody, 1 μl antibody to 60 μl 0.2% Triton X-100 in physiological buffer solution). After the antibody-antigen complexes for both μ -opioids and NK1 were incubated, they were localized by avidin (Vector ABD Elite) and developed with diaminobenzoate.

A seventh unoperated goat was euthanized, and the extracted and preserved medulla was sectioned in the sagittal plane. The sections were Nissl stained for visualization of the rostral-caudal orientation of major respiratory nuclei in goats.

NK1- and μ -opioid-immunoreactive neurons were visualized under a microscope and were counted over the distance 2.0–4.6 mm rostral to obex. Counts were made in the nucleus ambiguus and in an area of 1.5×1.5 mm just ventral to the nucleus ambiguus that we presume to include the preBötzC. Counts were also made in a 1.5×1.5 -mm area ventral to the presumed preBötzC.

Finally, after the physiological studies were completed in goats of *groups 1* and *2* they were euthanized, and then the medulla was

harvested and processed (Nissl stain) to verify the correct placement of the microtubules within the preBötzc area.

Data analysis.

Respiratory airflow and EMG signals were processed and analyzed (Windaq, DATAQ Instruments). Raw EMG data were full-wave rectified and passed through a moving time average (time constant 0.1 s) to obtain an integrated EMG signal. Pulmonary ventilation [inspiratory minute ventilation (V_i); l/min], f (breaths/min), tidal volume (V_t ; l), and Dia and Abd muscle activity (mV) were computed on a breath-by-breath basis and averaged into 5-min bins. All bins were divided by the 15-min control mean for normalization (% of control). Arterial blood was sampled in duplicate, and arterial partial pressure of O_2 (Pa_{O_2} ; Torr), arterial partial pressure of CO_2 (Pa_{CO_2} ; Torr), and pH (pH units) (model 248 Rapid Lab) were averaged. Breath-to-breath variations in breathing parameters were quantified by determining the coefficient of variation (CV) over all breaths at 5-min intervals. The data from all trials per animal were averaged. For *group 1* animals, the mean data were then statistically analyzed by one- or two-way ANOVA for repeated measures to determine significance ($P \leq 0.05$) of the physiological variables and expressed as SE about the mean. Individual significant differences were examined with the Bonferroni post hoc test. No statistical analysis was performed on *group 2* and *3* animals, given the small sample size.

Results

Histology.

Shown in Fig. 1A is a parasagittal section of a control goat's medulla that illustrates the approximate location of the presumed preBötzc relative to the nucleus ambiguus and the facial nucleus. Figure 1B shows a transverse section of a goat's medulla (3 mm rostral to obex) that is stained with the secondary but not the primary antibody (negative control). Shown in Fig. 1, C and D, are transverse sections of a control goat's medulla that illustrate μ -opioid- and NK1-expressing neurons 2.9 and 3.1 mm rostral to obex, respectively.

Fig. 1. Identification of neuronal groups in the medulla of goats. *A*: parasagittal Nissl-stained section ($\times 1$) 4.9 mm lateral to the midline that identifies the nucleus ambiguus (NA), the facial nucleus (FN), and the approximate location of the presumed pre-Bötzinger complex (preBötzC) (marked by box) which is ~ 3.0 mm rostral to obex. D, dorsal; V, ventral; R, rostral; C, caudal. *B*: transverse section ($\times 4$) of a goat 3.0 mm rostral to obex stained with the secondary, but not the primary, antibody (negative control). *C* and *D*: transverse sections ($\times 4$) 2.9 and 3.1 mm rostral to obex immunostained to identify μ -opioid and neurokinin-1 (NK1) receptor-expressing neurons respectively. *Insets* in *B–D*: $\times 40$ images of single control and μ -opioid- and NK1 receptor-expressing neurons, respectively. Dotted circle and square in *B–D* demarcate NA and presumed preBötzC, respectively. L, lateral; M, medial.

As in a previous study,³⁴ we found in four control goats a distinct increase ($P < 0.001$) in the number of NK1-positive neurons in a 1.5×1.5 -mm region just ventral to the nucleus ambiguus and 2.5–3.5 mm rostral to obex (Fig. 2A). We presume the preBötzC is included in this region of the goat medulla. NK1-positive neurons were also present in the nucleus ambiguus (Fig. 2B) and ventral to the presumed preBötzC (Fig. 2C), but there was no distinct rostral-caudal increase in NK1-positive neurons in these areas. In the presumed preBötzC region 2.5–3.5 mm rostral to obex, there was an abundance of μ -opioid-immunoreactive neurons (Fig. 2D), but there was no rostral-caudal change in the μ -opioid profile. There were many immunoreactive μ -opioid neurons in the nucleus ambiguus (Fig. 2E) and ventral to the presumed preBötzC (Fig. 2F).

Fig. 2. Quantization of NK1- and μ -opioid-expressing neurons 2–4.6 mm rostral to obex in the ventral medulla of goats. *A*: no. of NK1-immunoreactive neurons for a 1.5 $\times$ 1.5-mm region just ventral to nucleus ambiguus (NA); high density of positive neurons 2.5–3.5 mm rostral to obex indicates that this region likely includes the preBötzC. *B* and *C*: no. of NK1-positive neurons in NA and ventral to presumed preBötzC, respectively. *D–F*: no. of μ -opioid-positive neurons for presumed preBötzC, NA, and area ventral to preBötzC, respectively. Note that there are both NK1- and μ -opioid-positive neurons in all 3 regions, but only presumed preBötzC shows a rostral-caudal region of increased density of NK1-positive neurons.

Figure 3 shows a hemisection of one goat's medulla 3.2 mm rostral to obex. The microtubule tract is visible 4.45 mm from the midline and 3.35 mm from the ventral surface. For *group 1* and *2* goats, the average ($\pm$ SE) placements were 2.9 ± 0.6 mm rostral to obex, 4.6 ± 0.2 from the midline, and 3.4 ± 0.3 mm from the ventral surface.

Fig. 3. In *group 1* and *2* goats, microtubules were placed just ventral to the dorsal edge of NA into the preBötzC. *Left* hemisection is a Nissl stain of a control goat's medulla 3 mm rostral to obex, and the box outlines presumed preBötzC. *Right*

hemisection is a Nissl stain of a goat with bilaterally implanted microtubules. The tract of the microtubule is indicated by the area devoid of tissue. The extensive tissue disruption ventral to the tip of the microtubule is due primarily to the neurotoxin injected over weeks after completion of the [d-Ala,² N-Me-Phe,⁴ Gly⁵-ol]-enkephalin (DAMGO) studies. This conclusion is based on our laboratory's previous finding (see Figs. 4 and 5 in Ref. 8) in goats in which we followed the same design as in the present study and found that the tissue damage ventral to the microtubule implanted into the raphe nucleus was 5 times greater in goats that had an injection of ibotenic acid (IA) than in goats that did not have an injection of IA.

Glutamate injections confirm correct placement of microtubules.

Confirmation of correct microtubule placement in the goats studied here was provided by the characteristic tachypnea (Figs. 4 and 5B) in the goats when IA was injected into the preBötzC in increasing volumes at weekly intervals after the completion of the DAMGO studies. As the volume of the bilateral injections increased from 0.5 to 5 μ l, the tachypnea was dose dependent, increasing from 25% to 250% above control ($P < 0.05$), but the response to the unilateral 10- μ l injections was less than the response to the 5- μ l injections (Fig. 4). The reduced responses to 10 μ l may have been a result of neuronal destruction caused by the preceding injections. When IA was injected into one goat in which the microtubules were implanted dorsal and lateral to the preBötzC, no tachypnea was elicited (Fig. 4).

Fig. 4. Group ($n = 10$) average representation of the tachypnea induced by IA injections into the preBötzC of awake goats (■). The 0.5-, 1.0-, and 5- μ l injections were bilateral, while the two 10- μ l [left (L) and right (R) sides] injections were

separated by 1 wk. All injections induced a significant ($P < 0.05$) tachypnea. We speculate that the tachypnea is less at the two 10- μ l injections because 1) the 10- μ l injections were unilateral 1 wk apart or 2) the 0.5-, 1.0-, and 5- μ l injections had destroyed a significant number of neurons. □, Data from 1 goat in which microtubules were placed lateral and dorsal to preBötzc. f, Respiratory frequency.

Fig. 5. Inspiratory flow (V_i) and raw diaphragm (Dia) and external oblique abdominal (Abd) muscle activity in an awake goat during control conditions (A and C) and after injection of IA (B) or DAMGO (D and E) into presumed preBötzc. Note in all panels that Dia activity is in phase with V_i and Abd activity occurs in intervals between Dia activity. Also note that IA increased respiratory frequency but DAMGO had no effect on respiratory frequency or pattern.

Physiological measurements during eupneic breathing conditions.

In goats during eupnea in the awake state, the Dia contracts throughout inspiration and the Abd contracts during the expiratory period (Fig. 5, A and C). This muscle activation pattern was not altered by injection of DAMGO into the presumed preBötzc (Fig. 5, D and E, and Fig. 6). In *group 1* goats, there were no significant effects on V_i , Dia and Abd muscle activity (Fig. 6), f , V_t , resting P_{aCO_2} , MABP, or HR during eupneic breathing conditions after the 0.5 μ l, 1 nM ($n = 7$, 8 trials), the 0.5 μ l, 100 nM ($n = 7$, 13 trials), or the 10 μ l, 100 nM ($n = 7$, 11 trials) bilateral DAMGO injections ($P > 0.05$). Similarly in *group 2* goats, there was no consistent change beyond the variation during the control periods in V_i and Dia and Abd muscle activity after injection of 0.5 μ l, 1 μ M (Fig. 7); 10 μ l, 1 μ M; or 10 μ l, 10 μ M DAMGO. In this

group of goats, there also were no changes in any measured physiological variable after the DAMGO injections. In *group 1* and 2 goats, there was no significant ($P > 0.10$) or notable change after the DAMGO injections in the CV for all the measured physiological variables. Finally, when DAMGO was injected into the goat with the microtubules dorsal and lateral to the preBötzC, there was no effect on any measured physiological variable (data not shown).

Fig. 6. Inspiratory minute ventilation (V_i) and Dia and Abd muscle activity before and after bilateral injection of 0.5 μ l or 10 μ l of 100 nM DAMGO into preBötzC of awake goats. Injection time is indicated by vertical line. Data are expressed as % of control (before injections) and are average values for *group 1* goats ($n = 7$). There were no statistically significant effects of the injections on V_i or the activity of either muscle at both volumes.

Fig. 7. Vi and Dia and Abd muscle activity before and after bilateral injection of 0.5 µl, 1 µM DAMGO into preBötzc area of awake goats. Injection time is indicated by vertical line. Data are expressed as % of control (before injections) and are average values for group 2 goats ($n = 3$). Values after injections did not consistently differ from the variation during control conditions.

Physiological measurements during hypoxic breathing conditions.

During hypoxia without prior injections of DAMGO, the hypoxic (10.8% O₂) ventilatory response consisted of a sharp, almost immediate increase in breathing within the first 1–2 min of hypoxia, followed by a ventilatory plateau for 6–8 min, and finally another period of increased ventilation for the remainder of the hypoxic period

(Fig. 8; Ref. 13). After the 10 μ l, 100 nM DAMGO injection in *group 1* goats, the ventilatory response was significantly decreased ($P < 0.05$) from the control hypoxia study during the final 10 min of the hypoxic response (Fig. 8). In other words, the effect of DAMGO was on the third phase of the normal response to hypoxia. Also in *group 2* goats, the hyperpnea at 25 min of hypoxia was less than the control hypoxia after prior injection of 0.5, 1 μ M; 10 μ l, 10 μ M; and 10 μ l, 10 μ M DAMGO (Fig. 9).

Fig. 8. Bilateral 10 μ l, 100 nM DAMGO injection into the preBötzc of awake goats significantly attenuated the ventilatory (V_i) response during hypoxia (10.8% inspired O_2) ($P = 0.001$, $n = 7$, 5 trials). Data (on *group 1* goats) were averaged in 1-min intervals and expressed as % of control. Vertical lines delineate room air control, hypoxia, and room air recovery. Asterisks at *top* indicate time points during hypoxia significantly different ($P < 0.05$) between control and post-DAMGO responses.

Fig. 9. Bilateral injection of 3 different doses of DAMGO decreased the ventilatory (V_i) response to 10.8% O_2 in *group 2* goats. Individual data are presented for the 5th and 25th minutes of hypoxia.

Physiological measurements during hypercapnic breathing conditions.

When inspired CO_2 was increased without prior injection of DAMGO, there was an almost immediate increase in $\dot{V}_i$ due to an increase in V_t at 3% inspired CO_2 , and then an increase in f at 5% and 7% inspired CO_2 . In *group 1* goats, the only significant effect on this response was an attenuation of the tachypnea during 5% and 7% CO_2 after the 0.5 μl , 100 nM DAMGO injection (Fig. 10). In *group 2* goats, there were no consistent effects on CO_2 responses by the DAMGO injections.

Fig. 10. The 0.5 μl , 100 nM injection of DAMGO significantly reduced the respiratory frequency response during 5% and 7% CO_2 ($P = 0.028$, $n = 7$, 7 trials). Data were averaged in 1-min intervals and expressed as % of control. Dashed lines indicate the start of each level of CO_2 . Asterisks denote time points during hypercapnia significantly ($P < 0.05$) different between control and post-DAMGO conditions.

Discussion

Major conclusions.

In contrast to our first hypothesis, a bilateral injection of the μ -opioid receptor agonist DAMGO into the preBötzc of awake adult goats

had no significant effect on respiratory rhythm or pattern during eupneic conditions regardless of volume or concentration. However, consistent with our second hypothesis, DAMGO did alter aspects of breathing during hypoxic and hypercapnic conditions.

Anatomic and functional definition of the preBötzC.

Several studies have attempted to define the boundaries of the preBötzC. Studies in adult rats found a distinct increase in the density of NK1-positive neurons in an area just ventral to the nucleus ambiguus.^{4,6,26,31} Data from other studies suggested that somatostatin-positive neurons in this area defined the boundaries of the preBötzC.^{26,28} To further explore "the necessary and sufficient boundaries for a functional preBötzC," studies were recently completed on neonatal rat medullary slices of different thicknesses.^{22,23} The data indicate that there is a core of rhythmogenic neurons 0.5 mm caudal to the facial nucleus essential for respiratory rhythmogenesis, with neurons caudal and rostral to the core that contribute to the eupneic and sigh rhythms, respectively. Although these data define the preBötzC in a neonatal rat, the exact boundary of the preBötzC, or boundary of the medullary neurons responsible for the eupneic respiratory rhythm, has not been established in adult mammals. However, it is generally accepted that the preBötzC includes an area ventral to the nucleus ambiguus that has a high density of NK1- and somatostatin-expressing neurons.

Previous data from our laboratory,^{13,33} as well as data in Fig. 2 of the present study, substantiate a distinct increase in the density of NK1-positive neurons 2.5–3.5 mm rostral to obex in goats. This area of increased NK1-positive neurons is just ventral to the nucleus ambiguus. Thus we implanted the microtubules 2.5–3.5 mm rostral from obex and then advanced them to the ventral border of the nucleus ambiguus and the dorsal border of the preBötzC (Fig. 3).

Functionally, the preBötzC has been defined by the distinct, unique tachypneic and dysrhythmic response^{13,17,25,31,33} to glutamate receptor agonist injections into the area of an increase in NK1R-positive neurons.^{4,6,13,26,31,33} Indeed, weeks after the completion of the DAMGO injections in the present group of goats, there was prominent tachypnea and dysrhythmia when the glutamate receptor agonist and

neurotoxin IA was injected through the microtubule into the region of high-density NK1R-expressing neurons (Figs. 4 and 5). These findings corroborate a previous study in which a profound tachypnea and dysrhythmia were also observed after injection of IA in the presumed preBötzC of awake goats.³⁴ These functional data, and the above-mentioned histological data, provide evidence of the presumed location of the preBötzC in goats even though the exact boundaries have not been established.

μ-Opioid receptor immunohistochemistry.

It has been well documented that μ-opioid receptors are widely distributed throughout the ventral respiratory group and the preBötzC.^{2,4,7,14,16} As stated above, after the completion of the present study these goats were further used in studies that nearly destroyed the entire presumed preBötzC. Accordingly, we did not immunostain for the presence of μ-opioid receptors in these goats that received DAMGO. Consequently, μ-opioid immunostaining was completed in two unoperated control goats. Positive μ-opioid receptor staining was found in the preBötzC (Fig. 1C and Fig. 2D) and in the nucleus ambiguus (Fig. 2E) and the area ventral to the preBötzC (Fig. 2F).

Neurons exposed to DAMGO in the present study.

Accuracy in calculating the diffusion of substances injected into the brain is dependent on the validity of several assumptions. The validity of the assumption is a major issue, particularly, as pointed out by Thorne et al.²⁹ that “the intercellular spaces between neurons and glia contain an amorphous, negatively charged extracellular matrix (ECM) with the potential to shape and regulate the distribution of many diffusing ions, proteins, and drugs.” This statement emphasizes a limitation of the present study, specifically, that we do not have definitive evidence of the diffusion of DAMGO and thus the neurons affected by DAMGO. However, it is highly likely that the injected DAMGO diffused to affect preBötzC neurons. This conclusion is based on the finding that tachypnea and dysrhythmia occurred when a glutamate receptor agonist was subsequently injected through the microtubule into the tissue (Fig. 4). This unique response is characteristic of activation of preBötzC neurons.^{13,17,25,31,33} It is also

highly likely that preBötzC neurons were not selectively affected by the DAMGO injections. This conclusion is based on the extensive tissue damage (Fig. 3) and deficit in neurons (Table 2 in Ref. 13) beyond the presumed preBötzC as a result of the injection of the neurotoxin days after the DAMGO injections. The finding that the DAMGO injections affected the responses to hypoxia and hypercapnia (Figs. 8–10) clearly indicates that DAMGO diffused to affect neurons that are part of the network controlling breathing. Therefore, we are confident that the doses of DAMGO used in the present study were sufficient to affect preBötzC rhythmogenic neurons, as well as neurons in the region surrounding the preBötzC.

Effect of DAMGO during eupneic breathing conditions.

A bilateral injection of DAMGO into the preBötzC of awake adult goats had no significant effect on respiratory rhythm or pattern during eupneic breathing conditions. In agreement with our data, Montandon and Horner¹⁹ recently showed that microdialysis of 100 μ M fentanyl into the preBötzC of awake adult rats had no effect on respiratory rate. However, when they microdialyzed fentanyl in the anesthetized or non-rapid eye movement (NREM) sleep states respiratory rate was decreased 43% and 30%, respectively, but quantal slowing was not observed. In contrast, Lonergan et al.¹⁶ found that 10 mM endomorphin-1 microinjected into the preBötzC of anesthetized adult rats increased respiratory rate by 16–45%. Similarly, Mustapic et al.²¹ found that 100 μ M DAMGO injected into the preBötzC of adult decerebrate dogs increased respiratory rate by 44%. In both the studies of Montandon and Horner¹⁹ and Mustapic et al.,²¹ intravenous administration of a μ -opioid agonist depressed breathing. None of the in vivo studies on adult mammals has found quantal slowing of respiratory rate when a μ -opioid was administered to the preBötzC. Accordingly, even though there is no consensus among these in vivo studies on adult mammals, it seems unlikely that the preBötzC is the primary site within the brain for the depression of breathing that occurs with intravenous administration of opioids.

The findings summarized above for adult in vivo conditions contrast with the data from several in vitro studies. Indeed, opioids cause quantal slowing of respiratory rhythm in a brain stem-spinal

cord preparation containing the preBötzC and the preinspiratory neurons of the more rostral pFRG^{3,10,18} hypothesized to generate expiratory muscle activity.^{11,12} However, when DAMGO is applied to a more isolated preparation that lacks a functional pFRG, there is a more pronounced slowing of respiratory rhythm.^{18,22} From these data, Mellen et al.¹⁸ concluded "these findings suggest that opioid-induced quantal slowing results from transmission failure of rhythmic drive from pre-I neurons to preBötzC I networks, depressed below threshold for spontaneous rhythmic activity."

We are not aware of a definitive explanation for the differences in the effects of DAMGO in in vivo adult mammals and the in vitro neonatal rat preparation. One possible explanation, particularly in the awake goat, is that the respiratory control network was largely intact (except for neurons destroyed by the implanted microtubules). Thus sources of excitatory inputs to the preBötzC, and the capability for compensation for the presumed depressant effect of DAMGO on preBötzC inspiratory neurons, was greater than in the isolated in vitro preparations. Since studies were not completed in anesthetized goats, we have no direct evidence of greater compensation after DAMGO injections in the awake versus anesthetized state. Certainly, compensation or plasticity can occur within the respiratory network, as indicated by our findings (after the DAMGO studies) of a normal respiratory rhythm and pattern in this same group of goats after near-total, incremental destruction of the presumed preBötzC area.¹³ The capability for compensation may, however, be limited or differ between conditions that could account for our findings in the awake goat that DAMGO attenuated the ventilatory responses during the stressed conditions of hypoxia and hypercapnia. The possibility of greater compensation in the awake state is also suggested by the findings in adult rats that administration of a μ -opioid to the preBötzC slowed respiratory rate in anesthetized and NREM sleep states but not in the awake state.¹⁹ If indeed compensation occurs with depression of the preBötzC, it appears unlikely that compensation occurred through increased contribution of expiratory muscles, because abdominal expiratory muscle activity did not change after the injection of DAMGO (Figs. 6 and 7).

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References

- ¹ Ballanyi K, Lalley PM, Hoch B, Richter DW. cAMP-dependent reversal of opioid- and prostaglandin-mediated depression of the isolated respiratory network in newborn rats. *J Physiol* 504: 127–134, 1997.
- ² Chen Z, Hedner J, Hedner T. Substance P induced respiratory excitation is blunted by delta opioid receptor specific opioids in the rat medulla oblongata. *Acta Physiol Scand* 157: 165–173, 1996.
- ³ Feldman JL, Mitchell GS, Nattie EE. Breathing: rhythmicity, plasticity, chemosensitivity. *Annu Rev Neurosci* 26: 239–266, 2003.
- ⁴ Gray PA, Rekling JC, Bocchiario CM, Feldman JL. Modulation of respiratory frequency by peptidergic input to rhythmogenic neurons in the preBotzinger complex. *Science* 286: 1566–1568, 1999.
- ⁵ Greer JJ, Carter JE, Al-Zubaidy Z. Opioid depression of respiration in neonatal rats. *J Physiol* 485: 845–855, 1995.
- ⁶ Guyenet PG, Sevigny CP, Weston MC, Stornetta RL. Neurokinin-1 receptor-expressing cells of the ventral respiratory group are functionally heterogeneous and predominantly glutamatergic. *J Neurosci* 22: 3806–3816, 2002.
- ⁷ Hassen AH, Feuerstein G, Faden AI. Selective cardiorespiratory effects mediated by mu opioid receptors in the nucleus ambiguus. *Neuropharmacology* 23: 407–415, 1984.
- ⁸ Hodges MR, Opansky C, Qian B, Davis S, Bonis J, Bastasic J, Leekely T, Pan LG, Forster HV. Transient attenuation of CO₂ sensitivity after neurotoxic lesion in the medullary raphe area of awake goats. *J Appl Physiol* 96: 1815–1824, 2004.
- ⁹ Janczewski WA, Onimaru H, Homma I, Feldman JL. Opioid-resistant respiratory pathway from the preinspiratory neurones to abdominal muscles: in vivo and in vitro study in the newborn rat. *J Physiol* 545: 1017–1026, 2002.
- ¹⁰ Janczewski WA, Feldman JL. Distinct rhythm generators for inspiration and expiration in the juvenile rat. *J Physiol* 570: 407–420, 2006.

- ¹¹ Janczewski WA, Feldman JL. Novel data supporting the two respiratory rhythm oscillator hypothesis. Focus on "respiration-related rhythmic activity in the rostral medulla of newborn rats." J Neurophysiol 96: 1–2, 2006.
- ¹² Krause KL, Forster HV, Davis S, Kiner T, Bonis JM, Pan L, Qian B. Focal acidosis in the pre-Bötzinger complex area of awake goats induces a mild tachypnea. J Appl Physiol 106: 241–250, 2009.
- ¹³ Krause KL, Forster HV, Kiner T, Davis S, Bonis JM, Qian B, Pan LG. Normal breathing pattern and arterial blood gases in awake and sleeping goats after near total destruction of the presumed pre-Bötzinger complex and the surrounding region. J Appl Physiol 106: 605–619, 2009.
- ¹⁴ Lalley PM. Mu-opioid receptor agonist effects on medullary respiratory neurons in the cat: evidence for involvement in certain types of ventilatory disturbances. Am J Physiol Regul Integr Comp Physiol 285: R1287–R1304, 2003.
- ¹⁵ Lalley PM. Opiate slowing of feline respiratory rhythm and effects on putative medullary phase-regulating neurons. Am J Physiol Regul Integr Comp Physiol 290: R1387–R1396, 2006.
- ¹⁶ Lonergan T, Goodchild AK, Christie MJ, Pilowsky PM. Mu opioid receptors in rat ventral medulla: effects of endomorphin-1 on phrenic nerve activity. Respir Physiol Neurobiol 138: 165–178, 2003.
- ¹⁷ McCrimmon DR, Monnier A, Hayashi F, Zuperku EJ. Pattern formation and rhythm generation in the ventral respiratory group. Clin Exp Pharmacol Physiol 27: 126–131, 2000.
- ¹⁸ Mellen NM, Janczewski WA, Bocchiaro CM, Feldman JL. Opioid-induced quantal slowing reveals dual networks for respiratory rhythm generation. Neuron 37: 821–826, 2003.
- ¹⁹ Montandon G, Horner RL. Role of the pre-Botzinger complex in opioid-induced respiratory depression in adult rats in vivo (Abstract). FASEB J 23: 960.5, 2009.
- ²⁰ Murakoshi T, Suzue T, Tamai S. A pharmacological study on respiratory rhythm in the isolated brainstem-spinal cord preparation of the newborn rat. Br J Pharmacol 86: 95–104, 1985. [
- ²¹ Mustapic S, Radocaj T, Stucke AG, Stuth EA, Dogas Z, Zuperku EJ. Local microinjection of mu-opioids into the pre-Botzinger complex region produces opposite effects on breathing rate to systemic mu-opioid infusion in decerebrate dogs (Abstract). FASEB J 23: 960.6, 2009.
- ²² Ruangkittisakul A, Scharzacher SW, Secchia L, Poon BY, Ma Y, Funk GD. High sensitivity to neuromodulator-activated signaling pathways at physiological $[K^+]$ of confocally imaged respiratory center neurons in on-line-calibrated newborn rat brainstem slices. J Neurosci 26: 11870–11880, 2006.

- 23 Ruangkittisakul A, Scharzacher SW, Secchia L, Ma Y, Boboccea N, Poon BY, Funk GD, Ballanzi K. Generation of eupnea and sighs by a spatiochemically organized inspiratory network. *J Neurosci* 28: 2447–2458, 2008.
- 24 Santiago TV, Edelman NH. Opioids and breathing. *J Appl Physiol* 59: 1675–1685, 1985.
- 25 Solomon IC, Edelman NH, Neubauer JA. Patterns of phrenic motor output evoked by chemical stimulation of neurons located in the pre-Botzinger complex in vivo. *J Neurophysiol* 81: 1150–1161, 1999.
- 26 Stornetta RL, Rosin DL, Wong H, Sevigny CP, Weston MC, Guyenet PG. A group of glutamatergic expressing high levels of both neurokinin-1 receptors and somatostatin identifies the region of the pre-Bötzinger Complex. *J Comp Neurol* 455: 499–512, 2002.
- 27 Takeda S, Eriksson LI, Yamamoto Y, Joensen H, Onimaru H, Lindahl SG. Opioid action on respiratory neuron activity of the isolated respiratory network in newborn rats. *Anesthesiology* 95: 740–749, 2001.
- 28 Tan W, Janczewski WA, Yang P, Shao XM, Callaway EM, Feldman JL. Silencing preBotzinger complex somatostatin-expressing neurons induces persistent apnea in awake rat. *Nat Neurosci* 11: 538–540, 2008.
- 29 Thorne RG, Lakkaraju A, Rodriguez-Boulan E, Nicholson C. In vivo diffusion of lactoferrin in brain extracellular space is regulated by interactions with heparin sulfate. *Proc Natl Acad Sci USA* 105: 8416–8421, 2008.
- 30 Tseng LF, Collins KA. Different mechanisms mediating tail-flick inhibition induced by β -endorphin, DAMGO and morphine from ROB and GiA in anesthetized rats. *J Pharmacol Exp Ther* 257: 530–538, 1991.
- 31 Wang H, Stornetta RL, Rosin DL, Guyenet PG. Neurokinin-1 receptor-immunoreactive neurons of the ventral respiratory group in the rat. *J Comp Neurol* 434: 128–146, 2001.
- 32 Weil JV, McCullough RE, Kline JS, Sodal I. Diminished ventilatory response to hypoxia and hypercapnia after morphine in normal man. *N Engl J Med* 292: 1103–1106, 1975.
- 33 Wenninger JM, Pan LG, Klum L, Leekley T, Bastastic J, Hodges MR, Feroah T, Davis S, Forster HV. Small reduction of neurokinin-1 receptor-expressing neurons in the pre-Botzinger complex area induces abnormal breathing periods in awake goats. *J Appl Physiol* 97: 1620–1628, 2004.
- 34 Wenninger JM, Pan LG, Klum L, Leekley T, Bastastic J, Hodges MR, Feroah TR, Davis S, Forster HV. Large lesions in the pre-Botzinger complex area eliminate eupneic respiratory rhythm in awake goats. *J Appl Physiol* 97: 1629–1636, 2004.