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

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Krause KL, Forster HV, Kiner T, Davis SE, 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. First published December 18, 2008; doi:10.1152/japplphysiol.90966.2008.—Abrupt neurotoxic destruction of >70% of the pre-Bötzinger complex (preBöC) in awake goats results in respiratory and cardiac failure (Wenninger JM, Pan LG, Klum L, Leekley T, Bastasic J, Hodges MR, Feroth JR, Davis S, Forster HV. J Appl Physiol 97: 1629–1636, 2004). However, in reduced preparations, rhythmic respiratory activity has been found in other areas of the brain stem (Huang Q, St. John WM, J Appl Physiol 64: 1405–1411, 1988; Janczewski WA, Feldman JL. J Physiol 570: 407–420, 2006; Lieske SP, Thoby-Brisson M, Telgkamo P, Ramierz JM. Nature Neurosci 3: 600–607, 2000; St. John WM, Bledsoe TA. J Appl Physiol 59: 684–690, 1985); thus we hypothesized that, when the preBöC is destroyed incrementally over weeks, time-dependent plasticity within the respiratory network will result in a respiratory rhythm capable of maintaining normal blood gases. Microtubules were bilaterally implanted into the presumed preBöC of seven goats. After recovery from surgery, studies were completed to establish baseline values for respiratory parameters. At weekly intervals, increasing volumes (in order 0.5, 1, 5, and 10 μl) of ibotenic acid (IA; 50 mM) were then injected into the preBöC. All IA injections resulted in an acute tachypnea and dysrhythmia featuring augmented breaths, apneas, and increased breath-to-breath variation in breathing. In studies at night, apneas were nearly all central and occurred in the awake state. Breath-to-breath variation in breathing was greater (P < 0.05) during wakefulness than during non-rapid eye movement sleep. However, one week after the final IA injection, the breathing pattern, breath-to-breath variation, and arterial blood gases and pH were unchanged from baseline, but there was a 20% decrease in respiratory frequency (f) and CO2 sensitivity (P < 0.05), as well as a 40% decrease in the ventilatory response to hypoxia (P < 0.001). In subsequent histological analysis of the presumed preBöC region of lesioned goats, it was determined that there was a 90 and 92% reduction from control goats in total and neurokinin-1 receptor neurons, respectively. Therefore, it was concluded that 1) the dyssrhythmic effects on breathing are state dependent; and 2) after incremental, near total destruction of the presumed preBöC region, time-dependent plasticity within the respiratory network provides a rhythm capable of sustaining normal arterial blood gases.

plasticity; respiratory rhythmogenesis

THE SITES(S) WITHIN THE BRAIN STEM and the mechanisms of respiratory rhythm and pattern generation remain controversial. For several decades, one major theory, as proposed by Lumsden in the 1920s (20, 21), has been that respiratory rhythm and pattern generation are mediated by a pontine-medullary circuit. Subsequently, this view has been confirmed and extended by the studies by St. John et al. (41–45), which demonstrated that a presumable respiratory rhythm is sustained in the mylohyoid branch of the trigeminal nerve, after the phrenic rhythm is abolished by chemical lesions in the medulla in decerebrate cats (15, 42). Thus it has been hypothesized that, in isolation, the medulla is only capable of generating the gasping rhythm, and that the rhythm originating in the rostral pons from the trigeminal nerve “may underlie the neurogenesis of eupnea” (42).

Another major theory is that respiratory rhythm and pattern are generated uniquely by pacemaker or a network of rhythmicogenic neurons in the pre-Bötzinger complex (preBöC) in the medulla. A landmark in vitro study supporting this view by Smith et al. (35) found that serial transections of a neonatal rat medulla eliminate respiratory rhythm when the preBöC is abolished. Subsequently, some (19, 23, 48, 49), but not all (40, 52), investigations found that the preBöC is critical for respiratory rhythm in reduced preparations. As a result, these data have led to different opinions concerning the role of the preBöC in the control of breathing.

Recent findings in awake goats (51) and rats (47) appear consistent with the concept of a unique role of the preBöC in the generation of the eupneic breathing rhythm and pattern. For example, Wenninger et al. (51) found in adult, awake goats that abrupt neurotoxin-induced lesions, which destroyed 70% of the preBöC, resulted in a severe disruption of respiratory rhythm and pattern. Ultimately, these goats died of cardiac and respiratory failure or had to be euthanized due to severe hypoventilation, arterial hypoxia, and hypercapnia (51). More recently, Tan et al. (47) used a novel approach to rapidly silence somatostatin-expressing neurons in the preBöC of awake rats, which also “induced a persistent apnea without any respiratory movements to rescue their breathing.” However, it has also been found that ventilatory support was required for ~20 h in order for cats to recover from injections of a neurotoxin into the ventrolateral solitary nucleus (2). In addition, apneas and irregular breathing occurred in awake goats after neurotoxic lesions in the facial, vestibular, and raphe nuclei (7, 14). Accordingly, it is not clear whether the apneas or hypoventilation caused by abrupt lesions within the preBöC establish that the preBöC has a unique role in the generation of eupnea.
To gain insight into the concept that eupnea is generated uniquely by the preBo¨tzC, the present study aimed to destroy the preBo¨tzC in a stepwise, incremental manner over several weeks. The rationale for our hypothesis was based on the evidence that multiple areas within the brain stem appear capable of generating rhythmic respiratory activity (15, 17, 19, 42) and also the evidence that the respiratory network has the capacity for plasticity (2, 6, 10, 27, 40 – 42). Accordingly, we hypothesized that, when the preBo¨tzC was destroyed in a stepwise, incremental manner over several weeks, time-dependent plasticity within the respiratory network would result in a respiratory rhythm and pattern capable of sustaining normal arterial blood gases during both awake and asleep states.

METHODS

Data were obtained on 13 adult female goats, weighing 43.1 ± 2.91 kg. Only female goats were studied. No differences have been found between female and castrated male goats in changes in breathing after lesions within the preBo¨tzC (50, 51). Seven goats were implanted with microtubules (MTs) within the preBo¨tzC, one goat was implanted with a MT outside the preBo¨tzC, and five goats were not implanted with any medullary MTs and were utilized for histological control purposes only. 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 surgery, the goats were anesthetized with a combination of ketamine and xylazine (15 mg/kg and 1.25 mg/kg, respectively) and intubated. During surgery, they were mechanically ventilated with 1.5% halothane in 100% O2. All surgeries were performed under sterile conditions. The initial surgery on eight goats involved the following: 1) implanting electrodes into the diaphragm (DIA), abdominal (ABD), and three different upper airway (UAW) muscles to monitor respiratory muscle activity; 2) implanting electrodes for electroencephalogram (EEG) and electrooculogram (EOG) recordings; and 3) elevating a 5-cm segment of each carotid artery subcutaneously for eventual insertion of a catheter. Cefitifur sodium (Naxcel) (8 mg/kg) was administered daily (intramuscularly) postoperatively to minimize infection.

After at least 2 wk of recovery, seven goats underwent a second surgery to bilaterally implant 70-mm-long (1.27 mm outer diameter, 0.84 mm inner diameter) stainless steel MTs into the preBo¨tzC. In an eighth goat, a MT was implanted into a nonrespiratory medullary area, located near the dorsal surface and lateral to the nucleus of the solitary tract. To implant the MTs, an occipital craniotomy was created, and the dura mater was excised to expose the posterior cerebellum and the dorsal view of the caudal medulla. The MTs were implanted into the preBo¨tzC based on previously delineated coordinates (50) (2–3 mm rostral to obex, 4–5 mm lateral to the midline, and 4–6 mm from the dorsal surface). The MTs were chronically fixed using stainless steel screws and dental acrylic.

The goats were continuously monitored at least 24 h after brain surgery, or until the goat reached stable conditions. Buprenorphine hydrochloride (Buprenex, 0.005 mg/kg) was administered for pain, as needed. Following the brain surgery, the goats were placed on a regimen of medication to minimize infection (chloramphenicol, 20 mg/kg iv, 3 times/day for 3 days) and swelling (dexamethasome, intravenously, 3 times/day for 7 days, starting with 0.4 mg/kg and decreasing 0.05 mg·kg−1·day−1). Every day thereafter until death, the goats were medicated with cefitifur sodium and gentamycin (Gentamax 100; 6 mg/kg im, once per day).

Previous studies have demonstrated that injections of the neurotoxin ibotenic acid (IA) into the preBo¨tzC area can cause obstructive apnea (51), most presumably due to damage of nucleus ambiguus (NA), a nucleus known to contain motoneurons of the UAW muscles. Thus, in the present study, when the goats exhibited signs of UAW obstruction, a third surgery was performed to create a tracheostomy (n = 6). In five goats, the tracheostomy was performed 5 days after the injection of 5 μl of IA into the preBo¨tzC. In the sixth goat, the tracheostomy was performed 2 wk after the implantation of the MT into the preBo¨tzC area. A 5-cm midline incision was made longitudinally caudal to the cricoid cartilage, and then three to four cartilage rings were cut at the midline. The skin was sutured to the cartilage rings to prevent closure. After the tracheostomy, the goats were aerosolized everyday with 10% gentamycin in sterile water to help prevent infection.

Experimental procedures and protocol. Studies commenced after a minimum 2-wk recovery period from the brain surgery. Before the present study, this same group of animals was used to study respiratory chemosensitivity in the preBo¨tzC utilizing a microdialysis-injected acidosis, as well as to study the effects of μ-opioid receptor agonist microinjections in the preBo¨tzC. For the present study before any IA injections were made, the goats were initially studied for several days to establish normal eupneic breathing rhythm and pattern while awake, as well as to assess ventilatory sensitivity to CO2 and hypoxia. Then, to determine the goats’ normal physiological variation of the ventilatory variables (see Physiologic variables below), seven goats were monitored during room air breathing conditions for 5 h during the day, and six of the seven were also monitored for 5 h at night. Thereafter, the design for all goats consisted of several injections of IA (50 mM) to destroy the preBo¨tzC area in a stepwise manner. The IA injections were made at weekly intervals, starting with 0.5 μl, and progressing to 1.0, 5.0, and 10 μl. After a 15-min control period, a unilateral injection of IA was made into the preBo¨tzC through the MT during eupneic breathing conditions. Physiological variables were monitored for 1 h, and then an identical 0.5 μl, 1-μl, or 5-μl injection was made into the contralateral MT. However, due to previous findings that a bilateral injection of 10 μl of IA caused cardiac and respiratory failure (51), the injection of 10 μl into the left and right preBo¨tzC was separated by 1 wk. Blood gases were sampled every 0.5 h. This exact schedule was also followed in the goat that had a MT implanted in the medulla outside of the preBo¨tzC. Throughout these studies during the day, the goats were loosely tied in a stanchion so that they could not resume sternal recumbency, which is the normal sleep posture for goats.

After each 5-h IA study outlined above, the goats were permitted to eat and drink freely for 5 h. However, in an attempt to consolidate sleep, the goats were not allowed to assume sternal recumbency. Then, to determine whether IA had any effects on physiological variables during non-rapid eye movement (NREM) or rapid eye movement (REM) sleep, the goats (6 of 7) were allowed to assume sternal recumbancy, and, 10 h after the IA injections, they were monitored for another 5 h. This time frame corresponded to the normal sleep period for goats (9 PM to 2 AM). In addition, the goats were also studied during the same nighttime hours 5 days after each IA injection (1–2 days before the next larger volume of IA).

On several days between each injection, all physiological variables were monitored during eupneic, hypercapnic, and/or hypoxic breathing conditions. After a 15-min eupneic control period, CO2 sensitivity was assessed by sequentially increasing inspired CO2 to 3, 5, and 7% for 5-min intervals. Arterial blood was sampled over the last 2 min of each level of CO2. Hypoxia sensitivity was assessed after a 15-min control period by decreasing inspired O2 to 10.8% for 30 min, followed by a 15-min room air recovery. Arterial blood was sampled during minutes 6 and 26 of hypoxia and over the last 2 min of the recovery period.

Physiological variables. During all studies, inspiratory flow (Vt) was measured with a pneumotach by attaching a breathing valve to
either a custom-fitted mask secured to the goat’s muzzle (airway-intact goats), or an endotracheal tube (tracheostomized goats). During daytime studies, expired air was collected in a spirometer, allowing for determination of expired volume and the concentration of CO₂ and O₂ in expired air needed for metabolic rate calculations. VT, blood pressure (mean arterial blood pressure), heart rate, EMG activity, and during nighttime studies, EEG and EOOG were continuously recorded on both a Grass recorder and Codas software. Arterial blood was sampled in duplicate for blood-gas and pH measurements. Rectal temperature was monitored at regular intervals.

**Histological studies.** After completion of the experimental protocol, all goats, as well as the histological control goats, were euthanized (Beuthanasia), and the brains were perfused with physiological buffer solution and fixed with 4% paraformaldehyde. The medullas were excised and placed in a 4% paraformaldehyde solution for 24–48 h and then placed in a 30% sucrose solution. The medullas were frozen and then serial sectioned (25 µm) in the transverse plane (n = 12) or the sagittal plane (n = 1), and the sections were adhered to chrome alum-coated slides. For the transverse sections, one series was stained with hematoxylin and eosin for identification of living and dead neurons. Dead neurons have a strikingly different morphology (round) and color (pale pink) from live neurons. A second series in six goats (5 transverse and 1 sagittal) was used for Nissl staining to further document the total number of living neurons. A third series was used for neurokinin-1 (NK1) receptor immunoreactivity by complexing anti-NK1 receptor (primary antibody) with biotinylated anti-mouse (secondary antibody). After the antibody-antigen complex was incubated, it was localized by avidin (Vector ABC Elite) and developed (secondary antibody). The antibody-antigen complex was incubated, it was localized by avidin (Vector ABC Elite) and developed with diaminobenzene. Neurons were counted at a distance from 2.0–4.6 mm rostral to obex. Living and dead neurons and NK1-positive neurons were counted in NA and the presumed preBötC region (1.5 × 1.5 mm area just ventral to NA). Finally, neurons were also counted in a 1.5 × 1.5 mm region ventral and lateral to the presumed preBötC.

**Data analysis.** Pulmonary ventilation (VT; l/min), f (breaths/min), tidal volume (VT; liters), expiratory (Te; s) and inspiratory times (Ti; s), respiratory muscle activity (mV), and, in addition for night studies, the EEG and EOOG activity were analyzed on a breath-by-breath basis. The EEG and EOOG activity was utilized to visually distinguish between states of consciousness. Wakefulness was defined as a low-voltage, high-frequency EEG, with absent EOOG activity. NREM sleep was characterized by a low-frequency (<2 Hz), high-voltage EEG (2–3 times greater than that found in wakefulness), with absent EOOG. REM sleep was defined as having a low-voltage, high-frequency EEG, similar to wakefulness, but concurrent with frequent and distinct bursting activity (>30 µV) from the EOOG.

For the day studies, the breath-by-breath values were averaged into 5-min bins. All bins were divided by the average of the bins in the control period, and then the responses to each condition (CO₂, hypoxia and post-IA injection) were expressed and statistically analyzed on a percentage of control basis. The breath-to-breath coefficient of variation (CV) (SD/mean) was calculated for each 5-min bin. For each 5-h study, the number of augmented breaths (VT 2.5 times greater than average VT) and apneas (Te ≥ 2.5 times greater than average Te) were tabulated. Either a two-way ANOVA for repeated measures followed by the Bonferroni post hoc test, or a paired t-test was utilized to establish statistically significant (P ≤ 0.05) changes in measured variables.

For the night studies, each breath was categorized as awake, NREM sleep, or REM sleep. The breathed were then further categorized as “quiet,” regular breaths, or those that were “irregular/disturbed,” characterized by any respiratory muscle activity that was inappropriate for normal, eupneic breathing. These disruptions included swallowing, coughing, mastication, and eructation, as well as any abnormal patterns that could not be identified as a specific behavior. The rationale for this second categorization was as stated by Bolser et al. (4): “the presence of elements that are normally silent in close proximity to spontaneously active respiratory neurons raises the possibility that some interventions that are intended to study the neurogenesis of breathing might instead be influencing components of the holarchical system that are only conditionally active” (2); thus it was essential to make a delineation between the two types of breaths (quiet vs. disrupted). For each of the multiple categories of breaths, there were a limited number that were continuous; thus, rather than bin the data at specific time intervals as in the day studies, the data were binned and averaged for each 100 sequential breaths for each category. The CV of the breath-by-breath data was thus calculated on a 100-breath basis. Augmented breaths and apneas were tabulated as in the daytime studies. Statistically significant changes were established as outlined above.

**RESULTS**

**Histological verification of the preBötC.** As shown in Fig. 1, A–C, ventral to NA and 4–5 mm lateral to the midline of the medulla in goats, there are numerous NK1-positive neurons. In agreement with previous data (50), we presently 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 NA, 2.5 and 3.5 mm rostral to obex (Fig. 2A). We presume this region represents the preBötC for this species. Between 2.5 and 3.5 mm rostral to obex, there was no distinct increase (P > 0.10) in NK1-positive neurons in the regions lateral (Fig. 2B) or ventral to the presumed preBötC (Fig. 2C).

**Tachypnea and dysrhythmia during first 5 h after the injections of IA.** There was a range of responses among the goats in the magnitude and duration of the tachypnea after each injection of IA. Nevertheless, regardless of the volume of IA injected, f was significantly increased (P < 0.05, n = 7) after each injection (Fig. 3). Frequency increased in a dose-dependent manner after the 0.5-, 1-, and 5-µl bilateral injections, increasing maximally by 26 (P = 0.002), 48 (P < 0.001), and 159% (P < 0.001) above control, respectively. In each case, f increased significantly after the contralateral (second) injection was made and remained elevated for the entire duration of the 5-h study. Breathing f was also significantly increased after the 10-µl unilateral injections on the left (120%) and right sides (72%), but the maximal tachypnea was less severe than after the 5-µl bilateral injections, nor was the tachypnea sustained over the 5 h after the injections. This attenuated tachypnea may indicate the preceding IA injections had already destroyed the majority of the rhythmogenic neurons in the presumed preBötC region. There was no time-dependent increase in f during a 5-h control study on each of the seven goats (Fig. 3, P > 0.05), and, in a control goat, f did not change when IA was injected unilaterally into a medullary area dorsal and lateral to the preBötC (Fig. 3).

Some, but not all, other measured physiological variables were also affected after the IA injections. VT was not significantly elevated after the 0.5 µl (P = 0.235) and 1-µl (P = 0.998) injections, but was increased within 5–40 min after the 5-µl (P = 0.002), 10-µl left, and 10-µl right injections (P < 0.001). After the 1-, 5-, and 10-µl right injections, VT was significantly decreased by 24, 41, and 23% below control (P < 0.001), respectively, but was unaffected after the 0.5 µl (P = 0.532) or the 10-µl left injection (P = 0.678). Ti was also significantly decreased by 25–50% after every injection except the 0.5 µl (P < 0.05), as was Te (P < 0.05) except after the 0.5 µl and 1-µl volumes. Mean arterial blood pressure was signif-
Significantly increased only after the 1-μl (P = 0.003) and 5-μl injections (P < 0.001). There were no significant changes in heart rate, O₂ consumption, pH, arterial PCO₂ (PaCO₂), or arterial PO₂ (PaO₂) (P > 0.05) during the 5 h after any injection of IA.

As illustrated for one goat in Fig. 4, A, C, and E, before each injection, DIA contractions were in phase with VI, and ABD contractions were out of phase with VI. However, during the 5 h after the IA injections, there were periods of irregular/disrupted breathing that were not identical after each injection. The 500-nl bilateral injection caused the least severe effects; the DIA continued to contract appropriately in phase with VI, but there were periods in which there was strong tonic ABD activity throughout expiration and inspiration (Fig. 4B). After the 1-μl bilateral injection, both the tachypnea and disruptions became more severe, including periods of prolonged Tₑ, as well as large Vᵣ breaths that occurred with the simultaneous contraction of the DIA, ABD, and UAW muscles (Fig. 4D). The final 10-μl unilateral injection caused unusual markedly augmented breaths separated by large, gasp-like breaths that occurred when the DIA, ABD, and UAW muscles contracted simultaneously (Fig. 4F). We emphasize that, as shown in Fig. 4, C and E, there was a regular eupneic breathing pattern before each injection, indicating that the goat had recovered from the irregular breathing during the initial 5 h after the injection of IA 1 wk earlier.

These qualitative examples of the dysrhythmia were substantiated by the significant increase in the breath-to-breath CV of Tₑ (P = 0.06, data not shown), and by the increased number of augmented breaths (P < 0.01, Fig. 5A) and apneas (P = 0.06, Fig. 5B). In all goats after the first 10-μl injection of IA, and in six of seven goats after the 5-μl injection of IA, the number of augmented breaths and apneas was greater than during the 5-h control study. It should be noted that the apneas were primarily central (vs. obstructive), and the number increased as the volume of injected IA increased; there were 33 ± 6.3, 50 ± 9.8, and 65 ± 10.2% more central apneas after the 1-, 5-, and 10-μl injections, respectively, than during the control study.

Physiological functions 9–14 h after IA injections (normal sleep period). During the studies at night before any injections were made, the goats were awake 66% of the time, which was significantly (P < 0.05) greater than the 33% of time in NREM sleep and 1% of time in REM sleep. There was no significant
Ten to fifteen hours after the injections of IA, there were periods of quiet, regular breathing (Fig. 7) interspersed with periods of disrupted/irregular breathing patterns. For example, in one goat after the first 10-μl IA injection, the regular pattern (Fig. 7, A and B) was interspersed with periods when VT increased to ~2.5 greater than normal, which was coincident with a strong, simultaneous contraction of the ABD and UAW muscle and tonic DIA activity (Fig. 7C). Often, these large breaths during wakefulness (Fig. 7D) were followed by prolonged apneas, which were as long as 20 s in duration. During the 5 h of this night study, there were a total of 57 apneas, with 56 during wakefulness and 1 during NREM, and all but 1 was central in nature. In contrast, in this animal’s preinjection control study, no central apneas were observed, and five nights after the final injection, there were only six central apneas awake and two central apneas during sleep.

In a second goat after a 10-μl injection, there were periods of a gasp-like breathing pattern during wakefulness, characterized by a sharp inspiratory rise time and steep decrementing slope (Fig. 7, E and F). These breaths were immediately followed by a central apnea that lasted up to 20 s. During this 5-h night study, there were a total of 51 apneas, all of which were central, and all occurred during wakefulness. These apneas contrast with the absence of apneas in this goat’s control study. There were only 20 central apneas while awake and 3 during NREM sleep 5 days after the final IA injection.

On every night of sleep studies, a prominent finding was that the number of apneas and augmented breaths were greater ($P < 0.05$) during wakefulness than during NREM sleep (Fig. 8). A second prominent finding was that, as shown in Figs. 7, E and F, the apneas were primarily central at the higher volumes of IA injections, whereas, in contrast on the control night, a majority of the apneas were obstructive. The volume of IA that elicited the greatest number of apneas and augmented breaths was not consistent among the goats. Note that 5 days after the final IA injection, the number of apneas in both the awake and NREM sleep states had returned to control levels. The finding change in this sleep pattern with any injection of IA. Because REM sleep was not observed in all goats, or after all studies, breathing variables during REM sleep will not be reported herein. In contrast to the first 5 h after the IA injections, the goats were not tachypneic 10–15 h after the injections. During quiet, regular breathing on each night, $V_t$ (Fig. 6), $f$, and $V_T$ were significantly reduced during NREM sleep compared with the awake state by an average of 12.5 ± 0.06, 7.2 ± 0.10, and 5.8 ± 0.001%, respectively. The $T_i$ and $T_e$ were 4.8 ± 0.01 and 6.8 ± 0.02% greater ($P < 0.05$) during NREM sleep than while awake, respectively. $V_t$, $f$, $V_r$, and $T_i$ during wakefulness and NREM sleep did not change significantly ($P > 0.10$) over the course of the 11 studies at night, and the differences between the awake and NREM sleep states were not altered by the IA injections. In agreement with previous findings (22), arterial blood gases did not differ between awake and NREM sleep states on the control night, and there were no changes as the volume of IA injected was increased (Table 1).
that the disruptions in breathing pattern were primarily during the awake state was further substantiated quantitatively by the finding that the breath-to-breath CV for $V_t$, $T_i$, and $V_t$ (Fig. 9) were $32 \pm 0.8 (P = 0.02), 26 \pm 0.3 (P = 0.01)$, and $29 \pm 0.9\% (P = 0.03)$ greater, respectively, during quiet, regular wakefulness compared with quiet NREM sleep. There was no statistically significant change in the CVs ($P > 0.05$) over the several studies at night, but there was a trend of increased variability after each of the 10-μl injections, which then recovered to at or below preinjection control values 5 days after the injections (Fig. 9). A final indication of the state-dependent variability was that, during NREM sleep, 87% of the

Fig. 4. Representation of disrupted breathing and respiratory muscle patterns after IA injections into the preBo¨tz of 1 awake goat. A, C, and E: tracings taken from the control period before the injection of 0.5 μl (B), 1.0 μl (D), and 10 μl (F) of IA. Control panels A, C, and E show that the diaphragm (DIA) contracted in phase with inspiratory flow ($V_t$) and a smaller abdominal (ABD) muscle contraction occurred out-of-phase with $V_t$, which is the normal pattern for goats. Note in B, D, and F the increased $f$ after IA injections. B also shows irregular breathing after 0.5 μl IA injection with prominent ABD activity throughout inspiration and expiration. D and F both show irregular breathing and simultaneous contraction of the DIA and ABD muscle activity. Note in F the gasp-like $V_t$ followed by prolonged apneas. It is noteworthy that, as shown in C and E, there was a regular breathing and muscle activation pattern before each injection, indicating that the goat had fully recovered from the irregular patterns observed 1 wk earlier after the previous IA injection. BP, blood pressure; UAW, upper airway.
breathe quietly, regular breaths, whereas, in the awake state, only 64% were quiet, regular breaths.

*Chronic changes after the IA injections.* The day immediately following each IA injection study, all measured respiratory variables were unchanged from the baseline values measured the previous day before the injections of IA. Moreover, 1 wk after the first 10-μl injection, during awake and NREM sleep, Vt, Vt, Tt, arterial pH, PaO2, PaCO2 (Table 1, Fig. 10), and the CV of all respiratory variables were unchanged from the control studies before the initial injection of IA (P > 0.05). The f, however, was significantly decreased, and Tt was significantly increased (P < 0.03) (Table 1). Compared with studies before the lesions were created, CO2 sensitivity (change in minute ventilation/change in PaCO2) was significantly decreased from 2.025 ± 0.12 to 1.566 ± 0.13 (P < 0.05) (Fig. 10), and the peak ventilatory response to hypoxia was decreased from 244 ± 28 to 204 ± 60% of the prehypoxia control values (P < 0.001) (Fig. 11) 1 wk after the final injection. In the goat with MTs implanted outside the preBötzC area, CO2 and hypoxic sensitivity were not altered after the same schedule of IA injections.

Lesion size and medullary damage. As shown in Fig. 12, the tissue damage caused by the MT and the IA extended over the range of 2.5–3.5 mm rostral to obex. We quantitated the extent of the lesion in the region of the implanted MT by counting, at 50-μm intervals, the number of living neurons identified by the Nissl or the hematoxylin and eosin stains. There was excellent correlation between the counts of the two stains. For the presumed preBötzC region, NA, and the region ventral to the presumed preBötzC, the hematoxylin and eosin vs. Nissl counts for living neurons in the lesioned goats was 51 ± 19 vs. 46 ± 19, 12 ± 4 vs. 13 ± 7, and 89 ± 41 vs. 82 ± 40, respectively. We found a range of only 0–16 dead neurons in the presumed preBötzC region of the seven lesioned goats, and believe the small number of dead neurons reflect the scavenging of dead neurons that occurred over the month following the first IA injection.

Due to small differences in goat medullary morphology, not all of the MTs in goats were placed exactly in the same region, giving rise to the differences in living neurons remaining at the completion of the study. For example, in seven goats, the total number of living neurons remaining within the presumed preBötzC region ranged from 2 to 116 (Table 2). Similarly, in the region 0.4 mm caudal and 1.0 mm rostral to the presumed preBötzC region, the total number of living neurons ranged from 1 to 103 (Table 2). However, on average, for the preBötzC region, and the regions caudal and rostral to the preBötzC, there were only 10, 11, and 18% of the total living neurons remaining, compared with the control, unlesioned goats.

There was also variation between the goats in the number of living neurons in NA and the region ventral to the presumed preBötzC. For example, in one goat only 7 living neurons remained in NA between 2.5 and 3.5 mm rostral to obex, while the group average for living neurons was 14 (Table 2), or 22% of normal. Similarly, for the region ventral to the presumed preBötzC, the range of living neurons was 7–243 (Table 2), while the group average was 83 neurons, or 24% of normal.

For the seven goats, there was a range of 0–84 NK1-positive neurons remaining within the presumed preBötzC (Table 3). In four of the goats, there were less than 10 NK1-positive neurons remaining in the region (Table 3). On average, for the preBötzC, and the regions caudal and rostral to the preBötzC, there were only 8, 11, and 12% of the total NK1-positive remaining, compared with the control, unlesioned animals.

**DISCUSSION**

Major conclusions. The first prominent finding of this study is that all goats had a normal eupneic breathing pattern and normal arterial blood gases during both awake and NREM sleep states after an average >90% destruction of total neurons, and 92% destruction of NK1-positive neurons within the presumed preBötzC region. These findings are in marked contrast to previous findings of Wenninger et al. (51), who found that, after bilateral destruction of approximately only 70% of the preBötzC, three of three goats with intact airways died, and four of four tracheostomized goats had to be euthanized due to severe hypoventilation. The second prominent finding is that the acute disruptions of the normal respiratory rhythm and pattern from the IA injections are greater during the awake
state than during NREM sleep; however, in both states, a normal eupneic rhythm and pattern returned over days after the IA injections.

Anatomical and functional definition of the preBötzC. Several studies have attempted to define the boundaries of the preBötzC (12, 13, 32, 33, 46, 48). It has been shown in adult rats that there is a distinct increase in NK1-positive neurons within the ventral respiratory group (VRG), in a region just ventral to NA (12, 13, 46, 48). Subsequently, it was found that somatostatin-positive neurons in the region of the VRG supposedly define the boundaries of the preBötzC (46, 47), while others have found that, in neonatal rats, there is a core of rhythmogenic neurons 0.5 mm caudal to the facial nucleus essential for respiratory rhythmogenesis (32, 33). From these studies, the general consensus is that the exact boundary of the preBötzC or boundary of the medullary neurons responsible for the eupneic respiratory rhythm in adult mammals is not precisely established, nor is there a single anatomic marker that accurately defines the preBötzC. However, it has been generally accepted that the preBötzC includes a region within the VRG that has a high density of NK1- and somatostatin-expressing neurons.

Previously published data (50) and data in Fig. 2 of the present study substantiate that, in goats, there is a distinct increase in NK1-positive neurons between 2.5 and 3.5 mm rostral to obex, which, as in rat, is just ventral to NA. Accordingly, the MTs were implanted from the dorsal medullary surface to the ventral border of NA and the dorsal border of the presumed preBötzC between 2.5 and 3.5 mm rostral to obex (Fig. 12).

Several laboratories found that microinjection of a glutamate receptor agonist along the rostral-to-caudal direction of the VRG in anesthetized rats or cats elicits a tachypneic and dysrhythmic phrenic nerve response only when injected into the preBötzC (23, 37, 49). This laboratory has also previously reported (51) and currently corroborated, that injections into the preBötzC region of the neurotoxin IA (a glutamate receptor agonist that binds irreversibly and preferentially to N-methyl-D-aspartate receptors; Ref. 26), induces a significant tachypnea (Fig. 3) and dysrhythmia (Figs. 4 and 7). Injection of IA into a nonrespiratory medullary area does not induce these changes; thus these findings support the conclusion that the MTs were placed correctly within the preBötzC.

Acute apneahypoventilation and irregular respiratory rhythm in awake and sleeping goats after injection of IA into the preBötzC. This laboratory has previously demonstrated that phasic DIA activity is completely abolished within 2 h after a bilateral 10-μl injection of IA into the preBötzC of adult awake goats, and a profound hypoventilation, generated by phasic expiratory muscle activity, results in severe arterial hypercapnia and hypoxemia (51). These goats either died or had to be euthanized, as there was no evidence of recovery within 5 h after the injections. Histological analysis indicated that 70% of neurons in the presumed preBötzC region were destroyed in these goats. More recently, Tan et al. (47) showed that abrupt silencing of

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Table 1. Average values for arterial blood gases and pH and breathing variables before any (pre-IA) and 1 wk after all IA injections (post-IA)

<table>
<thead>
<tr>
<th></th>
<th>(P_{\text{ACO}_2}), Torr</th>
<th>(P_{\text{AO}_2}), Torr</th>
<th>(\text{pH})</th>
<th>(V_i), l/s</th>
<th>(f), breaths/min</th>
<th>(V_t), liters</th>
<th>(T_i), s</th>
<th>(T_e), s</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Daytime</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-IA</td>
<td>41.0±0.5</td>
<td>100.8±2.9</td>
<td>7.430±0.01</td>
<td>6.83±0.55</td>
<td>22.8±2.4</td>
<td>0.338±0.03</td>
<td>1.15±0.14</td>
<td>1.96±0.27</td>
</tr>
<tr>
<td>Post-IA</td>
<td>40.6±0.6</td>
<td>97.9±2.5</td>
<td>7.444±0.01</td>
<td>5.1±0.58</td>
<td>18.1±1.84*</td>
<td>0.308±0.02</td>
<td>1.36±0.17</td>
<td>2.46±0.23*</td>
</tr>
<tr>
<td><strong>Nighttime</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-IA awake</td>
<td>41.6±0.4</td>
<td>97.6±1.8</td>
<td>7.424±0.006</td>
<td>7.25±1.63</td>
<td>20.4±2.1</td>
<td>0.420±0.14</td>
<td>1.38±0.28</td>
<td>2.00±0.24</td>
</tr>
<tr>
<td>Post-IA awake</td>
<td>41.1±0.9</td>
<td>95.7±6.9</td>
<td>7.411±0.015</td>
<td>5.30±0.91</td>
<td>16.6±1.5</td>
<td>0.323±0.02</td>
<td>1.31±0.07</td>
<td>2.50±0.32</td>
</tr>
<tr>
<td>Pre-IA NREM</td>
<td>40.6±0.7</td>
<td>98.0±2.8</td>
<td>7.425±0.004</td>
<td>5.08±0.76</td>
<td>19.9±2.3</td>
<td>0.271±0.04</td>
<td>1.13±0.07</td>
<td>2.07±0.32</td>
</tr>
<tr>
<td>Post-IA NREM</td>
<td>39.3±1.2</td>
<td>101.0±5.1</td>
<td>7.422±0.003</td>
<td>4.75±0.84</td>
<td>15.8±1.64</td>
<td>0.304±0.03</td>
<td>1.36±0.08</td>
<td>2.59±0.28</td>
</tr>
</tbody>
</table>

Values are average ± SE. IA, ibotenic acid; NREM, non-rapid eye movement; \(P_{\text{ACO}_2}\), arterial PCO2; \(P_{\text{AO}_2}\), arterial PO2; pH, arterial pH; \(V_i\), inspired pulmonary ventilation; \(f\), breathing frequency; \(V_t\), tidal volume; \(T_i\) and \(T_e\), inspiratory and expiratory time, respectively. One week after the final injection, \(f\) was significantly decreased \((P < 0.05)\) and \(T_i\) was significantly increased \((*P < 0.05)\), but all other values were not changed from the pre-IA values.
Fig. 7. There were prolonged central apneas primarily during the awake state in goats 10–15 h after injections of IA into the preBo¨tzC. A–D: recordings from one goat; E and F: recordings from a second goat. All recordings are for periods 10–15 h after injections of 10 μl of IA. A and B: regular breathing during awake and NREM sleep states, respectively. C and D: gasp-like breaths coincident with strong contraction of the ABD and an UAW muscle. Also note the prolonged central apnea in D. Similarly, in another goat, there are also gasp-like breaths followed by prolonged central apneas (E and F). EEG, electroencephalogram; EOG, electrooculogram.
somatostatin-expressing neurons in the preBötC of rats also "induced a persistent apnea without any respiratory movements to rescue their breathing."

The present study has shown that, after a series of smaller volume injections of IA, the 10-μl IA injection into the preBötC also resulted in periods of abnormal breathing patterns and apneas over the first 5 h after the injections (Fig. 4) and also when the goats were monitored 10–15 h after the injections (Fig. 7). However, unlike the earlier study (51), the present study did not find any profound and prolonged hypoventilation and severe arterial hypercapnia and hypoxemia. Most striking was that none of the goats died or had to be euthanized within 5 h after the injections. The difference in responses to 10-μl injections in the previous and present study likely reflects that the lower volume IA injections in the present study initiated plasticity within the respiratory control network, which was critical to the goat’s survival after the 10 μl injection. Twenty-four hours to 1 wk after the IA injections, the goats were healthy, with normal arterial pH and blood gases, normal rhythmic DIA and ABD muscle activity, and a normal eupneic breathing pattern during both awake (Fig. 4, C and E) and NREM states. Furthermore, 1 wk after the completion of the entire IA injection series, the only significant changes from the pre-IA injections were a decreased f and increased TE. This recovery occurred, despite an average of only 45 ± 15.3 living neurons remaining in the presumed preBötC region, or 10% of the normal (Table 2), whereas 30% of normal remained in the earlier study. Moreover, nearly 80% of the neurons in NA 2.5–3.5 mm rostral to obex were also destroyed (Table 2). Finally, in the region ventral to the presumed preBötC, there were, on average, only 83 living neurons, or 25% of normal (Table 2).

The greater variability of breathing during wakefulness than NREM sleep is consistent with the previous findings that 7–14 days after injection of saporin conjugated to substance P (SAP-SP) into the preBötC of goats resulted in abnormal breathing only during wakefulness and not sleep (50). It is noteworthy that the greater variability while awake was not due to more frequent disturbances of breathing by nonrespiratory behaviors, as these were excluded from the regular breathing.

The greater variability of breathing during wakefulness than NREM sleep is consistent with the previous findings that 7–14 days after injection of saporin conjugated to substance P (SAP-SP) into the preBötC of goats resulted in abnormal breathing only during wakefulness and not sleep (50). It is noteworthy that the greater variability while awake was not due to more frequent disturbances of breathing by nonrespiratory behaviors, as these were excluded from the regular breathing.
category. Equally noteworthy is that nearly all of the apneas occurred in the awake state, and a large majority of the apneas were central, indicating dysfunction of rhythm-generating mechanisms. The difference in apnea frequency between wakefulness and NREM sleep cannot be due to a relatively less excitatory input to the rhythm generator, such as serotonergic, adrenergic, and cholinergic inputs, because these decrease during NREM sleep compared with wakefulness (1). Thus it seems that findings of this present study reflect the possibility that preBo¨tzC has a state-dependent role in respiratory rhythmogenesis, or as has been postulated by others (34, 36), the site and/or mechanism of rhythm generation is state dependent.

Implications of rhythmogenic plasticity within the respiratory network. The normal breathing after the gradual, stepwise destruction of the preBo¨tzC in contrast to the sustained apnea after abrupt destruction of the preBo¨tzC indicates that time-dependent plasticity occurs within the respiratory network after gradual destruction of this region. It seems unlikely that this plasticity occurred solely within the presumed preBo¨tzC region, because, in four goats, only 2, 4, 22, and 26 living neurons were found in this region. A second possibility is that the preBo¨tzC is not accurately defined by the region of a high density in NK1-positive neurons, 2.5–3.5 mm rostral to obex. In other words, the rhythm-generating neurons may not be restricted to this region. Indeed, if neurons expressing NK1 receptors are rhythmogenic, then the region ventral to the presumed preBo¨tzC might be rhythmogenic, as this region has a relatively high number of neurons expressing NK1 receptors. However, in two goats, only 7 and 31 living neurons remained in the entire region 2.4–4.6 mm rostral to obex. Moreover, in the one goat with only 2 living neurons in the presumed preBo¨tzC region, only 11 living neurons were found in NA and only 18 were found in the region ventral to the presumed

![Figure 11](http://jap.sagepub.com/content/106/1/615/F1.large.jpg)

**Fig. 11.** IA injections decreased the ventilatory response to hypoxia (10.8% O₂), Vl (P < 0.001), but not f or VT (P > 0.05), was reduced after the series of IA injections were completed. The data (means ± SE) were averaged in 1-min intervals and expressed as a percentage of control. The dashed lines delineate room air control, hypoxia, and room air recovery periods.
Despite these examples of near total destruction of the presumed preBötzC region, as well as the large or complete destruction in the surrounding tissue, it remains conceivable that plasticity occurred in the few living neurons within and surrounding the presumed preBötzC (19, 32).

Another possible explanation of plasticity could be that a reconfiguration occurred elsewhere within the respiratory network (19, 32). It could be that rhythmogenic neurons within the parafacial respiratory group (pFRG), which have been hypothesized to generate an expiratory rhythm in juvenile rats (6, 16, 17), are reconfigured to generate an inspiratory rhythm. However, there are no known data on adult mammals documenting the type of preinspiratory neurons in the pFRG that are rhythmogenic as in neonatal rats. On the other hand, since, in adult rats, neurons within the Bötzinger complex that are expiratory during normal oxygenation are reconfigured to a preinspiratory pattern during hypoxia (9), it is conceivable that similar reconfiguration occurs in the Bötzinger complex or pFRG after destruction of the preBötzC.

There is evidence that neurons within the pontine respiratory group (PRG) have a role in respiratory timing, and there are projections from the PRG to the DIA (3, 5, 15, 20, 21, 41–45). Moreover, in decerebrate cats, a presumed respiratory rhythm is sustained in the mylohyoid branch of the trigeminal nerve after phrenic rhythm is abolished by chemical lesions in the medulla (42). It has also been shown that lesions in the PRG result in disruptions in respiratory rhythm and pattern that are more profound during NREM sleep than in the awake state (5).

Table 2. Total number of living neurons for each individual animal within the NA, presumed preBötzC, and area ventral to the preBötzC, from 2.0 to 4.6 mm rostral from obex, 1 wk after the final 10-μl IA injection

<table>
<thead>
<tr>
<th>Goat No.</th>
<th>2.0–2.4 mm Rostral From Obex</th>
<th>2.5–3.5 mm Rostral From Obex</th>
<th>3.6–4.6 mm Rostral From Obex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NA Caudal preBötzC Ventral area</td>
<td>NA Presumed preBötzC Ventral area</td>
<td>NA Rostral preBötzC Ventral area</td>
</tr>
<tr>
<td>I</td>
<td>11 22 129</td>
<td>30 87 138</td>
<td>80 103 143</td>
</tr>
<tr>
<td>2*</td>
<td>10 2 46</td>
<td>16 4 49</td>
<td>23 1 25</td>
</tr>
<tr>
<td>3</td>
<td>0 0 29</td>
<td>17 61 88</td>
<td>17 12 53</td>
</tr>
<tr>
<td>4</td>
<td>9 10 19</td>
<td>11 2 18</td>
<td>0 19 14</td>
</tr>
<tr>
<td>5</td>
<td>0 0 32</td>
<td>12 26 37</td>
<td>19 32 58</td>
</tr>
<tr>
<td>6</td>
<td>0 0 33</td>
<td>0 22 7</td>
<td>7 64 54</td>
</tr>
<tr>
<td>7</td>
<td>16 56 156</td>
<td>9 116 243</td>
<td>31 83 251</td>
</tr>
<tr>
<td>Control</td>
<td>38±1.0 133±2.4 272±10.5</td>
<td>62±1.0 428±1.1 347±1.1</td>
<td>79±2.5 255±3.2 430±4.3</td>
</tr>
</tbody>
</table>

On average, between 2.5 and 3.5 mm rostral to obex, there were only 22, 10, and 24% of the total neurons remaining within the nucleus ambiguus (NA), presumed pre-Bötzinger complex (preBötzC) region, and region ventral to the preBötzC, respectively, compared with control, unoperated goats. *Not used in sleep studies.
Lesioning the preBo¨tzC attenuates CO2 and hypoxia sensitivity. The preBo¨tzC has also been implicated in both CO2/H+ and O2 chemosensitivity. Indeed, evidence from our laboratory has shown that a microdialysis-induced focal acidosis of the preBo¨tzC increased V1, solely as a function of increased f, by 10% (18). Moreover, it has already been shown that the ventilatory response to elevated inspired CO2 is significantly decreased in awake rats when the preBo¨tzC is lesioned with SAP-SP (11). Furthermore, Nattie and Li (28, 29) and Hodges et al. (14) have shown that destroying NK1-positive neurons at other chemosensitive sites, specifically the retrotrapezoid nucleus/parapyramidal region and the medullary raphe, also decreases the ventilatory response to elevated inspired CO2 in awake rats and goats. In the present study, CO2 sensitivity and the ventilatory response to hypoxia were also significantly attenuated after the IA injection in the preBo¨tzC. Thus it is conceivable that the destruction of NK1 neurons, as well as other potential chemosensitive and rhythmogenic neurons in the preBo¨tzC, is responsible for the significant decrease in the response to both hypercapnia and hypoxia. In other words, the data from this study appear to support previous data that there are both CO2-H+ (18, 38) and hypoxic (39) chemoreceptors within the preBo¨tzC. Moreover, the present data also are consistent with the concept of a distributed and not specialized areas of CO2-H+ chemoreceptors within the brain (30). Finally, because there was a normal respiratory pattern in response to these breathing conditions, albeit diminished, it appears that the redundant chemosensitive sites within the respiratory network were able to reconfigure to a new respiratory rhythm generator.

Conclusions. In support of previous studies (2, 6, 8, 10, 27, 40–42), data herein demonstrate the remarkable plasticity of the respiratory control system; there was time-dependent normalization of respiratory rhythm and pattern after near total destruction of a site (preBo¨tzC), which, when abruptly destroyed in the awake state, results in highly irregular breathing and/or prolonged/terminal apneas. The neural substrate of the plasticity likely incorporates areas beyond the presumed preBo¨tzC area; in two lesioned goats, there were only 7 and 31 living neurons within and surrounding the preBo¨tzC region. However, an alternative conclusion would be that only a few neurons in the preBo¨tzC region are required to mediate the complex functions of rhythm and pattern generation across multiple states and conditions. The data herein suggest the site and/or mechanism of rhythm and pattern generation are state dependent; 10–15 h after lesioning the preBo¨tzC, prolonged central apneas were nearly exclusively in the awake state. Accordingly, either the rhythm-generating mechanisms within the preBo¨tzC region are state dependent, or another brain stem site has a major role in generating the rhythm during NREM sleep. Finally, in contrast to the recent focus on the preBo¨tzC, which has been based largely on data from reduced preparations, the data from this study during physiological wakefulness and NREM sleep emphasize that regions in the brain stem other than the preBo¨tzC conceivably have or are capable of a major role in respiratory rhythm and pattern generation.

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GRANTS

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