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THE ROLE OF THE NUCLEUS ISTHMI IN RESPIRATORY PATTERN FORMATION IN BULLFROGS

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Summary

The nucleus isthmi (NI) is a mesencephalic structure of the amphibian brain located between the roof of the midbrain and the cerebellum. From a neuroanatomical perspective, the NI can be compared with the pons which, in mammals, contributes to the control of breathing pattern. This study tested the hypothesis that the NI plays a critical role in breathing pattern formation in the bullfrog. More specifically, we postulated that this nucleus was the site responsible for clustering breaths into distinct episodes of breathing. This hypothesis was tested by comparing the respiratory motor output of decerebrate, paralyzed and artificially ventilated bullfrogs before and after bilateral lesions of the NI by pressure microinjections of lidocaine or kainic acid (KA) into this area. Bilateral microinjections of lidocaine or KA into the NI transformed the breathing pattern from episodic (many breaths per episode) to one of evenly spaced single breaths, without affecting the amplitude of the fictive breaths. These changes in breathing pattern were associated with an overall decrease in breathing frequency and a reduction in CO_2-chemosensitivity. Breathing episodes of more than one breath reappeared during hypercarbia (3.5 % CO_2 in air) after KA lesioning. Bilateral lesions to the NI did not affect the changes in the timing or the amplitude of the respiratory-related bursts elicited by pulmonary stretch receptor feedback, indicating that mechanoreflexes do not require NI input. We conclude that the NI is not responsible for the genesis of breathing episodes, but provides a tonic excitatory input to respiratory centers in the lower brainstem. The NI also plays an important role in either CO_2 chemodetection or, more probably, integration of CO_2 chemoreceptor information. This, in turn, contributes to the production of episodes of more than one breath.

Key words: CO_2 chemoreceptors, control of ventilation, amphibian, episodic breathing, nucleus isthmi, control of breathing, bullfrog, *Rana catesbeiana*.

Introduction

Episodic breathing is characterized by the occurrence of clusters of breaths separated by non-ventilatory periods of variable duration where each breathing episode can consist of any number of individual breaths, depending on the level of respiratory drive (Kinkead and Milsom, 1994, 1996, 1997; for reviews, see Boutilier, 1988; Kinkead, 1997; Milsom, 1991). This particular temporal distribution of breaths characterizes the breathing pattern of many species of ectothermic vertebrates (for a review, see Milsom, 1991). Furthermore, some species of mammals in states normally associated with metabolic depression also breathe episodically (McArthur and Milsom, 1991*a*,*b*; Castellini *et al.* 1994). The broad occurrence of this breathing pattern amongst vertebrates suggests that episodic motor output may be a fundamental property of the respiratory control system under conditions of low respiratory drive. This is reinforced by the fact that recent investigations of the respiratory-related motor output produced by brainstem–spinal cord preparations of bullfrogs (Kinkead *et al.* 1994), turtles (Douse and Mitchell, 1990) and neonatal rats (Hilaire *et al.* 1989) have shown that episodic breathing also occurs *in vitro*, despite the absence of sensory afferent (except central chemoreceptor) and descending inputs. These results indicate that the production of breathing episodes is an intrinsic property of the central respiratory control system, rather than a result of the processing of afferent inputs. The sparse literature in this area of respiratory physiology makes it difficult to elaborate on the neural basis of episodic breathing. Most investigations performed on species that typically breathe episodically were designed to test respiratory reflexes or find the ‘noeud vital’, the center responsible for generating the respiratory rhythm. The majority of this work involves progressive transection of the brain in the rostral–caudal axis and, unfortunately, only a few reports document the changes in breathing pattern associated with each transection. In those

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reports, brainstem transection between the optic lobes and the cerebellum eliminates breathing episodes of more than one breath in bullfrogs (Oka, 1958*a*,*b*) and caimans (Naifeh *et al.* 1971*a*,*b*); the breathing pattern then appears to consist of evenly spaced single breaths. These data indicate that the production of breathing episodes is under the influence of specific sites independent of the one producing the respiratory rhythm; i.e. there are groups of neurons in the brainstem that cluster breaths into distinct episodes.

In bullfrogs, the area where brainstem transections affected breathing pattern included the nucleus isthmi (NI). This nucleus is located between the roof of the midbrain and the base of the cerebellum (Senn, 1972) in a position similar to that of the pontine respiratory group in mammals (Feldman and Smith, 1995). It is one of the most conspicuous structures of the anuran brain and relays visual information between the lobes of the optic tectum (Udin, 1987). The NI goes through substantial cellular arrangement and differentiation during amphibian metamorphosis (Senn, 1972), a period also associated with the onset of episodic lung breathing in bullfrogs (Burggren and Infantino, 1994). Together, this circumstantial evidence leads us to postulate that the NI plays a role in the clustering of breaths in this species. This hypothesis was tested in decerebrate, paralyzed and unidirectionally ventilated bullfrogs (an *in situ* preparation) by comparing the 'fictive' breathing pattern before and after bilateral lesions to the NI produced by microinjection of the local anesthetic lidocaine or the neurotoxin kainic acid (KA). A secondary objective of the present study was to determine whether the functional elimination of the NI affected respiratory reflexes produced by chemo- and mechanoreceptor afferent inputs.

Materials and methods

Experiments were performed on 25 adult bullfrogs (*Rana catesbeiana* Shaw) of either sex weighing between 165 and 469 g (mean mass ± S.E.M. = 295±20 g), obtained from a commercial supplier. The animals were maintained indoors in fiberglass tanks continuously supplied with running water. Air temperature was maintained at 20–22 °C. Frogs were exposed to a 12 h:12 h L:D photoperiod and fed live locusts at least once a week. The animals were not fed for 2 days before surgery was performed.

Animal preparation

The reduced frog preparation used in this study was similar to the one developed by Kogo *et al.* (1994). A detailed description of this method has also been reported previously (Kinkead and Milsom, 1997). Briefly, the frogs were anesthetized by immersion in a mixture of cold water and crushed ice bubbled with O_2 for an hour, or until the toe pinch reflex was abolished (Stehouwer, 1987). A small hole was made on the dorsal surface of the skull to expose the telencephalon. The brain was transected between the optic tectum and the rostral forebrain with a blunt spatula and the entire forebrain was aspirated with a suction device. The decerebration procedure usually took less than 5 min. The dura covering the area between the cerebellum and the optic tectum was removed to facilitate the penetration of the microinjection pipette. Small cotton pellets soaked with physiological saline were placed over the area to prevent desiccation. The frogs were then paralyzed by a pancuronium bromide injection in the dorsal lymph sacs (Pavulon, 0.5 mg 100 g^{-1}, 2 mg ml^{-1}).

The left femoral artery was cannulated occlusively with polyethylene tubing (PE 50) to monitor blood pressure. Polyvinyl cannulae were inserted into both lungs at the apex *via* small incisions in the body wall of the flanks to permit unidirectional ventilation (UDV) of the lungs. The skin covering the mandible was cut and retracted, and the mandibular branch of one trigeminal nerve was located and isolated. A piece of 5-0 gauge silk was placed around the nerve before it was cut distally. The glottis was sealed with two wound clips to prevent gas leaking from the artificially ventilated lungs. The vagus nerve was located on one side from a dorsal approach. An incision was made above the scapula, the muscles were cut and the scapula retracted to isolate the laryngeal branch of the vagus nerve. A piece of 5-0 gauge silk was placed around the nerve before it was cut. A custom-made waterbath bubbled with gas was positioned under the animal, and wet toweling was draped over the frog with both ends in the bath to act as a wick to keep the skin of the frog moist.

Experimental procedures

Once the surgery had been completed, the frogs were unidirectionally ventilated with air at a flow rate of 70 ml min^{-1}. Prior to delivery to the lungs, the inflowing gas was humidified by bubbling through an Erlenmeyer flask half-filled with water. Pulmonary pressure was monitored by installing a T-piece, connected to a pressure transducer, in the inflow cannula. The isolated branches of the trigeminal and vagus nerves were each positioned on bipolar platinum hook electrodes and covered with a 1:1 mixture of Vaseline and mineral oil. Electrical nerve activity recorded from the bipolar electrodes was amplified [filter settings: 200 Hz (high pass) and 10 kHz (low pass)], full-wave-rectified and integrated in 67 ms intervals. The raw and integrated nerve signals were viewed on an oscilloscope and stored on a polygraph recorder and on a computer disk with a data acquisition system (AC Codas). The sampling rate of the analog-to-digital conversion was 2500 Hz. The animal was allowed to recover from cold anesthesia for at least 30 min before the onset of experiments. Henderson and Burtsaert physiological saline (in mmol l^{-1}: 118 NaCl, 2.5 $CaCl_2$, 4.7 KCl, 1.1 KH_2PO_4, 24 $NaHCO_3$, 1.2 $MgSO_4$, 4.5 glucose) or adrenaline (0.05 mg ml^{-1} in physiological saline) was injected into the arterial cannula when mean arterial blood pressure dropped below 0.25 kPa.

Microinjections of kainic acid (KA, 4.7 mmol l^{-1} in saline), lidocaine hydrochloride (1 % in NaCl), saline (0.7 % NaCl) and Fast Green FCF (10 % solution in saline) into the area of the NI were made with triple-barrelled pipettes built from

microfilament capillary glass (A-M Systems, Inc.). Each barrel had an inner diameter of 0.6 mm and an outer diameter of 1.2 mm. The glass was pulled with a Narashige pipette puller and broken back until the total tip diameter measured roughly 40 μm. Each barrel was two-thirds filled with its respective solution so that each meniscus was approximately the same height. A piece of polyethylene tubing (PE 10, Clay Adams) was placed inside each barrel of the pipette and then secured with 5 min epoxy resin. A 27 gauge needle was inserted in each piece of PE tubing before being connected to the pressure ejection system (Picospritzer II, General Valve Corp.). The injection volumes were calculated from the measured radius of the pipette and the change in height of the fluid.

Experimental protocol

The effects of bilateral lesions of the nucleus isthmi on breathing pattern

This series of experiments assessed the role of the NI in the formation of breathing episodes by comparing the breathing pattern of intact frogs with that of animals after bilateral lesions of the NI. The micropipette was positioned from a dorsal approach into the region of the NI. This region lies 1.5–2.0 mm below the level of the cerebellum and 0.8 mm from the midline at the level of the junction between the optic tectum and the cerebellum. Each experiment began with bilateral sham injection of 175 nl each of physiological saline (0.7 % NaCl) using a series of 1–5 rapid pressure injections (550 kPa, 400–800 ms duration) until the meniscus moved the appropriate distance. Preliminary experiments have shown that a similar volume of KA produced the changes in breathing pattern that will be described below. Saline microinjections had no effect on respiratory-related motor output in any of the frogs tested (see Fig. 1A). Thus, saline microinjections were followed by bilateral injections of a similar volume of KA, a glutamate analog that selectively lesions neuronal cell bodies while sparing axons of passage (Coyle *et al.* 1978; Denavit-Saubié *et al.* 1980). Unilateral microinjection of KA in this area initially provoked an increase in bursting frequency and amplitude from both nerves, as well as in the level of tonic discharge mainly in the vagus nerve. The increased activity was followed by a period of quiescence. Fictive breathing reappeared on average 45 min after bilateral KA lesioning and became stable within 30 min. At this point, the experimental protocol for assessment of chemo- and mechanoreceptor reflexes described below was begun.

In four animals, 175 nl of lidocaine hydrochloride was microinjected into this region to demonstrate the effects of reversible bilateral lesions of the NI. Once the effects of lidocaine were completely dissipated (approximately 30 min), KA was injected into the NI. In all experiments, each KA injection was followed by an injection of 175 nl of Fast Green for subsequent localization of the injection site. The KA experiments were successful in nine animals out of 17 (see below).

The effects of bilateral lesions to the nucleus isthmi on respiratory reflexes

This series of experiments was designed to evaluate the role of the NI in other aspects of respiratory control, specifically the reflexive changes in respiratory motor output elicited by chemo- and mechanoreceptor afferent inputs. These experiments assessed the effects of changes in tonic pulmonary stretch receptor (PSR) feedback, *via* changes in lung pressure, under different levels of CO_2-related respiratory drive before and after bilateral lesions to the NI. The experiments involved two groups of bullfrogs; control frogs (N=8) in which no injections were performed and KA-injected frogs (N=9). The animals were placed on UDV with a gas mixture containing one of three different levels of CO_2 (1 %, 2 % and 3.5 % CO_2 in air). Since UDV with 2 % CO_2 in air maintains arterial blood gases at levels close to those measured in spontaneously ventilating bullfrogs (Kinkead and Milsom, 1994), the 1 % and 3.5 % CO_2 in air mixtures were used to induce hypo- and hypercapnia, respectively. The gas mixtures were administered randomly, and a 25 min equilibration period was allowed between each run with a new mixture. Once a level of respiratory drive had been established, PSR feedback was set at different levels by immersing the UDV outflow catheter in a water cylinder to change the outflow resistance of the UDV line and, thus, to increase or decrease pulmonary pressure accordingly. Pulmonary pressure (P_L) was set to 0, 0.2 or 0.5 kPa at random.

The effects of vagotomy after bilateral lesions of the nucleus isthmi

Because it is well established that the interaction between vagal and central nervous system (CNS) inputs plays a role in ventilatory pattern formation in mammals (for a review, see Milsom, 1991), this series of experiments compared the breathing patterns of four frogs in which the NI had been successfully severed before and after the vagus nerves were cut bilaterally at the cervical level. Since bilateral microinjections of KA significantly reduced breathing frequency (see Results), the comparison could only be made at the highest level of CO_2 used in this experimental protocol. The fictive breathing pattern was recorded at the different levels of tonic pulmonary pressure described previously before and after the vagus nerves were cut. The fact that changing pulmonary pressure after vagotomy had no effect on any of the variables confirmed that the vagotomy was successful at removing vagal input.

Analytical procedures

The gas mixtures administered to the animals were produced by mixing air with CO_2 using flow meters. The gas mixture fed to the UDV line and waterbath was sampled continuously from the outflowing gas line and monitored with a Beckman LB-2 CO_2 analyzer and OM-11 oxygen analyzer calibrated with gas mixtures produced by a Radiometer GMA2 precision gas supplier. Both pressure transducers were calibrated against a static water column.

The duration of the breathing cycle (T_{TOT}) and inspiratory duration (T_I) were estimated by measuring burst duration recorded from the laryngeal branch of the vagus nerve (Xl) and the mandibular branch of the trigeminal nerve (Vm), respectively. These assessments are based on the facts that (a) pulmonary ventilation in frogs can only occur while the glottis is open and (b) lung inflation (inspiratory phase) is produced by rapid elevation of the floor of the buccal cavity when the glottis is open (De Jongh and Gans, 1969; West and Jones, 1975; Sakakibara, 1984*a*), as previously demonstrated by others (Kogo *et al*. 1994; Kogo and Remmers, 1994). The peak of the full-wave-rectified and integrated trigeminal electroneurogram (ENG) was used as an index of tidal volume. Sakakibara (1984*a*) has correlated the pattern of respiratory nerve activity with the changes in buccal and pulmonary pressure that occur during the breathing cycle in the bullfrog. His study demonstrated that the compound trigeminal ENG recorded just proximal to the site where the nerve branches off to the respiratory muscles (the recording site in the present study) had activity corresponding to simultaneous elevation of buccal and pulmonary pressures. Sakakibara (1984*b*) subsequently demonstrated that, in the glottis-occluded frog, the peak integrated trigeminal activity was linearly related to peak buccal pressure, validating the use of peak integrated trigeminal nerve activity as an index of total inspiratory activity. This measurement compares with the use of peak integrated phrenic nerve activity as a correlate of tidal volume in mammals (Eldridge, 1975). Breathing frequency was quantified by analyzing the number of respiratory events (lung breaths) per unit time (f_L). Consequently, multiplying the peak integrated trigeminal activity ($V_{T_{index}}$) by breathing frequency (f_L) yielded an index of total ventilation ($\dot{V}_{index}$) reported here in arbitrary units.

Breathing episodes are designated on a subjective basis. Thus, the number of breaths within an episode was obtained by counting the number of large-amplitude fictive buccal movements occurring in succession with no pause longer than the length of two ventilation cycles between them. Note that, when breathing was continuous, no values were obtained for breaths per episode or episodes per minute. Recordings of respiratory variables were obtained only when the fictive breathing pattern was stable; this usually occurred within the first minute following a static change in pulmonary pressure.

Brain tissue histology

At the end of the experiment, the brain was removed from the bullfrog and fixed overnight in a cold 4 % paraformaldehyde solution in phosphate buffer neutralized to pH 7.9. The tissue was then transferred to a 30 % sucrose, 0.1 % sodium azide solution for 24 h before sectioning (50 μm) on a cryostat at −23 °C. The sections were then stained either with Cresyl Violet or with eosin–hematoxylin, according to standard procedures, and examined for Fast Green staining to determine the sites of the micropipette placements. Histological data were obtained from six bullfrogs.

Data analysis

Values for fictive breathing variables were obtained by analyzing a 5 min segment of data. Preliminary experiments have shown that unilateral lesions to the NI had no significant effect on respiratory motor output (data not shown). Thus, whenever possible, two criteria (anatomical and physiological) were used simultaneously to ensure that the KA microinjections resulted in bilateral lesions to the NI. More specifically, animals in which (1) Fast Green stains were located more than 40 μm away from the NI, on either side of the brain, and (2) bilateral KA injections did not change breathing frequency by 15 %, were not included in the data set. The pre–post bilateral NI lesion comparison for the physiological criteria was applied under ‘resting conditions’ i.e. F_{CO_2}=0.02, where F_{CO_2} is the fractional concentration of CO_2, and P_L=0.2 kPa. Note that because anatomical data was not available for each bullfrog, changes in motor output were the only criteria used for data selection in some cases. Animals that remained apneic for more than 1.5 h after the bilateral lesions were not included in the analysis.

All data are presented as means ± S.E.M. The results were analyzed statistically using a two-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls test (P<0.05).

Results

The effects of bilateral lesions of the nucleus isthmi on breathing pattern

The breathing pattern in intact bullfrogs and the in situ *preparation*

The fictive breathing pattern recorded before and after bilateral microinjections of saline into the NI was very similar to the breathing pattern recorded from intact bullfrogs (Kinkead and Milsom, 1994, 1996) and the pattern recorded from this *in situ* preparation in a previous study (Kinkead and Milsom, 1997). Lung ventilation was episodic, and the number of fictive breaths in each bout of activity varied according to the level of respiratory drive (see below). In most preparations, bursts of small amplitude were recorded from the trigeminal nerve between larger-amplitude bursts of activity (Fig. 1A). These smaller bursts, which occurred very rhythmically, represent the small-amplitude buccal oscillations described for intact frogs (West and Jones, 1975; Kinkead and Milsom, 1994, 1996). Comparable activity could rarely be recorded from the vagus nerve.

The breathing pattern following bilateral lesions to the NI

The breathing pattern observed following bilateral lidocaine injection consisted mainly of evenly spaced single breaths, and there were fewer fictive lung breaths per minute. Fictive buccal oscillations were still often observed during the non-ventilatory period (Fig. 1B). The effects of lidocaine usually lasted for approximately 20 min, after which breathing began to accelerate, and each episode of fictive breathing contained progressively more breaths.

In nine bullfrogs, bilateral KA injections had similar effects on breathing pattern to those observed following lidocaine injections (Fig. 1C). The brains of four of these nine animals were sectioned (Fig. 2A,B). In all four, both Fast Green markers were located inside, or in the immediate vicinity (<40 μm from) of, the NI bilaterally (Fig. 2C, filled circles). An important distinction between the effects of lidocaine and KA injections was that the effects of KA were not reversible. After an initial cycle of stimulation/inhibition, breathing was stable for the entire duration of the experiment.

Bilateral lesions of the NI prolonged the duration of the neural discharge associated with the ventilatory cycle (P=0.004), but had no significant effect on the relative duration of the inspiratory phase (P=0.217), as indicated by the pattern of vagal and trigeminal neural discharge, respectively (Figs 3, 7). It is noteworthy that, in some instances, the amplitude of the vagal bursts was reduced after NI lesions. In these cases, it was difficult to determine accurately the onset and termination of a burst (Fig. 3B).

The most striking effect of bilateral lesions to the NI was an overall reduction in $\dot{V}_{index}$ (P<0.001; Fig. 1B,C). This reduction in respiratory motor output was mediated by a reduction in f_L (P<0.001) which, in turn, was caused by a reduced number of fictive breaths per episode (a longer non-ventilatory pause; P<0.001) and fewer episodes of breathing per minute (P<0.001). Bilateral injection of KA had no effect on V_{Lindex} (P=0.073; Figs 4, 5). The breathing pattern observed after bilateral KA injections was one of evenly spaced single breaths. Note that the recording shown in Fig. 1C was obtained at a higher level of hypercarbic stimulation (3.5% CO_2; P_L=0.2 kPa) than those in Fig. 1 A,B (2% CO_2; P_L=0.2 kPa).

Bilateral injection of KA into the area of the NI had no effect on blood pressure (N=17; data not shown). In six animals, the bilateral injections had very little or no significant effect on the breathing output. The anatomical location of the Fast Green markers in one of these cases is shown by the open circles in Fig. 2C, which represents a cross section approximately 4 mm rostral to the obex. In this particular frog, only one of the markers was located in the NI, whereas the other was situated approximately 200 μm below the NI. In two experiments, the respiratory-related motor output remained silent 1.5 h after the KA injections. Brain tissue sections were obtained from one of these animals, and it was found that the markers were located approximately 50 μm above the NI (Fig. 2C; open triangles). Owing to the incomplete anatomical data, changes in motor output were the only criteria for data selection in some cases. Thus, none of these eight experiments was considered 'successful' and they were not included in the data set.

The effects of bilateral lesions to the nucleus isthmi on respiratory reflexes

Ventilatory responses to CO_2 in intact bullfrogs

In NI-intact bullfrogs, increasing the F_{CO_2} of the UDV gas mixture stimulated fictive breathing (P<0.001; Fig. 4A). This increase in $\dot{V}_{index}$ was mediated by an increase in f_L alone (P=0.021; Fig. 4B), since V_{Tindex} did not change significantly with the increase in respiratory drive (P=0.87; Fig. 4C). When P_L was 0 kPa, the increase in f_L observed under hypercarbic conditions was caused by a significant increase in the number of breaths in each bout of fictive ventilatory activity (P<0.033; Fig. 4D); the increase in the number of episodes of breathing per minute was not significant (P=0.187; Fig. 4E).

In this group, hypercarbia did not shorten either trigeminal (P=0.397) or vagal (P=0.593) burst duration (data not shown).

Ventilatory responses to CO_2 following bilateral lesions to the NI

Fictive breathing was not recorded in 55% (five out of nine) of the bullfrogs on UDV with a 1% CO_2 in air gas mixture at any level of P_L. All frogs with their NI intact produced

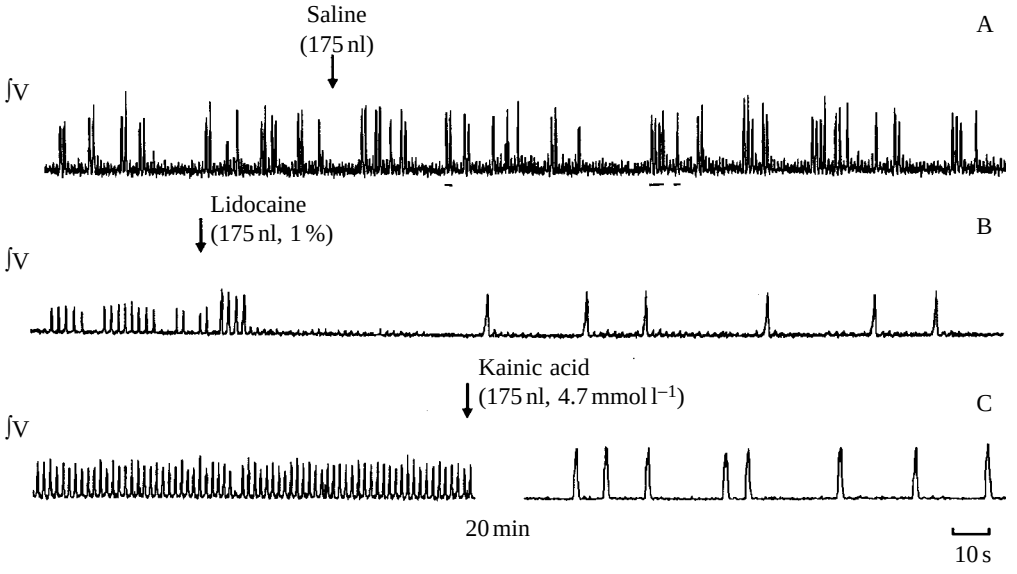

Fig. 1. The effects of bilateral microinjections into the nucleus isthmi (NI) of (A) saline, (B) lidocaine hydrochloride and (C) kainic acid on the fictive breathing pattern of decerebrate, paralyzed, unidirectionally ventilated bullfrogs, as shown by a recording of integrated trigeminal nerve activity (∫V). Owing to the reduced ventilatory output following bilateral lesions to the NI, the recording shown in C was obtained at a higher level of respiratory drive (3.5% CO_2; P_L=0.2 kPa) compared with those in A and B (2% CO_2; P_L=0.2 kPa). In A, the lines under the neurogram indicate (from left to right) breathing episodes of 2, 4 and 1 breaths. The arrow indicates when the second microinjection was given. P_L, pulmonary pressure.

respiratory-related motor output under these conditions. After bilateral NI lesions, fictive breathing was recorded in all bullfrogs only when the CO_2 level in the UDV gas mixture was raised to 3.5 %. Respiratory motor output stopped in two animals on UDV with 3.5 % CO_2 in air when P_L was increased to 0.5 kPa (see below). The ventilatory index recorded after bilateral lesions to the NI was significantly lower ($P<0.001$) than that of bullfrogs with their NI intact (Fig. 4A).

Increasing CO_2-related respiratory drive following KA microinjection had no significant effect on $\dot{V}_{index}$ ($P=0.021$). The breathing pattern of some animals was altered only when F_{CO_2} was 0.035 and P_L was 0.5 kPa (Table 1). The mean number of

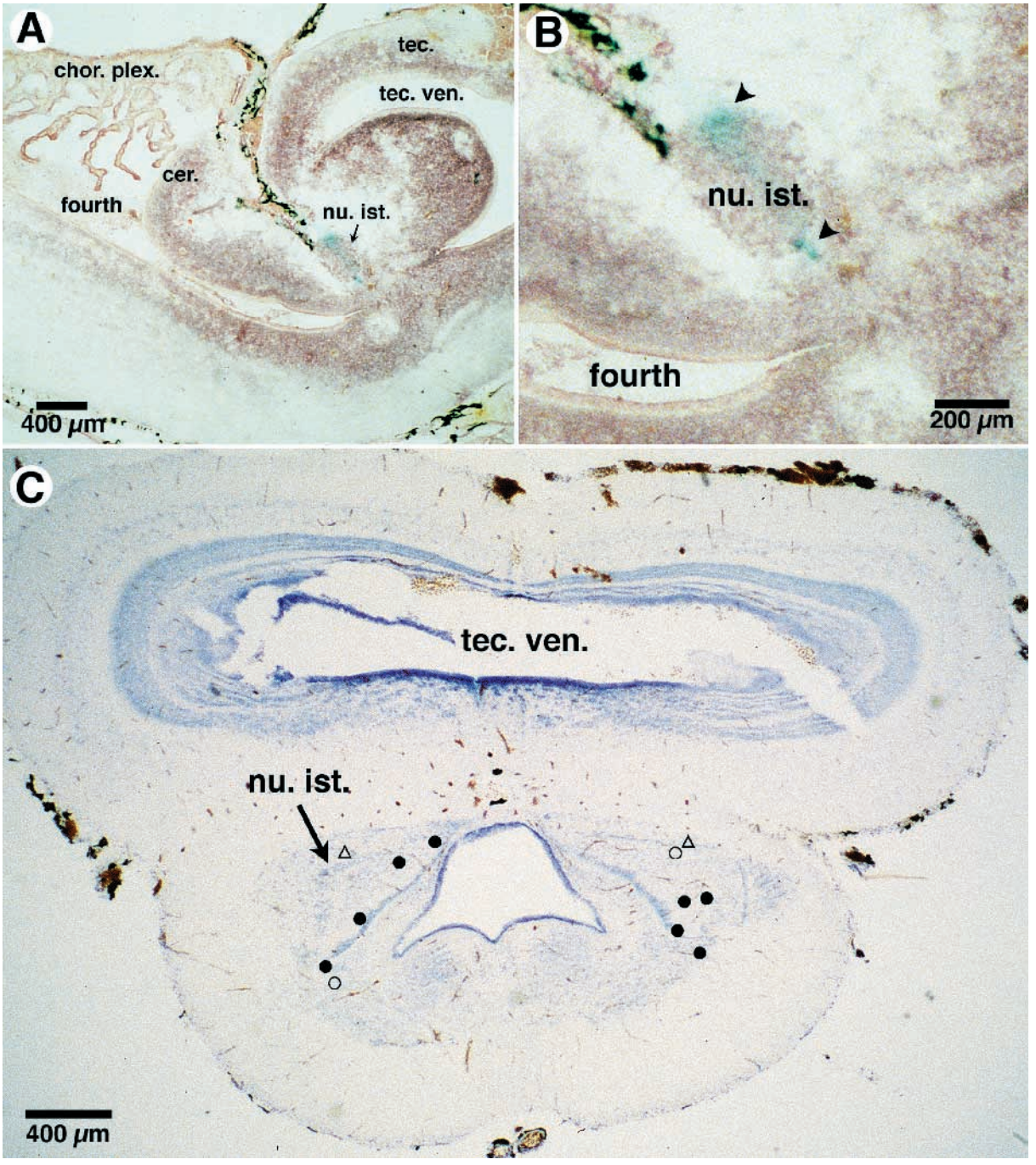

Fig. 2. (A) Sagittal section of the bullfrog brain showing the anatomical landmarks surrounding the nucleus isthmi. (B) Enlargement of the nucleus isthmi area showing the location of the Fast Green marker after a kainic acid microinjection. (C) Cross section of the bullfrog brain approximately 4 mm rostral of the obex showing sites of bilateral microinjections of kainic acid in the nucleus isthmi area. In four bullfrogs, the injections induced important changes in breathing pattern (filled symbols). In two other cases, kainic acid microinjections either had no effect (open circles) or completely eliminated bursting activity (open triangles). cer., cerebellum; chor. plex., choroid plexus; fourth, fourth ventricle; nu. ist., nucleus isthmi; tec., optic tectum; tec. ven., ventricle of the optic tectum.

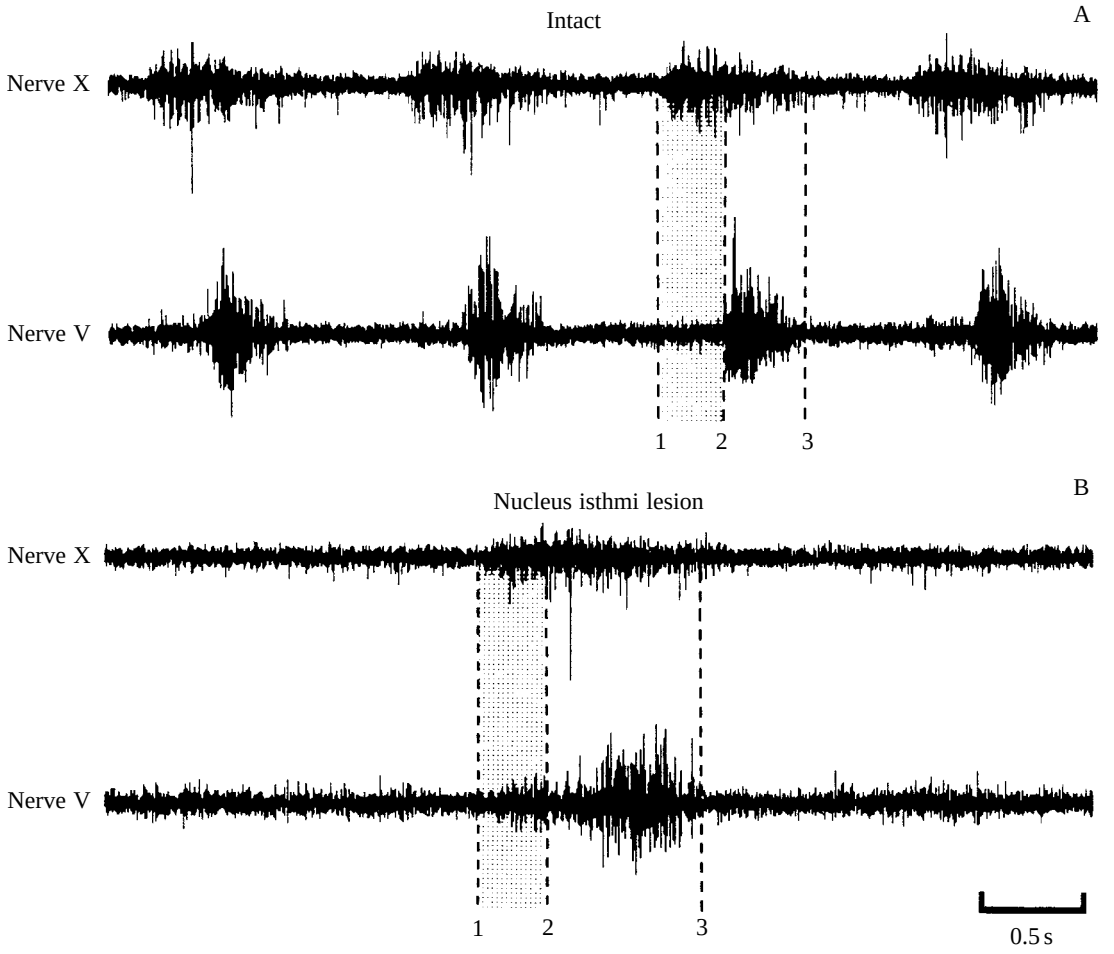

Fig. 3. Bursting activity of a typical 'fictive breath' recorded before (A) and after (B) bilateral microinjections of kainic acid into the nucleus isthmi. The simultaneous electroneurograms (ENG) are from the vagal (nerve X; top trace) and trigeminal (nerve V; bottom trace) nerves. These recordings were obtained from a single bullfrog unidirectionally ventilated with 3.5 % CO_2 in air at P_L=0.5 kPa. Broken lines indicate (1) the start of the vagal burst, (2) the start of the trigeminal burst and (3) the approximate termination of both vagal and trigeminal bursts. The stippled area indicates the expiratory phase of the breathing cycle. This particular case also illustrates the reduced amplitude of the vagal burst observed in some animals after nucleus isthmi lesions.

breaths per episode (P=0.269) and the number of episodes per minute (P=0.098) remained similar at all other combinations of P_L and F_{CO_2} (Fig. 4D,E). Changing the F_{CO_2} of the UDV gas mixture also had no effect on the vagal (P=0.532) or trigeminal (P=0.326) burst durations (data not shown).

Table 1 reports the number of breaths in a breathing episode observed after bilateral NI lesions in bullfrogs on UDV with 3.5 % CO_2 in air at a P_L of 0.5 kPa. These data show that, in two animals (F and G), breathing episodes of many breaths still occurred after the bilateral NI lesions, and the number of breaths in each episode increased as a function of F_{CO_2}. The breathing pattern recorded for animal G is shown in Fig. 5. In animals E and I, no breathing was recorded; three bullfrogs (B, C and D) still only took single breaths, and two frogs (A and H) took, on average, pairs of breaths at higher levels of F_{CO_2}.

Ventilatory responses to changes in pulmonary stretch receptor feedback in intact bullfrogs

Overall, tonic increases in P_L stimulated fictive breathing. For each level of respiratory drive, increasing P_L increased f_L and reduced $V_{T_{index}}$, resulting in a net increase in $\dot{V}_{index}$ (P<0.001 for all; Fig. 6A–C). The increase in f_L produced by increasing P_L was mediated by a change in breathing pattern. In bullfrogs on UDV with 2 % and 3.5 % CO_2 in air, increasing P_L above 0 kPa transformed the breathing pattern from episodic to continuous in most animals. In bullfrogs that did not breath continuously, however, increasing P_L augmented the number of breathing episodes per minute (P=0.003; Fig. 6E), but had no effect on the number of breaths in a

Table 1. *Individual values for the number of fictive breaths in a breathing episode that were recorded after nucleus isthmi lesions from bullfrogs unidirectionally ventilated with 3.5 % CO_2 in air at pulmonary pressure of 0.5 kPa*

Bullfrog	Mean number of breaths per episode
A*	2
B	1
C	1.1
D	1
E	–
F*	18
G*	11
H	2
I*	–

Note that no breathing was recorded for bullfrogs E and I.
* indicates bullfrogs for which histological data were obtained.

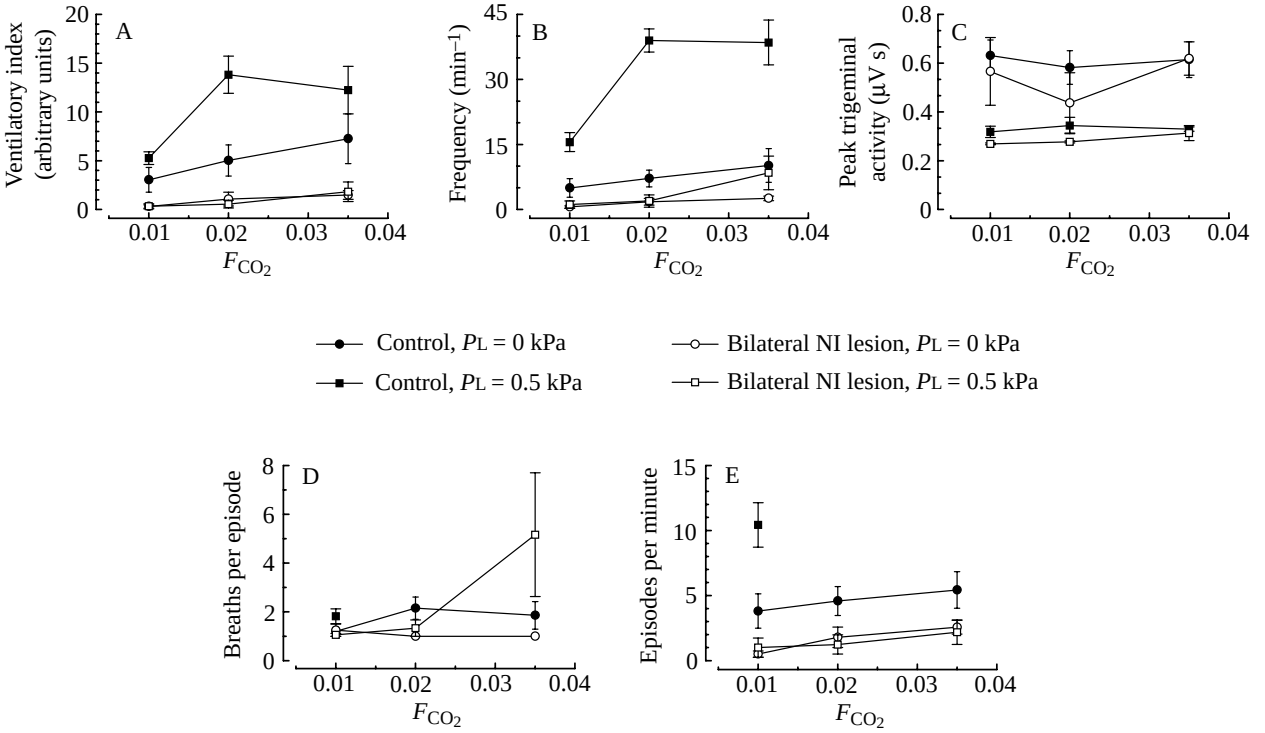

Fig. 4. The relationship between hypercarbic respiratory drive (F_{CO_2}) and (A) ventilatory index ($\dot{V}_{index}$), (B) breathing frequency (f_L), (C) peak integrated trigeminal nerve activity ($V_{T_{index}}$), (D) breaths per episode and (E) episodes per minute in bullfrogs with (N=8; filled symbols) and without (N=9; open symbols) their nucleus isthmi intact. Each curve represents a different level of tonic pulmonary stretch receptor feedback (circles, P_L=0 kPa; squares, P_L=0.5 kPa). Note that, in control animals (filled symbols), breathing became continuous when F_{CO_2} was raised above 0.01 kPa. Thus, there were no data for (D) breaths per episodes and (E) episodes per minute. Data are given as means ± S.E.M., P_L, pulmonary pressure.

breathing episode (P=0.187; Fig. 6D). The effects of P_L on f_L were directly proportional to the magnitude of the hypercarbic respiratory drive, as there was a significant interaction (P=0.036) between these variables.

Tonic increases in P_L had no significant effect on the duration of the vagal bursts (P=0.191). This means that when P_L was 0 kPa, the trigeminal burst began at the same time or before the onset of the vagal burst, and both nerves became silent at approximately the same time. With increases in P_L, however, trigeminal burst duration was shortened (P<0.001) such that the fraction of the gas exchange cycle during which gas would be expelled from the lungs was prolonged and the lung inflation phase was shortened (Fig. 7A).

Ventilatory responses to changes in pulmonary stretch receptor feedback following bilateral lesions to the nucleus isthmi

After bilateral NI lesions, tonic increases in P_L no longer had any significant effect on f_L (P=0.054), $\dot{V}_{index}$ (P=0.472), the number of breaths per episode (P=0.242) or the incidence of fictive episodes of breathing (P=0.559; Fig. 6; open symbols). It should be mentioned, however, that in four bullfrogs on UDV with 3.5 % CO_2 in air, increasing PSR feedback did increase the mean number of breaths in a breathing episode (Table 1). $V_{T_{index}}$ was the only variable consistently affected by changes in PSR feedback (P=0.046; Fig. 6C). Changes in tonic levels of PSR feedback could still affect the timing of the fictive breaths in KA-microinjected bullfrogs, since progressive increases in P_L shortened trigeminal burst duration (P=0.002) but, unlike bullfrogs with their NI intact, it also prolonged the duration of the vagal bursts (P=0.002; Fig. 7B). This means that, at higher values of P_L, bullfrogs without their NI would have a greater proportion of their breathing cycle devoted to expiration and, conversely, a shorter inspiratory phase than bullfrogs with their NI intact.

The effects of bilateral vagotomy after bilateral lesions of the nucleus isthmi

Following bilateral lesions to the NI, bilateral vagotomy reduced f_L further and reduced the number of breaths in an episode to one in animals that could still produce breathing episodes of more than one breath (Fig. 5D). This experiment also demonstrated that the effects of complete elimination of vagal feedback on breathing pattern did not differ from those caused by lung deflation. The data for vagotomized bullfrogs were never significantly different from the values recorded at a P_L of 0 kPa (Fig. 8).

Discussion

Critique of methods

The NI is a relatively large structure (radius approximately

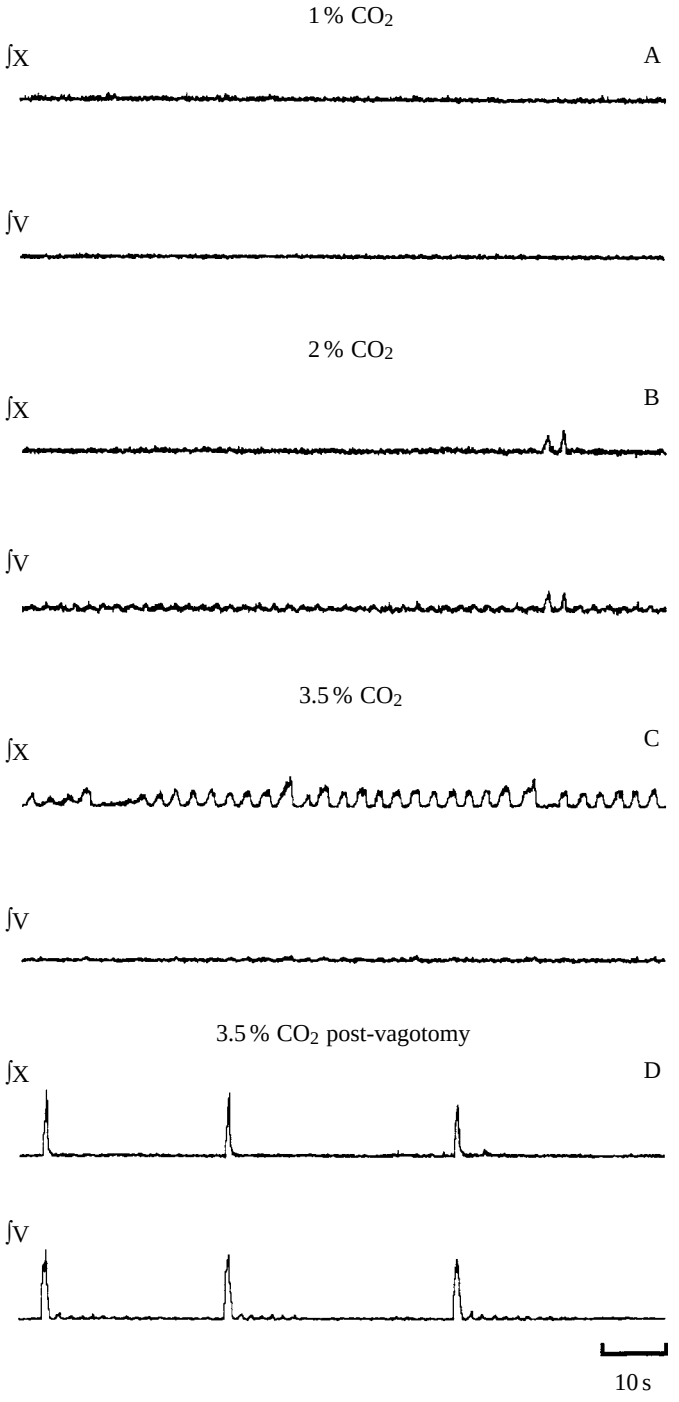

Fig. 5. Simultaneous electroneurogram recordings of full-wave-rectified, integrated vagal (∫X) and trigeminal (∫V) nerve activity obtained from a single bullfrog (bullfrog G, see Table 1) following bilateral lesions of the nucleus isthmi at three levels of respiratory drive: (A) 1 % CO_2 in air, (B) 2 % CO_2 in air and (C) 3.5 % CO_2 in air. D shows the effects of bilateral vagotomy in this particular animal when unidirectionally ventilated with 3.5 % CO_2 in air.

200 μm) in which the cell bodies are densely arranged at the periphery of the nucleus (cortex), while the center of the NI consists mainly of fibers (medulla) (Larsell, 1924; Senn, 1972). Thus, to reach a significant portion of the cell bodies, relatively large volumes of KA or lidocaine had to be injected. Volumes could not be too large, however, since in preliminary experiments, bilateral injections of KA caudal to the NI sometimes diffused to the trigeminal motor nucleus and eliminated ENG activity, while vagal activity remained unaffected. The difficulty of confining large injection volumes to a specific area may explain why microinjections within the NI area had no effect on breathing in six animals, but suppressed breathing in two other cases. Because unilateral lesion to the NI had no significant effect on fictive breathing at different levels of F_{CO_2} and P_L (R. Kinkead, M. B. Harris and W. K. Milsom, unpublished observations), the lack of effect of KA on breathing pattern reported for six bullfrogs is probably due to an incomplete NI lesion on one or both sides of the brain. The incomplete histological data makes it difficult to provide a clear anatomical correlation for the different effects exerted by KA microinjections. The absence of breathing activity following KA microinjection in two animals is also difficult to explain. It is possible that the frogs required more time to recover than the period allowed (roughly 1.5 h). Alternatively, since the histological data from one animal showed that the micropipette was located on the more dorsal area of nucleus, perhaps that KA diffused outside the injection site and exerted undesired effects on other key respiratory neurons. Yet, for the four bullfrogs from the 'successful' group (N=9) for which brain sections were obtained, the markers were located bilaterally in the immediate vicinity of the NI, indicating that the changes in breathing pattern were caused by bilateral lesions to the NI. The respiratory data from the four 'successful' animals for which anatomical data were available were analyzed separately. The mean results obtained from this subgroup of animals were virtually identical, albeit more variable, to those of the entire data set (N=9) presented here. This analysis confirmed that, in the present study, including/rejecting animals on the basis of physiological criteria did not inappropriately bias the data.

Lidocaine is a local anesthetic which affects both axons of passage and cell bodies, whereas KA is a potent glutamate analog which only affects cell bodies (Coyle *et al.* 1978; Denavit-Saubié *et al.* 1980). Once injected into the NI, both agents caused a severe reduction in breathing frequency and, for the most part, changed the breathing pattern to one of evenly spaced single breaths. These similar effects of the drugs indicate that the changes in $\dot{V}_{index}$ were the result of bilateral lesions to the NI, rather than disruption or impairment of neuronal pathways in this brainstem area.

The role of the nucleus isthmi in respiratory pattern formation

Following bilateral lesions to the NI, the transformation of the breathing pattern from episodic to evenly spaced single breaths was always associated with a reduction in f_L rather than a change in the temporal distribution of breaths. This raises the possibility either that the NI is responsible for producing

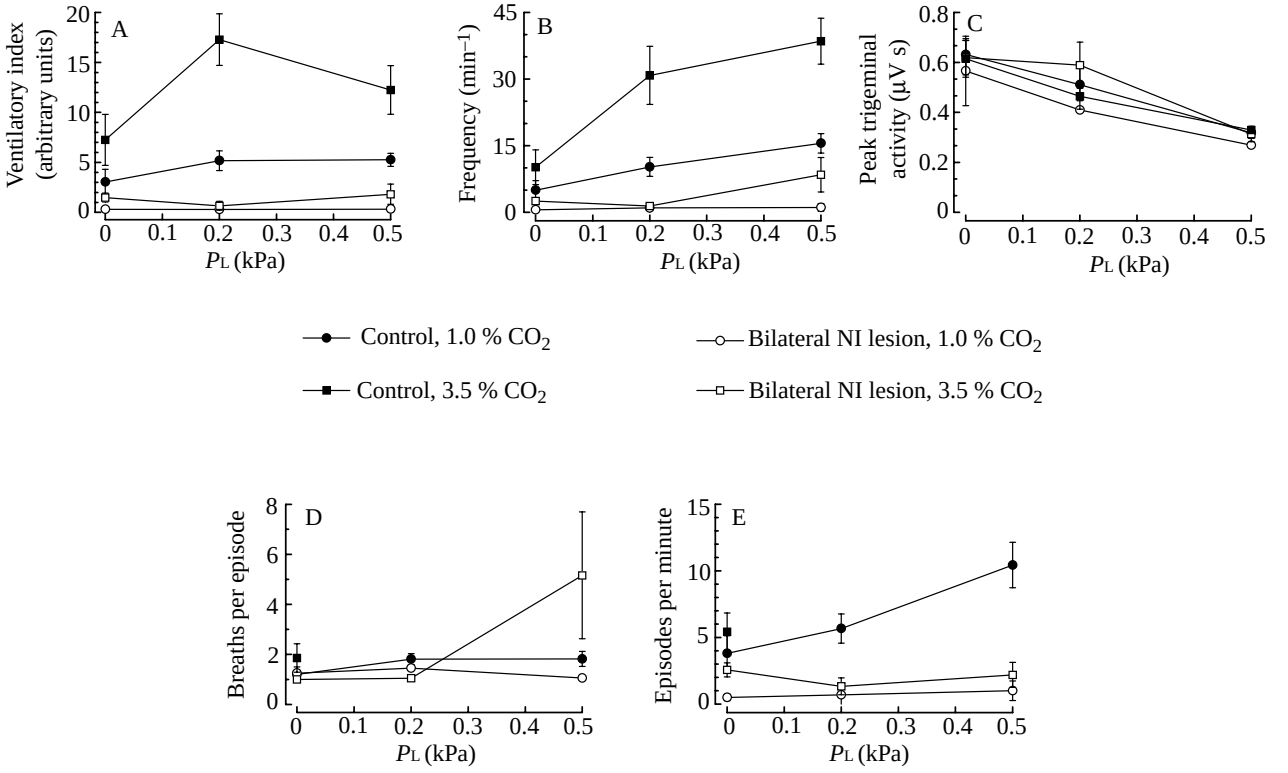

Fig. 6. The relationship between tonic changes in pulmonary pressure (P_L) and (A) ventilatory index ($\dot{V}_{index}$), (B) breathing frequency (f_L), (C) peak integrated trigeminal nerve activity ($V_{T_{index}}$), (D) breaths per episode and (E) episodes per minute in bullfrogs with (N=8; filled symbols) and without (N=9; open symbols) their nucleus isthmi (NI) intact. Each curve represents a different level of hypercarbic respiratory drive (circles, 1 % CO_2 in air; squares, 3.5 % CO_2 in air). Note that, in control animals (filled symbols), breathing became continuous when P_L was set above 0.2 kPa. Thus, there were no data for (D) breaths per episodes and (E) episodes per minute. Values are means ± S.E.M.

clusters of breaths or episodes or that the NI simply provides a tonic input and that episodes only occur when respiratory drive is high. Since breathing episodes of more than one breath were still observed in some animals following microinjections of KA in the area of the NI (Table 1; Fig. 5), and the overall reduction in $\dot{V}_{index}$ was roughly proportional to the reduction in chemosensitivity (see below), it would appear that the NI only provides tonic respiratory drive. Furthermore, the input provided by the NI is necessary to maintain eucapnic motor output and allow full expression of the CO_2 ventilatory response. Given this interpretation, the maximum levels of hypercarbia and PSR feedback utilized to stimulate breathing in this study (F_{CO_2}=0.035; P_L=0.5 kPa) produced episodes in only two frogs. Thus, the data do not support the hypothesis that the NI is responsible for clustering breaths into episodes,

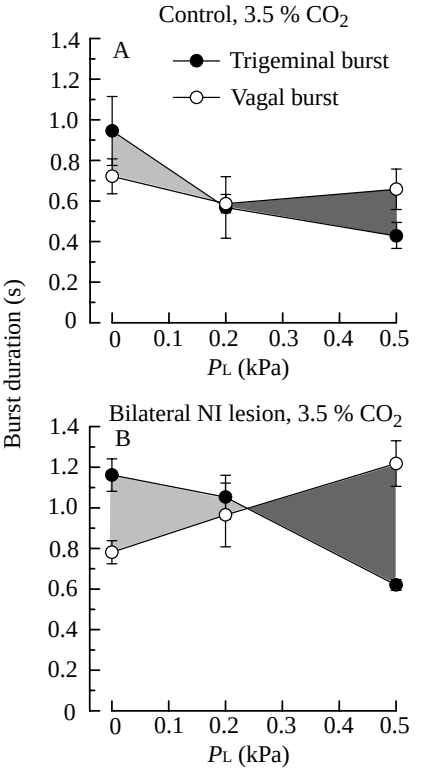

Fig. 7. The effects of changing pulmonary pressure (P_L) on the duration of the vagal (open circles) and trigeminal (filled circles) burst in bullfrogs with (A) (N=8) and without (B) (N=9) their nucleus isthmi (NI) intact. These data were obtained from animals artificially ventilated with 3.5 % CO_2 in air. Note that, when vagal burst duration equals or is less than trigeminal burst duration (light grey), no lung deflation would occur in spontaneously breathing bullfrogs. Conversely, the greater the extent to which vagal burst duration exceeds trigeminal burst duration, the more time is spent in expiration (dark grey). Data are given as means ± S.E.M.

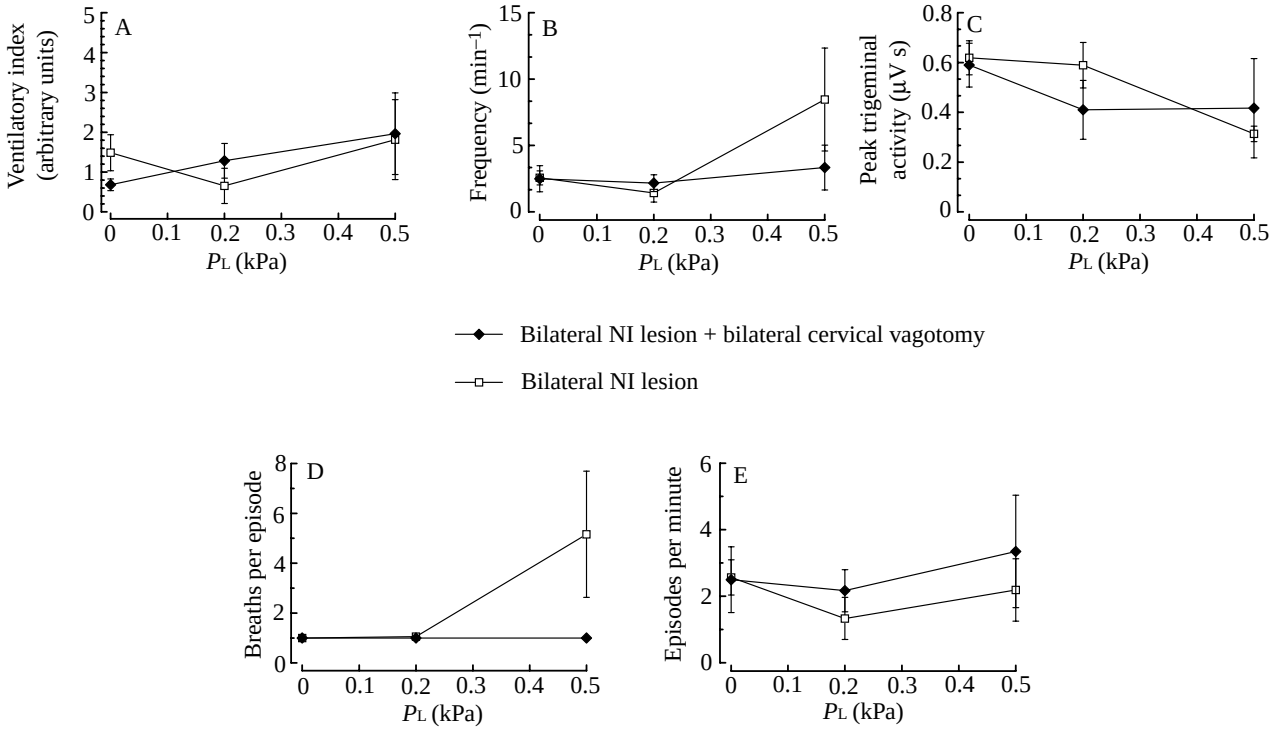

Fig. 8. The relationship between tonic changes in pulmonary pressure (P_L) and (A) ventilatory index ($\dot{V}_{index}$), (B) breathing frequency (f_L), (C) peak integrated trigeminal nerve activity (V_{Tindex}), (D) breaths per episode and (E) episodes per minute before (N=9; filled symbols) and after (N=4; open symbols) bilateral vagotomy in bullfrogs after bilateral lesion to the nucleus isthmi (NI). In this study, the bullfrogs were unidirectionally ventilated with 3.5 % CO_2 in air. Values are means ± S.E.M.

although they do suggest that, in addition to its role in visual function in frogs (Udin, 1987), the NI is an important site for the control of breathing and that glutamate is probably involved in its function.

The effects of bilateral lesions to the nucleus isthmi on respiratory reflexes

Effects on the hypercarbic response

The reduction of eucapnic ventilation and CO_2-chemosensitivity following bilateral lesions to the NI suggests that these neurons are important to chemoreception or integration of chemoreceptor information. These effects of NI lesions on breathing in frogs are similar to the effect of retrotrapezoid nucleus (RTN) lesions in anesthetized cats (Nattie and Li, 1990; Nattie *et al.* 1991) and awake rats (Akilesh *et al.* 1996). The RTN is a region of the rostral ventrolateral medulla ventromedial to the facial nucleus (Nattie, 1995). In these studies, unilateral lesions affected only the change in phrenic amplitude associated with CO_2 chemoreceptor stimulation and had no effect on the fictive breathing frequency. The net effect was a large reduction in sensitivity to hypercarbia because, in cats, the ventilatory response to hypercarbia is predominantly mediated by an increase in tidal volume, with breathing frequency remaining relatively constant (Schlaefke *et al.* 1979). Nattie and Li (1994) concluded that the role of the retrotrapezoid area was in the regulation of tidal volume rather than breathing frequency and that this area facilitated eucapnic ventilation and allowed full expression of CO_2-chemosensitivity. Although the RTN of mammals and the NI of frogs are not homologous structures, the analogous effects of lesions in these areas are striking.

From a neuroanatomical perspective, however, the NI may compare better with the pontine respiratory group (PRG), which is located more rostrally than the RTN. Lesions to the parabrachial and Kölliker–Fuse nuclei, which comprise the pontine respiratory group (PRG), reduce breathing frequency and increase tidal volume in rats (Fung *et al.* 1994) and cats (Denavit-Saubié *et al.* 1980). In cats, bilateral vagotomy after PRG lesions produces apneusis (a breathing pattern characterized by prolonged inspiratory efforts and short expiratory duration), or very slow, deep breaths, depending on the level of the lesion (Feldman and Gautier, 1976). These results indicate that both vagal feedback and the PRG provide a tonic input to eupneic breathing. Bilateral lesions to the NI had a similar effect on breathing frequency to PRG lesions in mammals and, in some cases, subsequent vagotomy slowed breathing further but never produced apneusis. The lack of information on the effects of PRG lesions on CO_2 responsiveness does not allow us to extend this comparison further. There is, nonetheless, an interesting parallel between the influence of the PRG and the NI on ventilatory control.

The discrepancies observed between the present and a previous study (Kinkead and Milsom, 1997) for data obtained in NI-intact bullfrogs were all related to the effects of

respiratory drive on breathing pattern. Overall, the intact animals behaved and responded to changes in P_L and/or respiratory drive in a fashion that closely resembled the responses described previously. For unknown reasons, the 1 % CO_2 in air mixture used in the present study did not always elicit changes in breathing pattern that were statistically significant, although a lower CO_2 level (air) did in previous experiments. This suggests that, unlike the effects of vagal inputs, the CO_2-responsiveness of the bullfrogs varied between the two studies.

Effects on the pulmonary pressure response

The effect of increasing PSR feedback on fictive breathing frequency was greatly reduced after bilateral KA microinjections. The shortening of the non-ventilatory pause provoked by increasing PSR feedback in frogs with their NI intact did not occur. This result was somewhat predictable given that PSR feedback and chemoreceptor input have previously been shown to interact to modulate f_L and breathing pattern in this species (Kinkead *et al.* 1994; Kinkead and Milsom, 1997) and that kainate lesions reduced the responsiveness of the frogs to CO_2/H^+. Thus, the only effect of changing P_L on breathing frequency was an increase in the number of breaths per episode that occurred at the highest P_L (0.5 kPa) and the highest level of chemoreceptor drive (F_{CO_2}=0.035). Bilateral NI lesions had no significant effect on the changes in $V_{T_{index}}$ or the timing of the fictive breaths elicited by PSR feedback, thus indicating that these reflexes do not require NI input.

Perspectives

The breathing pattern observed after NI lesions was very similar to that of premetamorphic tadpoles in many aspects. The ‘resting’ (normoxic, normocarbic) breathing pattern of tadpoles consists of single air breaths interspersed with gill ventilations. Also, in premetamorphic tadpoles prior to developmental stage XVI (staged according to the method of Taylor and Kollros, 1946), the lung frequency response to hypercarbia is poor (Infantino, 1989; Burggren and Infantino, 1994; Torgerson *et al.* 1996) and breathing episodes of more than one breath are rarely observed (Burggren and Infantino, 1994). The similarity of the breathing pattern of tadpoles and NI-lesioned adult bullfrogs is consistent with the correlation between the onset of episodic breathing, the emergence of CO_2 chemosensitivity and the developmental changes in the NI (Senn, 1972) that occur at metamorphosis in bullfrogs.

In summary, this study has demonstrated that bilateral lesions to the NI cause a reduction in respiratory motor output mediated only by a decrease in breathing frequency which is roughly proportional to the reduced sensitivity to hypercapnia and changes in pulmonary pressure. These findings lead to the conclusion that, in the bullfrog, the NI plays an important role in respiratory control by maintaining eucapnic motor output and allowing full expression of the CO_2 response, and that this function is likely to involve glutamate as a neurotransmitter. More complete anatomical, physiological and pharmacological characterization of this area of the amphibian brain will help us understand the function of these neurons in respiratory control.

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