Ultrasound measurements of fetal breathing movements in the rat

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Kobayashi, Koichi, Robert P. Lemke, and John J. Greer. Ultrasound measurements of fetal breathing movements in the rat. J Appl Physiol 91: 316–320, 2001.—The goal of this study was to determine when fetal breathing movements (FBMs) commence in the rat and to characterize age-dependent changes of FBMs in utero. These data provide a frame of reference for parallel in vitro studies of the cellular, synaptic, and network properties of the perinatal rat respiratory system. Ultrasound recordings were made from unanesthetized Sprague-Dawley rats from embryonic (E) day 15 (E15) to E20. Furthermore, the effects of respiratory stimulants (doxapram and aminophylline) and hypoxia on FBMs were studied. Single FBMs, occurring at a very low frequency (~8 FBMs/h), commenced at E16. The incidence of single FBMs increased to ~80 FBMs/h by E20. Episodes of clustered rhythmic FBMs were first observed at E18 (~40 FBMs/h). The incidence of episodic clustered FBMs increased to ~300 FBMs/h by E20, with the duration of each episode ranging from ~40 to 180 s. Doxapram, presumably acting to stimulate carotid body receptors, did not increase FBMs until E20, when the incidence of episodic clustered FBMs increased twofold. Aminophylline, a central-acting stimulant, caused an increase in episodic clustered FBMs after E17, reaching significance at E20 (3-fold increase). Exposing the dam to 10% O2 caused a rapid, marked suppression of FBMs (5-fold decrease) that was readily reversed on exposure to room air.

IN THE PAST DECADE, there has been an increasing use of perinatal rat models for examining the development of the central control of respiration. In particular, the advent of in vitro preparations has allowed for the detailed analyses of respiratory system development at the cellular, synaptic, and network levels. Such studies have demonstrated a major transformation of respiratory neuronal and muscle properties occurring prenatally (for review see Refs. 7 and 11). The relationship between respiratory system development and the inception and development of fetal breathing movements (FBMs) is of particular interest. Thus a clear understanding of the ontogeny of respiratory discharge during the prenatal period is required. Because of technical restraints, chronic instrumentation of the rat fetus for recording respiratory muscle electromyogram and tracheal pressure is not readily feasible. Rather, the basis for approximating the onset of fetal respiratory drive has been limited to in vitro recordings of phrenic nerve discharge in brain stem-spinal cord preparations (6, 9). Those data suggest that inspiratory drive transmission commences on embryonic (E) day 17 (E17) and that the frequency and amplitude of motor discharge progressively increase through birth (gestational period ~21 days). Whether the timing and age-dependent changes observed in vitro correlate with the naturally occurring behavior in utero was not known. Thus this study was undertaken to provide data that would characterize FBMs in utero from unanesthetized dams using ultrasound techniques. The FBMs were further characterized by monitoring the responses to the administration of respiratory stimulants (doxapram and aminophylline) and hypoxic gas mixtures, which have been shown to modulate FBMs in other mammalian species (4, 13, 15).

METHODS
Ultrasound recordings were performed on pregnant Sprague-Dawley rats during the period spanning gestational ages E15–E20 following procedures approved by the Animal Welfare Committee at the University of Alberta. The timing of dam pregnancy was determined from the appearance of sperm plugs in the breeding cages (designated E0) and confirmed by crown-rump length measurements taken from ultrasound images. Recordings were performed on each dam on consecutive days between 10 AM and 2 PM. A Hewlett-Packard (Andover, MA) SONOS 100CF machine with a 7.5-MHz mechanical sector probe was used for ultrasound recordings. Before the recording session, the dam was briefly anesthetized with halothane (1.2% delivered in 95% O2-5% CO2) while the abdominal hair was shaved, and the dam was placed into a Broome rodent restrainer (Harvard Apparatus, St. Laurent, PQ, Canada). The restrainer was modified by machining of an adjustable sliding opening for the insertion of the ultrasound probe adjacent to the dam’s abdomen (Fig. 1). The key to obtaining stable ultrasound recordings was the placement of a viscous acoustic standoff pad (Civco Medical Instruments, Calona, IA) between the dam’s abdomen and the ultrasonic probe. The recording sessions commenced 30 min after the cessation of anesthesia delivery and, unless otherwise stated, lasted for 30 min. A single fetus was fol-

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Fetal breathing movements in the rat

**RESULTS**

**Age-dependent changes in FBMs.** At E15, the only perceivable movement generated by the fetus was the beating of the heart. The first gross body movements were observed at E16. The first occurrence of sporadic single FBMs (7.8 ± 3.2 FBMs/h) interspersed between lengthy periods of inactivity was observed at E16. The incidence of isolated single FBMs increased with age, reaching 88 ± 9.0 FBMs/h by E20 (Fig. 2). By E18, episodes of clustered breathing movements were observed in two of the six animals (Fig. 2). The episodes of clustered FBMs, interspersed between times of no respiratory-related movements, became a consistent feature by E19 (6 of 6 cases). The total number of episodic clustered FBMs per hour increased markedly in an age-dependent manner (Fig. 2; 40.6 ± 22.8, 196.4 ± 89.8, and 301.1 ± 55.2 FBMs/h for E18, E19, and E20, respectively). Correspondingly, the total amount of time spent generating episodic clustered FBMs per hour increased with age (34.6 ± 22.6, 181.8 ± 74.2, and 269.6 ± 53.2 s for E18, E19, and E20, respectively).

We did not perform a detailed analysis of the pattern of FBMs within the episodic clusters at various ages. However, we did examine and quantify the patterns within episodic clusters of FBMs at E20 (n = 6), when FBMs were most robust. It was apparent that there were four basic patterns. The most consistent pattern (13 of 23 clusters analyzed, 56%) was that illustrated in Fig. 3A. The total duration of the FBM cluster was 57.8 ± 23.3 s (n = 13), and the frequency of FBMs followed a sinusoidal pattern within a given cluster. The pattern illustrated in Fig. 3B was observed 17.5% of the time, with an average duration of 177.3 ± 35.6 s (n = 4). The patterns shown in Fig. 3, C (duration 47.5 ± 9.2 s, n = 2) and D (duration 49.3 ± 7.6 s, n = 4), made up the remaining 10% and 17.5%, respectively, of episodic FBM clusters observed.

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Fig. 1. Methodology for measuring fetal breathing movements (FBMs) in the rat. The dam was placed into a modified rodent restrainer, and a 7.5-mHz ultrasound probe was used to monitor FBMs. A viscous acoustic standoff pad was positioned between the dam’s shaved abdomen and the probe. Images of single fetal rats were analyzed for ≥30 min and stored on videotape for later visual counts of clustered FBMs. A schematic representation is shown to illustrate that FBMs occurred as isolated single movements or as episodes of clustered movements lasting 40–180 s.

Fig. 2. Age-dependent changes in FBMs on embryonic (E) days 16–20 (E16–E20; n = 6). Episodes of clustered FBMs do not commence until E18, and there is a marked increase in their incidence through E20.
Effects of respiratory stimulants and hypoxia on FBMs.

The carotid body receptor stimulant doxapram did not affect FBMs until E20 (Fig. 4), when there was a significant increase in the total incidence (509.3 ± 95.5 vs. 267.7 ± 95.5 FBMs/h, n = 12) and duration (360 ± 77.4 vs. 158 ± 75.1 s, n = 12) of total FBMs. The doxapram-induced increase in FBMs persisted for 5–12 min. The centrally acting respiratory stimulant aminophylline caused an increase in FBMs that reached statistical significance at E20 (Fig. 5). Aminophylline induced the incidence (964.4 ± 155.5 vs. 394.1 ± 168.9 FBMs/h, n = 6) and duration (840 ± 136 s vs. 342 ± 179 s, n = 6) of total FBMs. The aminophylline-induced increase persisted throughout the 20-min recording session. The drug-induced increases in total FBMs were largely due to increases in the number of bouts of episodic clustered FBMs per hour. The numbers of single isolated FBMs were similar in control and doxapram- and aminophylline-treated animals (Figs. 4 and 5). No significant changes were observed in any parameters measured in response to saline injections.

We chose to examine the effect of hypoxia at E20, when FBMs were most prominent. As shown in Fig. 6, episodic clustered and single FBMs were dramatically reduced within 2 min of exposure to the hypoxic gas mixture. Specifically, the incidence and total duration of episodic clustered FBMs were decreased from 389.6 ± 171.4 to 77.6 ± 34.6 FBMs/h (n = 5, P < 0.05) and from 363.6 ± 162.2 to 78.1 ± 34.8 s (n = 5, P < 0.05), respectively. The incidence of isolated single FBMs was decreased from 78.2 ± 16.6 to 36.4 ± 12.7 FBMs/h (n = 5, P < 0.05). The suppression of FBMs...
DISCUSSION

FBMs have been observed in all mammals studied to date and appear to be necessary for normal fetal lung growth and maturation (16, 23). It has also been hypothesized that inspiratory drive transmission during the prenatal period is important for the functional development of respiratory neurons and muscle (7). The goal of this study was to ascertain when FBMs commence in the rat and to characterize the general age-dependent changes in FBMs prenatally. These data provide a frame of reference for parallel studies of age-dependent changes in the cellular, synaptic, and network properties of the perinatal rat respiratory system.

Methodological considerations. The resolution of the ultrasound recordings was sufficient to resolve the incidence of FBMs. However, because of technical constraints, the rat model does not lend itself to measurements of sleep and electroencephalographic state, which are critical determinants of FBMs (14). Furthermore, we could not follow the FBMs from a specific identifiable fetal rat for several days to obtain accurate measures of the total time spent generating FBMs. Nevertheless, by performing our recordings from a population of unanesthetized dams on a daily basis during the last trimester, we are confident that our recordings provide the data necessary for determining when FBMs commence in the rat and a general understanding of how the overall pattern changes prenatally. It should be noted that the dams were positioned in a restraining device during the recording sessions and appeared calm. However, the restraint may have caused a stress-induced increase in epinephrine levels and an ensuing increase in metabolism and the incidence of FBMs.

Characterization of rat FBMs. Four distinct movement patterns were discernable from our ultrasound recordings: 1) the beating of the fetal heart, 2) general gross body movements, 3) brief large-amplitude movements similar to movements characterized as fetal hiccups, and 4) lower-amplitude, periodic movements of the diaphragm and rib cage. At E15, only the heartbeat was observed. By E16, general body movements and hiccup-like movements were obvious. Movements characteristic of single FBMs were observed occasionally at E16 and more often by E17. Episodes of clustered FBMs commenced by E18 and increased in frequency in an age-dependent manner. Compared with other mammals studied, episodic FBMs in the rat commence at a relatively late stage of gestation. For comparison, FBMs commence at week 10 of gestation in humans and at day 50 in sheep (i.e., later in the 1st trimester) (10).

These ultrasound data are consistent with data derived from recordings of in vitro brain stem-spinal cord preparations isolated from fetal rats. In vitro, very-low-frequency, low-amplitude inspiratory discharge can be recorded from cervical ventral roots commencing at E17 (9). The frequency and amplitude of inspiratory discharge in vitro also increase markedly from E18 to E20 (6). The timing of the inception of FBMs is also consistent with the anatomic data showing that the descending bulbo spinal axons reach the cervical cord by E16 (17) and that diaphragm myotubes form by about E17 (1). Furthermore, the morphological and electrophysiological properties of phrenic motoneurons and the contractile properties of the diaphragm musculature undergo major transformations between E17 and E20, when FBMs become more robust (2, 18–21). The most obvious difference between the in vitro and in utero respiratory activity is the episodic nature of the in utero FBMs. However, the in vitro preparations lack supramedullary structures, which are thought to be important for regulating episodic, state-dependent control of FBMs (14).

Modulation of rat FBMs. The respiratory stimulants and hypoxic gas mixtures were administered to further characterize FBMs. The effects of administering respiratory stimulants on rat FBMs were similar to those reported in studies of other mammalian species (4, 13). Doxapram and aminophylline caused rapid increases in FBMs. The effects of doxapram were transient, which reflects the short half-life of the drug (3). In contrast, aminophylline remains in the circulation for hours and thus caused a prolonged increase in the incidence of FBMs. Doxapram, at the concentration we administered, is thought to stimulate respiration by acting on peripheral carotid body receptors, and it may be that the transduction mechanism underlying its action has not developed fully until E20. Alternatively, as evident from preliminary studies (22), the synaptic connections necessary for transmitting carotid body receptor signals to the respiratory rhythm-generating center could be underdeveloped before E20. Aminophylline acts centrally by a combination of antagonizing the actions of adenosine and stimulating intracellular cAMP levels (5, 12). The trend was for aminophylline to stimulate episodic clustered FBMs from E18 onward. However, the variation in the incidence of FBMs was such that statistical significance was not achieved until E20. It should be noted that we did not perform a dose-response study with the stimulants, and thus we cannot ascertain the limits of respiratory rhythogenesis at various stages of fetal rat development. However, in vitro studies have demonstrated that there can be a marked stimulation of respiratory rhythm in response to neuromodulators such as thyroid-releasing hormone as early as E17 (9).

Previous studies have demonstrated that exposure to acute hypoxia causes a rapid suppression of FBMs, electrooculal activity, and decrease of muscle tone (4). The precise mechanism underlying the hypoxia-induced suppression of FBMs is not known, but it has been established that centers located rostral to medul-
lary rhythm-generating centers are essential. We did not have a means of measuring the PO$_2$ in the fetal tissue, but the paradigm of exposing the dam to 10% O$_2$ is a standard protocol for producing a hypoxic response in utero. Indeed, there was a marked depression in the frequency of rat FBMs in the presence of hypoxic gas mixture delivered to the dam within minutes of exposure.

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