Laryngeal apnea in rat pups: effects of age and body temperature

Luxi Xia, James C. Leiter, and Donald Bartlett, Jr.

Department of Physiology, Dartmouth Medical School, Lebanon, New Hampshire

Submitted 5 July 2007; accepted in final form 19 October 2007

METHODS

The Institutional Animal Care and Use Committee of Dartmouth College approved the protocols in these studies. The experiments with the LCR were done using 35 Sprague-Dawley rat pups of both sexes, ranging in age from 3 to 21 days. The pups were kept with the mother and littermates before use, and the mothers had constant access to standard rat chow and water.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: J. C. Leiter, Dept. of Physiology, Dartmouth Medical School, Lebanon, NH 03756 (e-mail: james.c.leiter@dartmouth.edu).

First published October 25, 2007; doi:10.1152/japplphysiol.00721.2007.


© 2008 the American Physiological Society

Innovative Methodology

Because the apnea elicited by the LCR in neonates may be prolonged, even fatal in some anesthetized experimental animals (31), it has long been suspected that the LCR plays a role in some cases of the sudden infant death syndrome (SIDS). All infants regurgitate gastric contents from time to time (15), and the reflex apnea resulting from this material getting into the larynx may be enhanced by the low pH imparted by gastric acid (1, 35).

SIDS has been linked epidemiologically with heat stress. Infants are sometimes found in overheated rooms, wrapped excessively with blankets, and occasionally covered with sweat (9, 19, 26, 37). Prone infants lose heat to the environment more slowly than supine infants do (3), and some have speculated that heat stress may contribute to the higher incidence of SIDS in prone than in supine infants (12, 23).

Our laboratory has recently examined the interaction of the LCR and body temperature in unanesthetized, decerebrate piglets aged 3–16 days. The animals were vagotomized, paralyzed, and ventilated, and respiratory activity was recorded as phrenic nerve discharge. Under baseline conditions, the injection of 0.1 ml of water into the laryngeal airway through a transnasal catheter elicited an apneic period of 6–10 s. When body temperature was raised ~2°C by external heating, the same stimulus elicited a greatly prolonged apneic response: 46 s on average. This influence of body temperature was reversible and could be shown several times in the same animal (5). Further studies in piglets have shown that the response to whole body heating can be duplicated by focal warming of the brain stem in or near the nucleus of the solitary tract (NTS) (46) and that it can be reversed by microdialysis of antagonists of γ-aminobutyric acid (GABA) in the NTS region (47). Our laboratory has also studied the response to electrical stimulation of the SLN and found that the resulting respiratory disturbance, like that of the LCR, is greatly enhanced by hyperthermia (2).

Our piglet preparation has advantages for elucidating the mechanisms responsible for the thermal enhancement of laryngeal apnea, but we also need to develop a means for studying the phenomenon in animals with intact central nervous systems and for examining the influence of prenatal events, such as maternal exposure to cigarette smoke, and the influence of postnatal maturation. Accordingly, we have developed a method for studying laryngeal apnea in rat pups and have examined the influence of mild hyperthermia in animals of different ages.

RESEARCH DISCLOSURES

This work was supported by National Institutes of Health Grants HL34922, HL50499, and HL07557.

Address for reprint requests and other correspondence: J. C. Leiter, Dept. of Physiology, Dartmouth Medical School, Lebanon, NH 03756 (e-mail: james.c.leiter@dartmouth.edu).

Copyright © 2008 the American Physiological Society
Innovative Methodology

Each pup was anesthetized initially with 3–4% halothane. Once the animal was unconscious, urethane (1.0 mg/kg) and chloralose (20 mg/kg) were given by intraperitoneal injection. Halothane was then gradually withdrawn over the next 15–20 min so that by the time the study was begun, chloralose and urethane were the only active agents. A thermistor probe was inserted into the rectum to record body temperature, which was controlled initially at ~36°C (33) by means of a thermostatically regulated heating pad.

The animal was placed in the supine position, and hooked wire electromyogram (EMG) electrodes were placed in the trunk musculature to record diaphragm activity. Single-filament (0.002-in. diameter) stainless steel Teflon-coated EMG wires (A-M Systems, Everett, WA) were introduced into the intercostal muscles in the lowest part of the rib cage in the anterior axillary line, one on each side of the chest, using a 25-gauge needle in smaller animals (postnatal day 3 [P3–P10] and 0.015-in.-diameter braided fluorocarbon-coated EMG wire (Cooner Wire, Chatsworth, CA) introduced with a 23-gauge needle in older animals (>P10). A grounding wire was inserted subcutaneously into the skin over the abdomen. The EMG signal was amplified, moving water was made into the rostral trachea using a 100-μs time constant, and displayed on a monitor along with body temperature. A midline skin incision was made in the neck, and the cervical trachea was freed from adjacent tissues with the aid of an operating microscope (model OPMI, Zeiss, Germany). Care was taken to identify the recurrent laryngeal nerves and SLNs and avoid them in the dissection. The trachea was opened with a transverse incision that exposed the lumen but left the posterior tracheal wall intact. This permitted the animal to breathe freely through the unintubated caudal segment of the trachea. The rostral segment of the trachea was cannulated with polyethylene tubing, heat tapered at the tip to about PE10 outside diameter (0.6 mm). The tapered tubing was advanced until it was gently wedged into the trachea with its tip just caudal to the larynx and was secured in that position with a fine-silk ligature. The ligation (2-0 or smaller silk) may have damaged the recurrent laryngeal nerve; we made no effort to assess laryngeal muscle function. The animal was then tilted ~20° head down so that water injected into the rostral trachea and larynx would run out through the nose and mouth.

After recording stable breathing for at least 10 min, 0.1-ml injections of water were made into the rostral trachea by means of a 1-ml Hamilton syringe and a programmable infusion pump (model SP100i, WPI, Sarasota, FL), starting at the beginning of inspiration. The LCR is particularly easy to elicit during inspiration, and calculating the onset of the reflex is facilitated by giving the injection during inspiration. At least 5 min passed between each sequential test of the LCR. After three trials under baseline conditions, 23 of the animals were warmed to ~38°C, and another 3 trials were made. Finally, body temperature was lowered back to ~36°C, and a further three trials were performed. In five animals, the procedure was repeated after section of both SLNs to verify that the responses were mediated by these nerves. Time control experiments were performed with 12 pups following the same protocol, but with no heating.

An additional 17 animals (aged 4–21 days) were studied with a similar protocol but with electrical stimulation of an SLN rather than with intralaryngeal water. Both SLNs were identified and sectioned as close to the larynx as possible. The central cut end of one SLN was placed on a bipolar stimulating electrode in a mineral oil pool. Each trial consisted of a 5-s train of 0.3-ms constant-current pulses at a frequency of 25Hz. The current was started at a very low value and increased stepwise until peak integrated diaphragmatic EMG activity was decreased by at least 50% for at least two breaths: this was taken as the threshold current for the rat. The remainder of each experiment was conducted with stimulating current at 1.5 times the threshold current.

Because the response to laryngeal stimulation by either method is a complex mix of respiratory inhibition and activities that clear the airway, such as swallowing, we operationally defined the duration of each response as the period of respiratory disruption from the beginning of the stimulus until the onset of at least five regular breaths (43). We separately analyzed the duration of the longest period of apnea occurring during the response.

We analyzed each experiment using an ANOVA (ANOVA procedure or the general linear model procedure in SYSTAT, Chicago, IL) and the average response of each animal in each condition. We used a repeated-measures design in which body temperature was a within-subjects factor with three levels (control, test, and recovery). When we examined the effect of age on the response to laryngeal stimulation or SLN stimulation, age was treated as a random variable, and treatment group (time control, LCR, or SLN stimulation) was a categorical between-subjects factor. Specific preplanned comparisons were made using P values adjusted by the Bonferroni method when the ANOVA indicated that significant differences existed among treatment groups. Data are presented as means ± SE.

RESULTS

The laryngeal chemoreflex. As illustrated in Fig. 1, the LCR, however measured, was significantly prolonged when body temperature was increased from 35.8 to 38.7°C in this case. The thermal prolongation of the LCR was reversed when body temperature was reduced back to 36.2°C. The average responses of apnea duration and LCR or the duration of respiratory disruption following SLN stimulation and the average body temperature in each test condition are shown in Table 1 for the time control tests (no elevation of body temperature during the second test period), tests of the LCR and electrical stimulation of the SLN. The ANOVA indicated that significant differences existed among treatment groups (control, LCR, and SLN stimulation) and among thermal conditions (control, hyperthermia, recovery) for all three variables. Thus the apnea duration and the duration of the LCR were significantly longer when body temperature was elevated compared with the baseline control temperature condition (P < 0.05 for both variables). In addition and by design, body temperature was significantly elevated above the baseline value. However, all of these variables returned to baseline values when the animal...

Fig. 1. Responses of a 14-day-old male rat pup to intralaryngeal injection of water. From top to bottom, the tracings show respiratory responses under baseline conditions, during hyperthermia, and after recovery. BT, body temperature (in °C). Note the exaggerated response to intralaryngeal water during the hyperthermic trial.
was cooled, and none of the variables measured in the recovery period was significantly different from the initial control period. A similar pattern was apparent in the response to stimulation of the SLN; apnea duration and the period of respiratory disruption increased significantly ($P < 0.05$) when body temperature was elevated but not when the animal was cooled back to the control temperature. Apnea duration, LCR duration, and body temperature did not change significantly throughout the experimental protocol compared with the control condition in animals in the time control treatment group. There was no significant difference in any of the variables between the responses of male and female animals.

Apnea duration and the duration of respiratory disruption after laryngeal stimulation did not vary as a function of age in the control group. This indicates that the measurements of the LCR were stable among animals over the range of ages we studied. Moreover, apnea duration and respiratory disruption did not vary as a function of age in any of the test groups (control, LCR, and SLN stimulation) in the absence of hyperthermia. These findings indicate that the pattern of the LCR during normothermia did not vary as the rat pups matured: apnea and respiratory disruption were still present in the oldest animals that we studied. However, the thermal prolongation of the LCR and of the respiratory disruption after SLN stimulation was enhanced in young animals. This is demonstrated in Fig. 2, which shows the prolongation of the LCR duration by heating, relative to the average of the baseline and recovery values, as a function of age. The age dependence of the thermal prolongation of the reflex response was not different between the SLN stimulation group and the LCR group, and, therefore, the data from these groups were combined for this analysis. The slope of the regression line relating the thermal prolongation of apnea to animal age was significantly different from the control condition in the first data column, $P < 0.05$.

DISCUSSION

There are two important issues in these studies. The first issue is technical and pertains to the selection of animal models in studies of neonatal reflexes. The second issue pertains to the age dependence of the behavioral manifestations of the LCR and to the age dependence of the thermal prolongation of the LCR.

Studying the LCR in neonatal animals. Historically, animal studies of neonatal laryngeal reflexes have been conducted in piglets (5, 7, 22, 43) and lambs (11, 14, 36, 39) and less frequently in puppies and kittens (1, 13, 17). These choices have been driven primarily by the size of the animals; these neonates are large enough to accommodate the surgical instrumentation necessary to elicit and study the physiological details of the reflex response. However, piglets and lambs are precocial. They walk soon after birth, have adult patterns of thermoregulation within hours of birth, and have well-developed cycling between rapid eye movement (REM) and non-REM sleep (4, 43), to name but three of the many relatively mature responses they possess that are quite different from human infants at the same age. The duration of the neonatal period is, therefore, short.

For these reasons, we have developed methods to let us study a variety of cardiorespiratory reflex responses in neonatal rodents. Neonatal rodents, in contrast to piglets and lambs, are altricial; the neonatal period stretches over at least 3 wk. Neonatal rodents regulate body temperature poorly until non-REM sleep (4, 43), and inhibitory (8) neurotransmitter systems extend from human infants at 2–6 mo and are thus highly relevant to the pathogenesis and incidence of SIDS in this age range in human infants. For example, inhibitory neurotransmitter systems in the human cortex seem to mature within the first 3 mo of life (8), and other neurotransmitter...
systems also seem to mature in this time frame (18). Adult sleep patterns also begin to emerge from 3 to 12 mo of age in human infants (16). Thus many developmental milestones during the neonatal period in humans seem to occur in rodents in the third and fourth week of life may provide insight into infant development in humans after P15, and studies in rodents during the time of the peak incidence of SIDS.

Rodents are, therefore, advantageous for studies of neonatal maturation, but they are small. Three technical modifications to our usual study protocols permitted us to study the LCR in neonatal rats. First, we used the diaphragm EMG to monitor respiration while the animal breathed spontaneously. Second, each rat pup was tracheostomized by making an incision part way through the trachea below the larynx, but the distal trachea was not intubated. Finally, a beveled and heat-shaped piece of polyethylene tubing was fitted into the rostral trachea. This tube isolated the lower trachea and protected the lungs, but also it provided a means of stimulating the larynx with water. We should also note that the anesthesia, although not unusual, must be titrated with care. Any one of these changes is minor, but each was required to achieve a stable neonatal preparation in which we could effectively study the LCR over an extended period.

In planning these studies, the question arose as to what baseline body temperature should be chosen for the rat pups. We selected 36°C guided by the report of Schmidt et al. (33), who measured core temperatures in week-old rats under natural conditions, huddling with the mother and littermates. Body temperature measurements in individual rat pups also indicated that the “normal” body temperature was in the range of 36 ± 0.5°C from ages P3 to P21 (34). This relatively low body temperature is also a manifestation of the immaturity of thermoregulatory processes in rodents. We rejected higher temperatures based on this report and on preliminary trials indicating that some pups heated from 38 to 40°C did not survive.

One may also wonder whether the laryngeal stimulus we used to study the LCR (0.01 ml of water injected retrograde into the larynx) was a consistent stimulus across all the ages we studied. First, the tracheal dimensions did not change much between P3 and P21 animals; tubing tapered to the outside diameter of PE-10 tubing fit animals of all ages throughout this study. Second, we gave some animals larger fluid boluses, and we saw no consistent change in the duration of apnea or the LCR. Thus 0.01 ml of water seemed to elicit the LCR effectively in all the ages we studied.

**Age-dependence of the thermal prolongation of the LCR.** The results of these experiments demonstrate that the LCR can be elicited reproducibly in rat pups with an intact brain and that the enhancement of the response with increased body temperature, first identified in decerebrate piglets (5), is clearly demonstrable in the youngest animals and becomes less prominent with postnatal age. A similar response pattern exists for SLN stimulation, as first shown in canine puppies by Haraguchi et al. (13) and more recently in piglets (2).

The duration of the respiratory response to intralaryngeal water was briefer in these experiments than in our laboratory’s earlier studies with decerebrate piglets (5, 46, 47). This is partly attributable to the fact that in the rat pups, the drive to breathe increased throughout the apneic period due to progressive hypoxia and hypercapnia, whereas these stimuli remained constant in the artificially ventilated piglets. A further consideration is that normal cyclical cardiovascular and respiratory events are briefer in smaller animal species (40), and it may be that reflex events are briefer as well. Finally, more of the brain is present in the neonatal rats than in the decerebrate piglet studies, and these higher centers may contribute to the overall drive to breathe, tending to limit the duration of reflex apnea and the duration of the LCR (21). The rat pups were anesthetized with chloralose, which tends to prolong the LCR (22), but...
the net effect of all these factors was still a reduction in the duration of the LCR.

On the other hand, the duration of the LCR was remarkably consistent within the age range of animals that we studied, and one may ask whether a constant LCR duration represents the same degree of respiratory inhibition in animals of different ages. This question is not easily answered. One way to address this issue is to express the LCR duration as a fraction of an estimate of respiratory drive. The average respiratory frequency, which we can measure accurately, was $94 \pm 6$ breaths/min and constant across the developmental period we studied, findings similar to previous studies (6). Using the respiratory period (total time), to normalize the LCR duration leads to the conclusion that the LCR and degree of respiratory inhibition were constant across animals aged P3–P21. On the other hand, minute ventilation increases during this period of development as a result of an increase in tidal volume as the animal grows (6, 30, 32), and body weight did increase consistently as a function of age in our study [$body$ $weight$ ($g$) = $3.3 + 2.2 \times age$ (days); $r^2 = 0.75, P < 0.001$ for both intercept and slope]. The LCR duration is usually inversely related to respiratory drive (21, 22, 43), and the constant duration of the LCR as tidal volume and minute ventilation increase in older animals may imply that the LCR is actually relatively more potent in older animals. Thus the normalization schemes do not lead to a consistent conclusion, and the simplest hypothesis, given the data we have, is that the LCR was unchanged over the developmental period from P3 to P21. A better approach may be to measure the consequences of the LCR more effectively. Unfortunately, we did not measure the degree of hypoxemia or hypercapnia associated with apnea and the LCR in our studies, but these values might be more useful for judging the relative strength of the LCR among animals of different ages.

The temporal evolution of the manifestations of the LCR as animals mature has been studied in a variety of mammals, including humans (11, 22, 36, 41). Apnea, bradycardia and redistribution of blood flow are typical of immature neonatal animals, but cough and swallowing, which are present in the neonate as well, become the sole manifestations of the LCR in older animals and adults (41). The rate at which the neonatal pattern of the LCR wanes and is superceded by the adult pattern of coughing and swallowing also varies among species. In lambs delivered 13–16 days prematurely, apnea and bradycardia, although present at age P7, were absent when the LCR was stimulated at age P14 (36), and lambs born at term have little or no apnea and bradycardia at ages P4–P6 (35). Similarly, neonatal piglets >1 mo of age no longer demonstrated reflex apnea when the larynx was stimulated even though apnea was common in younger animals (22). In kittens, a more altricial species than pigs or sheep, apnea is present at ages P5–P14, swallowing appears as the major part of the reflex response by P28–P30, but the adult pattern of frequent swallowing does not emerge until P60–P70 (25). In puppies, apnea is very prominent during the first 10 days of postnatal life, but is not an important component of the response after P30 (1). In the rat pups that we studied, the apneic response was still present at age P21. We did not see any variation in the strength of the LCR over the age range we studied. We suspect that the apneic component of the LCR wanes in older rats and swallowing and cough emerge, but this transformation probably occurs after age P21.

Although apnea was present in all ages of rats that we studied, the thermal prolongation of apnea and respiratory disruption diminished over the entire age range. The youngest rats had the greatest thermal prolongation, and the thermal prolongation of the LCR had disappeared by P21 even though apnea was still evident at that age.

The mechanism by which mild hyperthermia enhances respiratory inhibition due to laryngeal stimulation is not addressed in these studies. The response, however, is very similar to that demonstrated in decerebrate piglets (5), shown to depend on warming in or near the NTS (46) and to be reversible by pharmacological antagonism of GABA either systemically (2) or in the region of the NTS (47). The new findings show that the influence of hyperthermia occurs in infant animals with intact central nervous systems and that it is importantly affected by age. Without knowing more about the central neuronal circuitry of the LCR, it is difficult to speculate about the processes whereby the thermal enhancement of the LCR and then the apneic responses of the LCR wane during maturation. However, these seem to be two separate processes with two distinct temporal patterns of maturation. The thermal enhancement of the LCR seems to depend on neural circuitry within the dorsal medulla (46, 47) and disappears beyond P21 in rat pups. Finally, the age-dependent decline in the potency of the thermal prolongation of the LCR is similar to the reduction in the strength of a variety of reflexes, including the LCR itself, that inhibit respiration in neonates (23).

GRANTS

This work was supported by National Institute of Child Health and Human Development Grants HD-36379 and HD-042707 and by a grant from the Flight Attendant Medical Research Institute.

REFERENCES

Innovative Methodology