Changes in respiratory timing induced by hypercapnia in maturing rats

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Abu-Shaweesh, J alal M., Ismail A. Dreshaj, Agnes J. Thomas, Musa A. Haxhiu, Kingman P. Strohl, and Richard J. Martin. Changes in respiratory timing induced by hypercapnia in maturing rats. J. Appl. Physiol. 87(2): 484–490, 1999.—Premature infants respond to hypercapnia by an attenuated ventilatory response that is characterized by a decrease in respiratory frequency. We hypothesized that this impaired hypercapnic ventilatory response is of central origin and is mediated via γ-aminobutyric acid-ergic (GABAergic) pathways. We therefore studied two groups of maturing Sprague-Dawley rats: unrestrained rats in a whole body plethysmograph at four postnatal ages (5, 16–17, 22–23, and 41–42 days); and ventilated, decerebrate, vagotomized, paralyzed rats in which phrenic nerve responses to hypercapnia were measured at 4–6 and 37–39 days of age. In the unrestrained group, the increase in minute ventilation induced by hypercapnia was significantly lower at 5 days vs. beyond 16 days. Although there was an increase in tidal volume at all ages, frequency decreased significantly from baseline at 5 days, whereas it increased significantly at 16–23, and 41–42 days. The decrease in frequency at 5 days of age was mainly due to a significant prolongation in expiratory duration (TE). In the ventilated group, hypercapnia also caused prolongation in TE at 4–6 days but not at 37–39 days of age. Intravenous administration of bicuculline (GABAA-receptor blocker) abolished the prolongation of TE in response to hypercapnia in the newborn rats. We conclude that newborn rat pups exhibit a characteristic ventilatory response to CO2 expressed as a centrally mediated prolongation of TE that appears to be mediated by GABAergic mechanisms.

THE HYPERCAPNIC VENTILATORY response is characterized by a sustained increase in minute ventilation (VE), which appears qualitatively similar between newborns and adults (32). Quantitative characterization of the hypercapnic response during postnatal maturation has not, however, generated consistent results. Although some investigators have shown that, per body weight, the ventilatory response to hypercapnia in term neonates is similar to that in adults (32), others have found that hypercapnic sensitivity increases with age in preterm infants (33) and preterm primates (15). Furthermore, premature, but not term, lambs or term piglets exhibit an attenuated hypercapnic response (8, 35), and newborn rats, being immature at birth, express a lower ventilatory response to hypercapnia at 2 compared with 8 days of life (26). It, therefore, appears that prematurity and/or immaturity are important contributors to the attenuated hypercapnic response in newborns of various species. Preterm infants exhibit an impaired hypercapnic response in response to hypercapnia (11, 29).

The mechanisms underlying impaired hypercapnic responses in preterm infants and premature animals have not been fully identified. Krauss et al. (23) observed simultaneous improvement in lung compliance in parallel with maturation of the hypercapnic response in infants. Respiratory inhibition could also be attributed to laryngeal reflexes triggered by inhaled high levels of CO2 (3) or an exaggerated Hering-Breuer reflex (26). Frantz et al. (13) suggested a central neural origin for this phenomenon. This impairment of the hypercapnic response could be mediated by inhibitory neurotransmitters that have been implicated in the control of breathing, such as γ-aminobutyric acid (GABA) (6). In the mammalian central nervous system, GABA is the main inhibitory neurotransmitter, where it exerts its effect via ionotopic (GABA_A) receptors to produce fast synaptic inhibition and through metabotropic (GABA_B) receptors to produce slow, prolonged inhibitory signals (31). We assumed that hypercapnia-induced changes in respiratory timing in preterm infants, namely prolongation in TE, could be mediated by release of GABA and activation of GABA_A receptors, causing fast synaptic inhibition of the initiation of a respiratory burst and resultant TE prolongation.

In the present study we sought to test the initial hypothesis that rat pups would exhibit prolongation of TE, resulting in an impaired ventilatory response to hypercapnia comparable to that observed in preterm infants. Furthermore, we hypothesized that central neural mechanisms underlie this response and that the inhibitory neurotransmitter GABA might be implicated. To avoid the confounding effects of anesthesia on the central respiratory-related network, studies were performed in nonsedated, free-moving rats. Furthermore, we excluded any reflex effects of intralaryngeal CO2 and of vagal input on breathing by performing a second series of experiments in decerebrate, vagotomized, intubated, and mechanically ventilated rats.

METHODS

We performed a series of studies in two groups of maturing rats. The first group consisted of unanesthetized, unre-
strained rats in which the hypercapnic ventilatory response was studied at different ages to characterize its maturation and the relative contributions of tidal volume (VT) and frequency to this hypercapnic response. The second group of rats consisted of decerebrate, vagotomized, paralyzed, tracheostomized, and mechanically ventilated rats in which the phrenic nerve response to hypercapnia was measured at different ages. The experiments in the ventilated rats allowed us to isolate the responses of central phrenic neural output in the absence of vagal afferents, upper airway reflexes, and changes in the mechanical properties of the lung. In a subgroup of ventilated rats, we also studied the effect of administration of a GABA inhibitor (bicuculline) on the phrenic nerve response to hypercapnia.

Unrestrained rats. All study protocols were approved by the Institutional Animal Care and Use Committee. These studies were initially performed sequentially in one litter of nine Sprague-Dawley rats at 5, 16–17, 22–23, and 41–42 days of life. The entire group survived the 6-wk duration of this experiment. Weights were as follows: 10.1 ± 0.5 (SD) g at 5 days, 33.9 ± 1.4 g at 16 days, 54.9 ± 2.0 g at 22 days, and 161.3 ± 6.2 g at 41 days. The older animals maintained their temperature without extra assistance. The temperature of the 5-day-old rats was monitored by an infrared temperature probe and was maintained by using a warming pad to maintain a stable environmental temperature of 33°C. In an additional group of newborn rat pups (n = 6), we measured esophageal temperature (Physitemp, model BAT-12, Sansorteck, Clifton, NJ) via a precalibrated probe while the animals were in a cage under the same environmental conditions as in the other experiments. Esophageal temperature was stable throughout the experiment at 35.6 ± 0.5°C and was not influenced by exposure to 5 min of CO₂.

The rats were acclimatized to the chambers in which ventilation was measured for 30–60 min before testing was started. The animals were then exposed to the test gases in the following order: room air, 5% CO₂ in room air for 5 min, followed immediately by 10% CO₂ in room air for 5 min. Exposure time did not include the 60-s period allowed for equilibration of gas in the box. The ventilatory parameters were recorded continuously throughout the exposure. VT and respiratory frequency were averaged from 20 consecutive breaths at the end of room air and hypercapnic exposures. At 16–17, 22–23, and 41–42 days of age, the animals were clearly awake during measurements. The sleep-wake state of the 5-day-old animals was difficult to identify.

Ventilation was measured by whole body plethysmography in chambers that were modified for the unanesthetized, unrestrained rats as previously described (4, 36). Round Plexiglas chambers of different sizes (0.6 liter at 5 and 16–17 days and 8.4 liters at 22–23 days and 41–42 days) were used. These chambers contained ports to measure the following parameters: flow rate of air or test gas flowing through the chamber, pressure swings in the chamber (Validyne transducer), and the fractional content of CO₂ and O₂ in the chamber. The flow rate through the chamber was calibrated as in previous studies to prevent CO₂ build up (6–10 times Ve) (36). The chamber was flushed rapidly with air or the test gas (2 l/min), and equilibrium was attained after 20 s. Calibration for VT changes was performed at the average spontaneous respiratory frequency, ~2 breaths/s. Ventilatory parameters were recorded both on strip chart and by an analog-to-digital converter coupled to a computer. Two setups were available, each using the same transducers and monitors so that simultaneous studies could be performed.

We subsequently performed a series of additional studies in unrestrained animals to confirm our initial findings in the single litter of animals. These studies allowed us to characterize the contributions of inspiratory duration (TI) and TE to the respiratory frequency response to hypercapnia at two different ages in a larger group of animals. The same equipment as already described was employed; however, only exposure to 5% CO₂ was performed. Studies were performed in rats at 5 and 22–23 days of age. Three litters comprising 29 rats were studied at 5 days, and two litters comprising 16 rats were studied at 22–23 days. VT, frequency, TI, TE, and Ve were averaged by sampling 50 breaths every minute in room air and 50 breaths every minute during exposure to 5% CO₂.

Ventilated rats. These experiments were performed in Sprague-Dawley rat pups at 4–6 and 37–39 days of age, with group sizes of eight per each protocol. The animals were decerebrated by using the following procedure. Initially, rat pups were anesthetized with 2% Metofane in oxygen and then were quickly removed from the anesthetic vessel and placed prone on a heating pad. The scalp was cut, and, with sharp forceps, a narrow fissure was made on the interparietal bone perpendicular to the sagittal plane, midway between the lambdoid and the occipital suture. A stainless steel wire (0.2-mm) loop was advanced through the fissure and brain tissue at an angle of ~70° until the tip touched the floor of the skull. The wire loop was swung gently left and right, completing transection of the brain stem at the midcollicular level. Thereafter, the loop was removed and skin edges approximated and closed with adhesive tape. This procedure ensured complete decerebration at the desired level with minimal blood loss. Completeness of decerebration and the level of brain stem transection were confirmed in each animal at the end of the study.

After decerebration the animals were intubated through a tracheostomy and ventilated with a volume ventilator (model 683, Harvard Apparatus) adjusted for small VT values. To avoid hypoxia, the animals were ventilated with 30% O₂-balance N₂. The frequency and VT were adjusted such that the animals were given Ve of 1 ml·g⁻¹·min⁻¹. The jugular vein was cannulated with PE-10 tubing for administration of fluids and drugs. Muscular paralysis was achieved by gallamine triethiodide (4–6 µg/g). Both vagi were dissected and cut at the cervical level. The body temperature was maintained between 36 and 36.5°C by means of a heating pad throughout the experiment. The phrenic nerve was dissected and cut, the proximal end was placed on fine platinum bipolar electrode, and the neural output was recorded. The electrical activity from the nerve was amplified, filtered (band pass 0.03–3 kHz, Grass 511I, rectified, and passed through a Paynter filter to produce a moving average neurogram. The neurogram was recorded continuously on a Gould chart recorder before and during hypercapnic exposure.

The animals were left to recover for 30 min before recordings were started. The moving average of phrenic nerve activity was recorded before and during 10-min exposure to a gas mixture of 5% CO₂ in 30% O₂-balance N₂. TI, TE, and frequency were measured on a breath-by-breath basis and averaged from 20 breaths in the control period and from 20 breaths at 2.5, and 10 min of hypercapnic exposure. Measurements of respiratory timing were based on the peak activity of the moving average traces. TI was defined as the interval between the onset of phrenic nerve discharge and its peak, and the TE was determined from the point of rapid decline of peak phrenic nerve activity to the onset of the next phrenic nerve discharge. Because of differences in the gain of amplifiers used to record moving average phrenic nerve discharge, changes in amplitude associated with hypercapnic exposure were not compared between different age groups.
In a subgroup of animals (n = 6), after the phrenic nerve response to hypercapnia was recorded, the effect of intravenous administration of the GABA_A blocker (bicuculline 2 mg/kg body wt) on the hypercapnic responses of phrenic nerve timing was examined. When given systemically, bicuculline crosses the blood-brain barrier (27). Our preliminary studies showed that blockade of GABA_A receptors lasts >30 min, which allowed sufficient time for completion of the experimental protocol.

Statistical analysis. One-way repeated-measures ANOVA was used to evaluate the overall effect of advancing age on the hypercapnic response, with Newman-Keuls test to compare ventilatory responses at specific ages. ANOVA was also used to evaluate \( V^e \) \( V_T \), frequency, Ti, and Te response to hypercapnia during the duration of hypercapnic exposure. Two-way ANOVA was used to compare ventilatory responses between the two different ages (5 and 22–23 days). Results are presented as means ± SE.

**RESULTS**

Unrestrained rats. In the first group of animals derived from a single litter, both 5 and 10% \( \text{CO}_2 \) caused an increase in \( V^e \) in each animal at all ages, with the response being higher with 10% \( \text{CO}_2 \). The percent change in \( V^e \) increased significantly with advancing age in response to both 5% (\( P < 0.0001 \)), as seen in Fig. 1. The percent increase in \( V^e \) increased significantly with advancing age in response to both 5% \( \text{CO}_2 \) and 10% \( \text{CO}_2 \) \( P < 0.0001 \), as seen in Fig. 1. The percent increase in \( V^e \) was significantly lower at 5 days compared with all other age groups \( P < 0.05 \); Newman-Keuls test), and there was no significant difference among the later three age groups (Fig. 1).

At all ages the increase in \( V^e \) was accompanied by an increase in \( V_T \) (Table 1). The percent increase in \( V_T \) to 5% \( \text{CO}_2 \) was comparable among all ages; however, 10% \( \text{CO}_2 \) caused a significantly lower \( V_T \) response at 5 days compared with the later three ages \( P < 0.01 \), with no difference among the later three ages. The frequency response did not change from baseline in the 5-day-old animals in response to either 5 or 10% \( \text{CO}_2 \). However, both 5 and 10% \( \text{CO}_2 \) caused a significant increase in frequency at 16–17 days \( P < 0.05 \) and at 22–23 and 41–42 days of age (both \( P < 0.0001 \)).

In the second group of animals studied, the ventilatory response to 5% \( \text{CO}_2 \) was similar in magnitude to that in the first group of rats. The increase in \( V^e \) was significantly lower at 5 than 22–23 days of age (\( P < 0.0001 \) between ages). There were 32 ± 4 and 81 ± 8% increases in \( V^e \) after 5 min of exposure to 5% \( \text{CO}_2 \) at 5 and 22–23 days of age, respectively.

The percent increase in \( V_T \) was similar between the two ages; however, frequency decreased significantly in response to 5% \( \text{CO}_2 \) at 5 days \( P < 0.0001 \), whereas it increased significantly at 22–23 days \( P < 0.0001 \), as seen in Fig. 2. The decrease in frequency at 5 days was accomplished by a significant increase in \( \text{Te} \) \( P < 0.0001 \) without a significant change in \( \text{Ti} \) (Fig. 3). The increase in frequency at 22–23 days was accomplished

<table>
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<tr>
<th>Age, days</th>
<th>%Change in ( V_T^* )</th>
<th>Frequency, breaths/min</th>
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<tr>
<td>5</td>
<td>86 ± 3</td>
<td>220 ± 10</td>
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<tr>
<td>16–17</td>
<td>99 ± 7</td>
<td>250 ± 15</td>
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<tr>
<td>22–23</td>
<td>123 ± 14</td>
<td>280 ± 20</td>
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<td>41–42</td>
<td>142 ± 16</td>
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**Table 1. Ventilatory responses to hypercapnia at different ages**

*Data are means ± SE. \( V_T \), tidal volume. *Percent increase in \( V_T \) significantly lower at 5 days vs. later ages only in response to 10% \( \text{CO}_2 \), \( P < 0.01 \) (see text). †Increase in frequency in response to both 5 and 10% \( \text{CO}_2 \) only significant at the later 3 ages (different at each age).
by a significant decrease in both T\(I\) (\(P < 0.01\)) and T\(E\) (\(P < 0.0001\)).

Ventilated rats. Hypercapnia caused a significant decrease in frequency of phrenic nerve discharge in the 4- to 6-day-old rats, from 50 ± 6/min at control to 35 ± 3/min after 10 min of CO\(_2\) exposure (\(P < 0.01\)) but did not affect the frequency at 37–39 days of age (40 ± 3/min during control vs. 43 ± 2/min 10 min after CO\(_2\)). As in the unrestrained group the decrease in frequency in response to hypercapnia in the newborn rats was associated with a significant prolongation of T\(E\) (\(P < 0.01\); Fig. 4), whereas T\(I\) did not change from baseline. At 37–39 days hypercapnia caused a significant decrease in T\(I\) (\(P < 0.001\)), whereas T\(E\) was not different from baseline.

A subgroup of newborn rats was exposed to hypercapnia before and after intravenous administration of bicuculline. In this subgroup hypercapnia also caused a significant prolongation of T\(E\) in the rat pups under controlled conditions. After administration of bicuculline, prolongation of T\(E\) time in response to hypercapnia was abolished (Fig. 5).

**DISCUSSION**

The major finding of our study is that rats exhibited a clear maturation of their hypercapnic ventilatory response secondary to centrally mediated changes in respiratory timing. To our knowledge, there has been no previous study that systematically evaluated the maturation of hypercapnic ventilatory responses in unrestrained, unanesthetized rats. Separate studies performed in both unanesthetized newborn rats and in adult rats have demonstrated hypercapnic ventilatory responses that are quantitatively comparable to those observed in our study and are highly suggestive of an increase in the hypercapnic ventilatory response with age (24, 34). Recently, Bamford et al. (2) have shown that 3- and 8-day-old rats expressed a lower ventilatory response to CO\(_2\) than did 18-day-old rats secondary to an impaired frequency response. Zhou et al. (38) have reported that phrenic nerve activity changes from irregular to regular between 7 and 10 days of life in unanesthetized decerebrate newborn rats. Although they reported no significant effect of maturation on phrenic responses to hypercapnia over this time, it is possible that the regularity of phrenic output observed by 10 days would have resulted in a greater ventilatory response at that time.

Ventilatory response to CO\(_2\) has been shown to increase with advancing postnatal age (13, 23, 33) and gestational age (13, 23) in preterm human infants and in unanesthetized premature primates (15). Furthermore, a recent study in lambs has demonstrated an attenuated hypercapnic response in preterm, but not term, newborn lambs (8). These data suggest that prematurity is associated with an attenuated hypercap-
Fig. 5. Effect of bicuculline on timing of phrenic nerve output at 4–6 days of age in ventilated rats (n = 8). Values are means ± SE. 5% CO₂ caused significant prolongation in expiratory time (B) under control conditions (*P < 0.01). Intravenous administration of bicuculline prevented this prolongation in expiratory time in response to hypercapnia. Hypercapnia did not affect inspiratory time (A) significantly under both conditions.

namic response, whereas mature newborns have hypercapnic responses that are more comparable to the corresponding adult responses (33). This is consistent with our present data and previously published results (2) that newborn rats exhibit such an attenuated hypercapnic response, because they are quite immature at birth (9), whereas newborn pigs (like lambs), which are more mature when delivered at term, exhibit a response that does not appear to so clearly differ in magnitude from the adult response (35).

The results from the second group of unrestrained rats confirmed our initial findings in a single litter and allowed further characterization of respiratory timing responses in a larger group of animals. As we have observed in unrestrained 5-day-old rat pups, the ventilated newborn rats expressed a time-dependent increase in Tₑ and reduction of phrenic nerve frequency in response to hypercapnia. Hypercapnia is known to induce a relatively sustained increase in Vₑ in human infants that is almost entirely due to an increase in Vₜ (23, 32) without consistent change in frequency. We have previously reported that premature infants exhibited an initial transient decrease in Tₑ over the first 2 min of exposure to CO₂ that then returned to control value (29). In a subsequent study of premature infants, hypercapnia was also associated with a decrease in frequency that was accompanied by prolongation in Tₑ (11). The hypercapnic response in the preterm infants in both studies appeared to be associated with increased braking (persistence of diaphragmatic activity during expiration) (11, 29). The increase in Tₑ, which we have also observed in the most immature animals, if associated with laryngeal adduction or diaphragmatic braking might serve to maintain a high end-expiratory lung volume and so optimize gas exchange. Further studies are clearly needed to characterize the role of expiratory upper airway muscles during the prolonged expiratory phase.

Our findings in vagotomized and decerebrate rat pups would appear discordant with previous studies performed in the neonatal brain stem preparation, in which the most frequently observed respiratory response to hypercapnia is a sustained increase in phrenic burst frequency (21, 30). Such differences could conceivably be due to the technique by which chemosensory neurons are exposed to CO₂. However, it is more likely that differences may be related to the relative tissue hypoxia of in vitro brain stem preparations and resultant inhibitory effects on respiratory-related chemosensory neurons. Local hypoxic loading of the ventrolateral medulla induces prolongation of expiratory duration and apnea (17), and increases in CO₂ tend to reverse the inhibitory effect of hypoxia (28).

We acknowledge that changes in the sleep-wake state might be a factor in respiratory changes seen in the newborn nonsedated animals. However, CO₂-induced prolongation in Tₑ was also observed in decerebrate rat pups, making it unlikely that changes in sleep-wake state play an important role in the observed age-related changes in hypercapnic ventilatory responses. In the present study, several differences exist in the ventilatory responses between the two preparations. Respiratory frequency in the ventilated animals was one-third of that seen in the spontaneously breathing rat. This is probably secondary to vagotomy and is consistent with previous results in vagotomized animals (12, 38). Furthermore, the older animals in the ventilated group did not have a significant decrease in Tₑ in response to hypercapnia. Similarly, in vagotomized adult cats and dogs, hypercapnia or topical application of cholinergic drugs and peptidergic compounds to putative chemosensory areas of the ventrolateral medulla did not affect Tₑ but increased activity of expiratory neurons and elevated respiratory drive (5, 16, 18).

Several mechanisms have been proposed to explain the attenuated hypercapnic ventilatory responses exhibited by premature and immature newborns of various species. Possibilities include changes in the mechanical properties of the lung, maturation of hypercapnia-induced upper airway reflexes, maturation in the peripheral or central chemoreceptors, or changes in the central integration of chemoreceptor or other neuronal signals. Bartlett et al. (3) showed that intralaryngeal CO₂ in bilaterally vagotomized cats caused a dose-related decrease in phrenic activity. The phrenic nerve response to intralaryngeal CO₂ was attenuated by systemic hypercapnia and completely abolished in vagally intact animals (3). A similar mechanism has been suggested for the decrease in ventilatory response to severe hypercapnia in preterm infants (1). Conceivably, the greater susceptibility to laryngeal inhibitory inputs
could explain the attenuated ventilatory response in newborn rats. However, it is unlikely that this mechanism is primarily responsible for our results because the CO₂-induced decrease in frequency and prolongation of Tₑ occurred in vagally intact, spontaneously breathing rats, as well as in vagotomized, mechanically ventilated animals in which endotracheal intubation bypassed the upper airways. Similarly changes in vagal input via maturation of the Hering-Breuer reflex (26) are an unlikely mechanism for our physiological findings, because these were observed in vagotomized rat pups. Frantz et al. (13) confirmed that ventilatory responses to CO₂ are decreased in premature infants and, by measuring end-expiratory occlusion pressures, suggested that decreased respiratory center sensitivity contributed to this phenomenon. Our present data in decerebrate, vagotomized, intubated and ventilated rats strongly indicate a primarily central origin for hypercapnia-induced changes in respiratory timing during maturation, namely prolongation of Tₑ.

Various neurotransmitters have been implicated in the control of breathing, of which GABA is considered to be the major inhibitory neurotransmitter. GABA was found to inhibit respiratory activity mainly via activation of GABAₐ receptors (19,20). In the studies reported here, bicuculline, a competitive inhibitor of GABAₐ receptors, significantly reduced the prolongation of Tₑ in response to hypercapnia, suggesting that hypercapnia activates fast synaptic inhibitory GABAergic pathways to reduce excitation of inspiratory-related neurons and delay initiation of their activity. This finding might point to relative overexpression of GABAₐ receptors in the newborn rats. Both structural and functional differences in GABAₐ receptors have been observed during development (7,22,25). Xia and Haddad (37) demonstrated that the newborn rat brain stem has a much higher GABAₐ-receptor density than does adult brain stem with a peak in receptor density at postnatal day 5 or 10 (37). These results do not exclude a possible role of metabotropic (GABAₐ) receptors in hypercapnia-induced respiratory depression. Future studies employing selective blockers could determine the importance of GABAₐ receptors in respiratory depression induced by CO₂ in premature and immature animals.

Immaturity of the hypercapnic ventilatory response has been associated with apnea of prematurity in human infants, although the biological mechanisms underlying this association are unclear (10,14). Recognizing the shortcomings of using an animal model to investigate human responses, we believe that the immaturity of newborn rats and the similarities of their hypercapnic ventilatory response to that of premature infants make them a useful model for further characterizing the biological mechanisms underlying instability of neonatal respiratory control.

In conclusion, we are describing for the first time that newborn rats exhibit an impaired hypercapnic response that is mediated by a decrease in respiratory frequency and prolongation in Tₑ. This ventilatory response is primarily central in origin and does not appear to be secondary to maturational changes in vagal nerve responses or mechanical properties of the lung. Furthermore, our data suggest that developmental changes in GABAergic mechanisms contribute to this maturation of the hypercapnic ventilatory response.

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