The Basomedial Hypothalamus Modulates the Ventilatory Response to Hypoxia in Neonatal Rats

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ABSTRACT

We sought to examine the role of the basomedial hypothalamus in the regulation of breathing in neonatal rats. Small basomedial hypothalamic lesions were produced by a lesioning agent, goldthioglucose, in 5-d-old male rat pups, and 2 d later, baseline ventilation and the ventilatory responses to hypoxia and hypercapnia were examined. When compared with vehicle-injected controls, goldthioglucose-lesioned rat pups had a significantly slower respiratory rate and longer expiratory time at baseline. Lesioned rats displayed an impaired increase in breathing frequency in response to hypoxia, and augmented increases in tidal volume and respiratory drive (the ratio of tidal volume to inspiratory time) during hypoxia relative to controls. Hypercapnic responses were not affected. These data demonstrate that cells

in a restricted area of the hypothalamus specifically influence the respiratory response to hypoxia. (*Pediatr Res* 53: 945–949, 2003)

Abbreviations

GTG, goldthioglucose
NPY, neuropeptide Y
PVN, paraventricular nucleus of the hypothalamus
 T_E , expiratory duration
 T_I , inspiratory duration
 T_{TOT} , length of respiratory cycle
 V_T , tidal volume

It is well established that ventilatory responses to hypoxia are mediated by discrete neural pathways involving peripheral chemoreceptors, primary afferents, and brainstem respiratory-related neurons. The characteristics of the response, however, depend on complex interactions at multiple levels of the neuraxis, including suprapontine structures. Several studies have suggested that the caudal hypothalamus modulates responses to hypoxia and hypercapnia (1, 2). For example, one study showed that 21% of hypothalamic units are activated by hypoxia and 31% by hypercapnic loading (3). Furthermore, hypoxia and hypercapnia stimulate neurons in various hypothalamic nuclei (paraventricular, supraoptic, and dorsomedial) to synthesize the transcription-regulating protein Fos (4–6). Binding of the adipocyte hormone leptin to receptors in either the hypothalamus or area postrema modulates respiration and may underlie the respiratory abnormalities observed in genetically obese rodents (7–9). Finally, electrolytic lesions that

destroy extensive amounts of perifornical cells in the posterior hypothalamus enhance the ventilatory response to hypoxia (10). However, the role of these structures in regulating ventilatory responses to chemical drive is not well understood.

Functional effects of relatively large electrolytic lesions of the hypothalamus are instructive, but are less helpful in pinpointing the precise location of hypothalamic cells critical for respiratory control. Likewise, unit recording or examination of Fos expression throughout the hypothalamus during hypoxia does not identify cells initiating a hypothalamic response to hypoxia because of multisynaptic projections among activated neuronal pools. These techniques also do not determine the functional role of hypothalamic cells in the regulation of respiratory responses to changes in chemical drive. One approach to this problem is to examine the effects of relatively restricted hypothalamic damage. This can be achieved *via* the systemic administration of a toxin, GTG, which diffuses into the hypothalamus *via* the permeable capillaries of the median eminence and which damages only the dorsal portion of the arcuate nucleus and a small portion of the ventromedial hypothalamic nucleus (11).

We hypothesized that the arcuate nucleus of the hypothalamus may be an important regulator of respiratory control. Arcuate neurons immunoreactive for NPY, proopiomelanocor-

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tin, and substance P project to diverse regions of the hypothalamus such as the PVN. The PVN, in turn, regulates respiratory drive (12). Also, arcuate neurons project directly to the dorsal medulla, an area that strongly influences the activity of respiration-regulating neurons of the medullary pre-Bötzinger complex (13–16). Finally, the arcuate nucleus contains numerous leptin-responsive neurons, and hence may be a major site of the influence of leptin upon respiratory control (7, 9).

METHODS

In this study, 5-d-old Sprague Dawley albino rat pups, weighing about 15 g, were injected intraperitoneally with either saline or with GTG (0.3 mg/g in sterile saline). Two days later, ventilatory responses to hypoxia (8% O₂, 92% N₂), were tested. The responses were measured using plethysmography for unrestrained, unanesthetized small rodents (Buxco Electronics, Troy, NY, U.S.A.), which is a modification of the body-box technique (15). Briefly, in the body-box technique, pressure changes caused by differences in the temperature of inhaled or exhaled air are sensed by a sensitive pressure transducer and recorded as a pressure curve. However, in a Buxco plethysmograph, because the box is open to the atmosphere, pressure changes are differential and the recorded curve is a flow curve. With appropriate software, recorded flow is integrated on a breath-by-breath basis to obtain V_T as well as T_I, T_E, and T_{TOT} (T_{TOT} = T_I + T_E).

The temperature in the plethysmograph was kept at ~33°C to minimize body heat loss in these small, immature rats. Body temperatures of five vehicle-treated and five GTG-treated rats were also measured before and after hypoxia with an esophageal probe to rule out any possible effects of GTG upon body temperature maintenance that could influence the outcome of the study. To prevent CO₂ buildup and a decrease in oxygen, a bias flow of room air (2 L/min) continuously passed through the plethysmograph. During exposure to hypoxia, a hypoxic gas mixture was introduced through the bias flow port after the animal was placed in the plethysmograph. Breathing activity was observed until a stable pattern was noted and recorded as baseline ventilatory activity. Then, the hypoxic mixture (8% O₂, 92% N₂) was introduced for 10 min.

Rats were subsequently (2 h later) exposed to 10 min of hypercapnia (5% CO₂, 30% O₂, 65% N₂). To derive ventilatory variables from the plethysmographic recordings, measurements for 20 breaths for each time point and each animal were averaged (20 breaths occurred over a time span no greater than 10 s). This approach allowed us to minimize between-breath variability when analyzing these data. Comparison of baseline ventilatory variables was made *via* an unpaired *t* test. Hypoxic responses between groups were compared by two-way ANOVA with repeated measurements. Data are expressed as mean ± SE. A *p* value of < 0.05 was considered statistically significant.

At the end of the experiment, five animals from the GTG-treated group and five of the control pups were deeply anesthetized (pentobarbital, 60 mg/kg) and wash-perfused with saline through the left ventricle followed by 10% formalin. Entire brains were immersed in 20% sucrose-formalin for

cryoprotection and then serial, coronal frozen sections (30 μ thick) of the hypothalamus and area postrema region were prepared using a sliding microtome. Brain sections were dried down onto subbed slides, stained with 0.1% thionin in acetate buffer, and inspected for GTG-induced lesions (11). All procedures for handling animals were approved by the Case Western University Institutional Animal Care and Use Committee.

RESULTS

Hypothalamic sections of brains from GTG-injected rats showed bilateral areas of necrosis, gliosis, and vascular damage that encroached upon the dorsal portion of the arcuate nucleus and ventral margin of the ventromedial nucleus, a pattern that is typical of GTG-induced damage (11). The GTG-induced lesion caused a partial collapse of affected tissue into a glial scar, resulting in a folding and distortion of the normally smooth contours of the adjacent ependymal layer enclosing the third ventricle (Fig. 1). Close inspection of the region bordering the area postrema of these rats, in contrast, revealed no signs of damage, which is consistent with previous

Figure 1. (A) Basomedial hypothalamus from a rat treated with GTG. GTG-induced damage was visible as pale-staining regions of gliosis (arrows) accompanied by focal accumulations of damaged blood vessels. Loss of neurons in portions of both the arcuate (a) and ventromedial (v) nuclei resulted in a partial collapse of the region and a folding and distortion of the normally smooth contours of the ependymal layer enclosing the third ventricle. (B) Hypothalamus from a saline-treated rat, demonstrating the normal structures at these sites. Scale bar = 200 μm.

results showing that this dose of GTG is less likely to affect other circumventricular regions as compared with the hypothalamus itself (11). Likewise, inspection of the dorsal portions of brain sections just anterior to the arcuate nucleus revealed no GTG-induced damage in the region of the subfornical organ. No histologic changes in hypothalamic structures were observed in saline-injected groups.

Table 1. Baseline respiratory variables in the two groups of rat pups

Respiratory parameter	Vehicle	GTG	<i>p</i> Value (unpaired <i>t</i> test)
T_I , msec	122 $\pm$ 4	132 $\pm$ 6	0.15
T_E , msec	295 $\pm$ 15	364 $\pm$ 23	0.02
Frequency, breaths/min	147 $\pm$ 6	126 $\pm$ 7	0.03
V_T , μ L	80 $\pm$ 4	80 $\pm$ 3	0.95
Minute ventilation, mL/min	12 $\pm$ 0.8	10 $\pm$ 0.6	0.08
V_T/T_I , μ L/msec	0.66 $\pm$ 0.04	0.61 $\pm$ 0.03	0.06

No significant between-group differences in body temperatures were found ($p = 0.7$). In vehicle-treated rats, body temperature before and after hypoxia equaled 35.6°C and 35.3°C, respectively, resulting in a nonsignificant fall in temperature of 0.3°C. In GTG-treated rats, body temperature before and after hypoxia was 35.5°C and 35.2°C, respectively, also amounting to a fall in body temperature of 0.3°C.

Baseline respiratory variables of the two groups are summarized in Table 1. Baseline breathing frequency in GTG-treated rats ($n = 14$) was significantly lower than in vehicle-treated animals ($n = 14$). This was associated with a significantly longer baseline T_E in the GTG-treated pups (Table 1). As shown in Table 1, there were no other significant differences in baseline ventilatory variables between groups. After exposure to hypoxia, the GTG-treated animals had a response of breathing rate significantly different from the vehicle-treated group, manifested by greater roll-off of frequency ($p < 0.01$, two-way ANOVA, Fig. 2A). There were significantly different responses of both inspiratory and expiratory times to hypoxia between

Figure 2. (A) Exposure to 8% O₂ resulted in significantly greater depression in breathing rate in GTG-treated rats when compared with controls. (B) At baseline, V_T did not differ between groups, however, during exposure to hypoxia, V_T increased significantly more in GTG-treated pups compared with control pups. (C) T_I initially shortened in both GTG-treated and control groups with the introduction of hypoxia. However, during hypoxic exposure, T_I of GTG-treated pups rose significantly more than in control pups. (D) Exposure to hypoxia caused a similar shortening of T_E in both groups. Subsequently, T_E showed significantly greater prolongation in GTG-treated pups vs controls.

groups, with less shortening of both T_I and T_E in GTG-treated pups during the course of hypoxic exposure ($p < 0.02$ for T_I and $p < 0.01$ for T_E , both two-way ANOVA, Fig. 2, C and D).

Although baseline values of V_T were comparable between groups, there was a significantly greater response of V_T to hypoxia in the GTG-treated rat pups ($p < 0.001$, two-way ANOVA, Fig. 2B). Also, GTG lesions enhanced the hyperpneic increase in V_T at 2 min of hypoxia, although other respiratory variables at this time did not differ between groups ($p < 0.01$). In addition, in spite of greater prolongation of T_I , the V_T/T_I ratio, an index of respiratory drive, was significantly greater in the GTG group during hypoxia than in control animals ($p < 0.01$, Fig. 3A). Finally, the percentage change in V_T times frequency (minute ventilation) was significantly greater in the GTG-treated rats than in the vehicle-treated rats during exposure to 8% oxygen ($p < 0.001$, Fig. 3B). However, there was no difference between groups in the roll-off of the V_T response to hypoxia.

No significant differences were observed between groups in response to hypercapnic loading (data not shown).

DISCUSSION

It is widely accepted that hypoxic exposure during early postnatal life results in a biphasic ventilatory response characterized by an initial increase and a subsequent gradual decrease in ventilation. Therefore, the current results are in agreement with previously published data in newborn animals and in preterm infants (14–17). However, the data from the present study show for the first time that GTG-induced hypothalamic lesions affect not only baseline respiratory timing variables, but also the profile of the ventilatory response to hypoxia. Lesioned rats displayed a greater depression of respiratory timing in response to hypoxia. However, their augmented V_T response led to an increase in ventilation in spite of a reduced breathing frequency. These data suggest that neurons within the basomedial hypothalamus activate a neuronal network that shortens inspiratory and expiratory time in response to hypoxia.

The greater depression of respiratory frequency in response to hypoxia in the lesioned pups mimics what we see in preterm babies (15). It is known from fetal and neonatal sheep experiments that descending inhibition to the medulla contributes to this phenomenon (18, 19). The current data support the assumption that there are also excitatory inputs from the basomedial hypothalamus to the brainstem. Maturation changes in these neurons may contribute to age differences in hypoxic depression.

In GTG-treated rats, exposure to hypoxic stress induced a larger increase in V_T than in the control group. During the first 2 min of hypoxia, this was unaccompanied by any other changes in respiratory variables. Subsequently, there was also a disproportionate lengthening of inspiratory duration in GTG-lesioned rats. These data show that the hypothalamic arcuate nucleus provides input to the brainstem respiratory-related network that regulates the gain of the ventilatory response to hypoxia. Such a network optimizes gas exchange at a minimal level of respiratory effort (work).

The mechanism of GTG lesion formation seems to involve localized diffusion of GTG from the median eminence and uptake of GTG into specialized astrocytes that possess high-capacity glucose transporter proteins (11, 20). After uptake into target cells, toxic effects of the GTG molecule seem to cause a generalized cell death of glial, neural, and vascular elements in the region of the lesion.

Because arcuate neurons have been found to directly project to the medulla, one plausible explanation for the observed effects of lesions was the destruction of these specific arcuate cells (14). Alternatively, destruction of arcuate neurons that project to the PVN or lateral regions of the hypothalamus could also affect respiration, because these sites also project to the medulla (12, 14).

A variety of subpopulations of arcuate neurons, each containing specific neurotransmitters, could contribute to the effects of lesions. Arcuate neurons containing substance P, for example, could be involved, because substance P affects respi-

Figure 3. (A) The V_T/T_I ratio, an index of respiratory drive, was similar in both groups during room air breathing. Exposure to hypoxia caused a significantly greater increase in V_T/T_I in GTG-treated pups compared with control pups. (B) Percentage changes in minute ventilation during hypoxia were greater in GTG-treated pups compared with control pups.

ratory motor output and breathing frequency, probably *via* an action upon the pre-Böttinger complex (21). Also, lesions may affect neurons that contain NPY and project to the PVN. Hence, a lesion-induced change in function of NPY arcuate neurons and a withdrawal of excitatory inputs to PVN neurons that project both to the pre-Böttinger complex and to phrenic nuclei could explain these results (12).

In accord with this general hypothesis, one study reported an increase in the mRNA for NPY after GTG lesioning (22). Other studies, however, have not replicated these findings, suggesting that other populations of arcuate neurons are responsible for lesion-induced functional changes (23–25). Thus, the exact roles of various arcuate neurotransmitters in lesion-induced hypothalamic dysfunction and changes in the ventilatory response to hypoxia await clarification.

Yet another possible explanation for functional effects of arcuate lesions is an interruption of fibers passing from the arcuate nucleus to the lateral hypothalamus. Fibers from NPY-containing arcuate neurons innervate orexin-immunoreactive neurons in the lateral hypothalamus, which in turn project to many brainstem regions regulating respiration and which also project back to arcuate NPY neurons (26, 27).

Based upon these data, it is possible that a maturation of hypothalamic circuits could contribute to changes in respiratory regulation that occur during the first week of development of the rat (28). It is known that levels of arcuate neurotransmitters do not reach adult levels until several weeks after birth (29). Also, axonal projections from arcuate neurons to the nearby median eminence become complete only during the last several days of development *in utero*; completion of projections to more distant structures like the medulla are likely completed postnatally (30).

The question of whether or not hypothalamic neurons themselves monitor blood levels of oxygen during hypoxia, either directly or *via* indirect input from peripheral chemoreceptive cells, is also germane to this study and could not be directly addressed using these methods. Hypothalamic neurons could either directly monitor oxygen levels or else simply modulate the activity of oxygen-sensitive neurons located elsewhere. Evidence for both viewpoints has been reviewed in a number of studies (3, 4).

The ventilatory changes reported here are somewhat analogous to those found after electrolytic lesions of the posterior hypothalamus (10). In that study, $\dot{V}_E$ ($V_T \times$ breathing rate) was increased in lesioned rats by about 17% during hypoxia. In our study, $\dot{V}_E$ overall was also increased. The considerably smaller hypothalamic lesions produced in our study thus had an effect upon respiration comparable to larger electrolytic lesions.

Finally, we speculate that alterations in diencephalic neuronal circuits may contribute to a number of clinical syndromes involving the hypothalamus that may manifest simultaneous abnormalities in appetite, body weight, sleep, and respiratory control during sleep (9, 31, 32).

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