Differential CO$_2$-induced c-fos gene expression in the nucleus tractus solitarii of inbred mouse strains

CLARKE G. TANKERSLEY,1 MUSA A. HAXHIU,3 AND ESTELLE B. GAUDA2

1Department of Environmental Health Sciences, Bloomberg School of Public Health, and 2Department of Pediatrics, School of Medicine, The Johns Hopkins University, Baltimore, Maryland 21205; and 3Department of Physiology and Biophysics, College of Medicine, Howard University, Washington, District of Columbia 20059

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Tankersley, Clarke G., Musa A. Haxhiu, and Estelle B. Gauda. Differential CO$_2$-induced c-fos gene expression in the nucleus tractus solitarii of inbred mouse strains. J Appl Physiol 92: 1277–1284, 2002.—Genetic determinants confer variation between inbred mouse strains with respect to the magnitude and pattern of ventilation during hypercapnic challenge. Specifically, inheritance patterns derived from low-responsive C3H/HeJ (C3) and high-responsive C57BL/6J (B6) mouse strains suggest that differential hypercapnic ventilatory sensitivity (HCVS) is controlled by two independent genes. The present study also tests whether differential neuronal activity in respiratory control regions of the brain is positively associated with strain variation in HCVS. With the use of whole body plethysmography, ventilation was assessed in C3 and B6 strains at baseline and during 30 min of hypercapnia (inspired CO$_2$ fraction = 0.15, inspired O$_2$ fraction = 0.21 in N$_2$). Subsequently, in situ hybridization histochemistry was performed to determine changes in c-fos gene expression in the commissural subnucleus of the nucleus tractus solitarius (NTS). During hypercapnia, breathing frequency and tidal volume were significantly ($P < 0.01$) different between strains: C3 mice showed a slow, deep-breathing pattern relative to a rapid, shallow phenotype of B6 mice. CO$_2$-induced increase in c-fos gene expression was significantly ($P < 0.01$) greater in NTS regions of B6 compared with C3 mice. In this genetic model of differential HCVS, the results suggest that a genomic basis for varied hypercapnic chemoreception or transduction confers greater afferent neuronal activity in the caudal NTS for high-responsive B6 mice compared with low-responsive C3 mice.

C3H/HeJ; C57BL/6J; hypoventilation; hypercapnic ventilation; control of breathing

HYPERCAPNIC VENTILATORY SENSITIVITY (HCVS) among human volunteers has been shown to vary across a continuum from low- to high-responsive subgroups. For example, Hirshman et al. (19) demonstrated a sixfold difference between low- and high-responsive human volunteers. Although phenotypes in these subjects varied positively with height, weight, and the ventilatory response to hypoxia, the feature of bimodality in HCVS distributions suggested that a major genetic determinant conferred individual variation in HCVS.

Our laboratory has demonstrated a similar continuum among many inbred mouse strains (34). This implies that genetic diversity, which determines differential HCVS, may be conserved across species and is the basis for comparative mapping studies (31). The C3H/HeJ (C3) and C57BL/6J (B6) inbred strains occupy the extremes of a strain distribution pattern for hypercapnic ventilatory traits (34); that is, with inspiratory challenges from 3–8% CO$_2$ in normoxia, C3 mice are characterized with a blunted HCVS phenotype (i.e., low gain) relative to the B6 phenotype (i.e., high gain) for HCVS (34, 35). With the use of quantitative genetic approaches, our studies further demonstrate that hypercapnic ventilatory traits, like tidal volume (V$_T$) and mean inspiratory flow, are inherited by offspring of C3 and B6 progenitors (33). Furthermore, the heritability of these traits may be conferred by as few as two major genes.

Hypercapnic challenge activates multiple genes, including the c-fos gene, which plays a significant role as a transcriptional factor and regulator of multiple cellular functions (20). Whether genes involved in determining ventilatory responses to CO$_2$ are linked to the regulation of c-fos gene expression is unknown. Therefore, the purpose of the present study was to 1) thoroughly characterize the inheritance pattern of HCVS phenotypes, by systematically measuring the HCVS responses in C3 and B6 parental strains and their first- and second-generation offspring, and 2) test the hypothesis that genetic diversity in HCVS between C3 and B6 strains involves concomitant c-fos gene expression differences, which are indicative of strain variation in integrating afferent inputs to the nucleus tractus solitarii (NTS) or in the signal transducing mechanisms mediated by intracellular changes associated with CO$_2$ responsiveness. In either case, if the findings support the hypothesis, then the genetic basis for C3 and B6 strain variation in HCVS involves mecha-

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nisms of differential neural processing under variable transcriptional regulatory control. Multiple signals are received, processed, and transmitted by way of the subnuclei of the solitary tract (NTS) to provide peripheral and central information to respiratory-related networks. For example, neurons within the caudal NTS mediate signals from the carotid bodies (14), which are activated by hypercapnic challenge (15). Furthermore, elevated CO2/H+ content of extracellular fluid stimulates subpopulations of NTS neurons, even after synaptic blockade (9, 18, 24). In addition, acidification of this region induces an increase in ventilatory drive (23). c-Fos protein expression is a technique that has been routinely used to map brain stem neuronal pathways activated by hypercapnic (and hypoxic) exposure in several mammalian species, including rats (4, 18, 29, 37), cats (10, 36), pigs (28, 30), and sheep (8). Neuronal activation is known to increase the level of c-fos gene expression in many neuroanatomic locations involved in mediating ventilatory responses to hypercapnic challenge (17). We presume that NTS neurons that respond to hypercapnia with expression of c-fos are activated by increases in CO2/H+ concentration in the extracellular fluid. We recognize that some of the c-fos gene-expressing cells within the NTS may be transsynaptically activated by chemosensory neurons. However, while hypercapnia may also trigger inhibitory processes that suppress activity of neurons that terminate expiration (1), these inhibited neurons do not express c-fos (17). Taken together, the available evidence indicates that differences in CO2-induced c-fos gene expression within the NTS may accurately reflect the strain variation in HCVS between C3 and B6 mice.

In the present study, CO2-induced c-fos gene expression is used as a measurement of variation in neuronal activity in the commissural subnucleus of the NTS between C3 and B6 mice. We postulate that CO2 exposure is likely to increase c-fos gene expression in the NTS in both strains; however, CO2-induced c-fos gene expression will be greater in B6 relative to C3 mice. The results from the present study are consistent with the hypothesis that differences in HCVS are heritable and determined by as few as two genes. Moreover, the phenotypic expression of these genes in response to CO2 exposure is positively associated with variation in c-fos gene induction in the NTS.

METHODS

Animals. Individual animals within a standard inbred strain are genetically identical and homozygous at every gene as a result of 20 or more generations of brother-sister matings (16). The animals used for determining the inheritance pattern of HCVS between C3 and B6 progenitors were either purchased from Jackson Laboratories (Bar Harbor, ME) or propagated from breeding colonies in the animal facilities at Johns Hopkins University. Male and female C3, B6, and B6C3F1/J (i.e., female B6 x male C3, F1) were paired to generate backcross (i.e., female B6 x male C1, B6BX; female C3 x male F1, C3BX) and intercross (i.e., B6C3F2 or F2) progeny. From the breeding colonies, animals were weaned within 4–5 wk, and randomly selected male backcross and intercross progeny were housed for an additional 6–12 wk before testing. These animals were used to establish the inheritance pattern for differential HCVS between C3 and B6 progenitors. A different group of male C3 and B6 mice (8 wk of age, 22–26 g in weight) were purchased to perform in situ hybridization experiments described below. All animals were provided water and chow (Agway Pro-Lab RMH 1000) ad libitum. The light/dark phases of the animal facility were controlled by a 12-h cycle, and each study was performed between 0900 and 1800. The environment before and during both experiments, as well as animal handling, was highly standardized. All animal protocols were reviewed and approved by the Animal Care and Use Committee of the Johns Hopkins Schools of Medicine and Public Health.

Whole body plethysmography. Ventilatory function was assessed at baseline and during both hypercapnic and normoxic inspiratory challenge protocols by using whole body plethysmography (34). By using unanesthetized and unrestrained conditions, each animal was permitted to acclimate in the chamber at least 30 min before ventilation measurement was obtained. Chamber temperature was maintained within the thermonutral zone for mice (i.e., 26–28°C) and was recorded with each ventilatory measurement by using a type T thermocouple. Compressed air was humidified (90% relative humidity) and directed through the chamber at a flow rate of ~300 ml/min. At a constant chamber volume, changes in pressure due to inspiratory and expiratory temperature fluctuations were measured with a differential pressure transducer (model 8510B-2, Endevco). Additional details regarding the plethysmographic method and the computation of other ventilatory traits have been reported elsewhere (34).

Inheritance pattern of HCVS in C3 and B6 mice. Minute ventilation [V5; is equal to the product of breathing frequency (f) and VT] was determined at baseline and during two levels of hypercapnic, normoxic challenge (inspired fraction of CO2 = 0.03 and 0.08 in inspired fraction of O2 = 0.21 balanced with N2). After V5 was normalized for body weight in each animal, least squares regression was performed to determine the slope (i.e., HCVS gain) of the Ve-to-inspired CO2 relationship (i.e., using 0, 3, and 8% CO2). Although arterial blood-gas determinations have been recently accomplished in mice (25), the required large number of animals needed to assess inheritance patterns among different offspring classes prohibited the routine use of blood-gas determinations in the present study. As an alternative, HCVS was designed to rapidly phenotype mice as an index of the change in ventilation per unit change in arterial PCO2.

The response distribution patterns of HCVS were then analyzed by the statistical package, Statistical Analysis for Genetic Epidemiology (SAGE) (28a), according to the methodology proposed by Elston (11). The first step of this analysis (using the program CLUSTR) tested whether or not the best power transformation to achieve normality and homoscedasticity (i.e., variance homogeneity and zero covariance) for the C3 and B6 parental strains (n = 20 mice per strain) and F1 (n = 14 mice) response distributions was significantly different from the best transformation to achieve normality alone. The next step (using the program BCROSS) analyzed the transformed response distributions of backcross (C3BX, n = 20 mice; and B6BX, n = 35 mice) and intercross (F2, n = 69 mice) progeny, comparing these data with the response distributions of the progenitors. In each case, 37 homoscedastic hypotheses were tested, including single locus, two loci, polygenic, and mixed single gene and polygenic hypotheses. For each hypothesis, Akaike’s information criterion (2) was calculated and used to select the best genetic hypotheses. To
establish the best fit of these hypotheses, approximate \( P \) values were calculated, assuming that twice the difference in log-likelihood between the unrestricted model and the best-fit hypothesis follows a \( \chi^2 \) distribution (with degrees of freedom equal to the difference in the number of independent parameters being estimated between the hypothesis and the unrestricted model).

In situ hybridization studies in C3 and B6 mice. Strain differences in magnitude and pattern of ventilation during \( \text{CO}_2 \) exposure to induce variation in \( \text{c-fos} \) gene expression in the NTS were also evaluated. Ventilatory measurements were obtained before exposure (0 min) and at 3, 7, 15, 20, 25, and 30 min during hypercapnic, normoxic exposure (inspired fraction of \( \text{CO}_2 = 0.15 \) in inspired fraction of \( \text{O}_2 = 0.21 \) balanced with \( \text{N}_2 \)) in a separate group of C3 and B6 mice.

Immediately after hypercapnic exposure protocol, each animal was anesthetized using methoxyflurane (Schering-Plough) and killed by decapitation. The brain of each animal was removed, quickly frozen, and stored at \(-70^\circ \text{C}\). From each brain, coronal frozen sections (i.e., 12 \( \mu \text{m} \) ) were serially cut, mounted on gelatin-coated glass slides, and stored at \(-70^\circ \text{C}\). Brain sections were obtained from regions corresponding to Bregma \(-7.48 \text{ mm} \) and \(-7.20 \text{ mm} \) (26) for the commissural nucleus of the NTS (see Fig. 4) and extended to \(-6.48 \text{ mm} \) to include the retrofacial region. Each slide was further processed by first warming it to room temperature, fixing it in 4% paraformaldehyde solution (0.9% saline) for 10 min, and incubating it in a fresh solution of 0.25% acetic anhydride in 0.1 M triethanolamine and 0.9% saline (pH 8.0) for 10 min. The slide-mounted sections were then dehydrated in a series of ascending concentrations of ethanol, delipidated for 5 min in two washes of chloroform, rehydrated, and air dried. Finally, all slides were stored at \(-20^\circ \text{C} \) until further processing for in situ hybridization histochemistry.

Radioactive ribonucleotide probes were generated by in vitro transcription of cDNA incorporating a radioactive \([^{35}\text{S}]\text{UTP}\). The cDNA to be transcribed was cloned into a polylinker site of a vector with a promoter for SP6, T7, or T3 RNA polymerase (Promega, Madison, WI). Before in vitro transcription, the DNA was linearized with the appropriate restriction enzyme and then extracted by phenol/chloroform and ethanol precipitation using standard methods. Ribonucleotide probes were generated using cDNA of the mouse \( \text{c-fos} \) gene (27, 38). Probes were labeled with \([^{35}\text{S}]\text{UTP} \), and \( 1.5 \times 10^6 \text{ dpm} \) were added to 100 \( \mu \text{l} \) of hybridization buffer [50% formamide, 300 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% dextran sulfate, 1 \( \times \) Denhardt’s, 100 \( \mu \text{g/ml} \) salmon sperm DNA, 250 \( \mu \text{g/ml} \) yeast total RNA (type XI), 250 \( \mu \text{g/ml} \) yeast tRNA, and 100 mM dithiothreitol]. Buffer was applied to slides containing 8–10 sections per slide. Hybridization was performed overnight at 55°C. The slides were then washed in 1 \( \times \) saline sodium citrate (0.15 M sodium chloride/0.015 M sodium citrate, pH 7.2) at room temperature. After treatment with RNase A (20 mg/ml), slides were washed at 60°C in 0.2 \( \times \) saline sodium citrate, rinsed in deionized water, and air-dried. Slides were then dipped in Kodak photographic emulsion, dried, and exposed in the dark at \(-20^\circ \text{C} \) for 12 wk. After exposure, the slides were thawed at room temperature, developed with Dektol (Kodak, NY), and counterstained with thionin, and coverslips were applied with Permount.

Data analysis of in situ hybridization experiment. Comparisons were only made from slides that were processed for in situ hybridization, hybridized, exposed, and developed together under the same conditions. Silver grains generated by \( ^{35}\text{S} \) in the emulsion were analyzed and quantified using a microscope and Macintosh image analysis program (NIH Image. W. Rasband, National Institutes of Health). The clusters of grains, identifying \( \text{c-fos} \) gene-expressing neurons, were observed within chemosensitive regions along the medulla oblongata. However, unlike the caudal NTS, the number of clusters was relatively low within the midline and retrofacial regions, which excluded these regions from relevant statistical analysis. After darkfield images of the caudal NTS were captured and digitized at \( \times 400 \), the number of grains was counted in 30 randomly selected fields. A 60-\( \mu \text{m} \) diameter circle was randomly placed over clusters of grains within the caudal portion of the NTS. The level of expression within the analyzed circle did not exclude the presence of two or more nuclei of \( \text{CO}_2 \)-activated neurons, and the signal intensity of the clusters was above background levels. The most abundant \( \text{c-fos} \) gene expression was found within the caudal NTS region extending from the calamus scriptorius to the upper portion of the area postrema. The analyzed region was localized dorsal and lateral to the central canal and dorsal to the dorsal motoneurons of the vagus nerve. This area includes the commissural subnucleus of the NTS.

The distribution of silver grains per neuron was summarized by generating cumulative histograms to evaluate the strain (C3 vs. B6) and treatment (\( \text{CO}_2 \) vs. room air) effects. Ventilatory measurements obtained during the 30-min hypercapnic protocol were reported as a function of time (means \( \pm \text{SE} \)). A two-way analysis of variance was used to determine the statistical significance between strain and treatment effects. Silver grain distributions and time-dependent ventilatory strain differences were further assessed using Duncan’s multiple-range test. The \( \alpha \)-level for statistical significance was set at 0.01.

RESULTS

Inheritance pattern of HCVS in C3 and B6 mice. In Fig. 1, HCVS responses are illustrated for C3 and B6 parental strains and their first- and second-generation offspring. The B6 progenitor was characterized by an HCVS phenotype that was significantly (\( P < 0.001 \)) greater than the C3 progenitor and the F1 offspring. While the range of HCVS responses of C3BX resembled the C3 and F1 mice, the response ranges of the B6BX and F2 progeny were much broader, extending across the range of responses for both progenitors.

![Fig. 1. Segregation plot of individual hypercapnic ventilatory sensitivity (HCVS). Significant (\( P < 0.001 \)) strain differences in HCVS are demonstrated among C3H/HeJ (C3; \( n = 20 \) mice) and C57BL/6J (B6; \( n = 20 \) mice) mice and their B6C3F1/J (F1; \( n = 14 \) mice) offspring. Individual HCVS responses from backcross (C3BX, \( n = 20 \) mice; and B6BX, \( n = 35 \) mice) and intercross (F2; \( n = 69 \) mice) progeny are illustrated. HCVS represents the slope of the relationship between minute ventilation and the level of inspired \( \text{CO}_2 \) at 0, 3, and 8% in normoxia.](image-url)
In Table 1, SAGE results are reported for HCVS responses among C3 and B6 progenitors and their progeny. With the use of a power transformation of 0.9152, the variances in HCVS response distributions were made homogeneous for C3, B6, and F1 mice. When this transformation was applied to the entire data set, including the backcross and intercross progeny, one-locus, mixed-locus, and polygenic models were rejected because the likelihood values associated with these models were significantly different from the unrestricted model (based on the $\chi^2$ goodness-of-fit test). Only two-locus models were shown not to be significantly different from the unrestricted model, indicating that the observed response distributions for HCVS met the expected criteria defining two interactive genes. With the use of Akaike’s information criterion (2), the most parsimonious model for HCVS responses among C3 and B6 progenitors and their progeny was a two-unlinked-equal and additive loci model.

In situ hybridization studies in C3 and B6 mice. As shown in Fig. 2, $V_T$ responses between C3 and B6 mice were significantly ($P < 0.01$) different 3 min after the onset of hypercapnic challenge. No detectable strain differences in $V_T$ were observed at baseline or after 3 min of inspired hypercapnia.

In Fig. 3, $f_T$, inspiratory time ($T_i$), and mean inspiratory flow (i.e., the ratio of $V_T$ to $T_i$) responses are depicted for responses at baseline and during a 30-min hypercapnic challenge. Consistent with previous observations (33, 34), the breathing pattern at baseline and during 30 min of hypercapnia was significantly ($P < 0.01$) different between C3 and B6 mice; that is, C3 mice demonstrated a slow, deep-breathing pattern relative to the rapid, shallow phenotype of B6 mice. The $T_i$ responses in C3 mice were significantly ($P < 0.01$) prolonged at baseline and throughout the 30-min hypercapnic exposure compared with that in B6 mice. Similarly, $V_T$-to-$T_i$ responses were significantly ($P < 0.01$) greater at baseline and during hypercapnia in B6 mice relative to C3 mice.

In Fig. 4A, atlas coordinates for the mouse brain stem were depicted for Bregma $-7.48$. Brain sections were obtained from this region and extending through to Bregma $-7.20$ mm. Distribution of silver grains in the field (within the box) containing the area postrema and the caudal NTS showed the most abundant CO$_2$-induced c-fos gene expression and, therefore, was the focus of the subsequent data analysis.

In Fig. 4B, representative high-powered, dark-field photomicrographs of differential c-fos gene expression in the commissural subnucleus of the NTS are shown for C3 and B6 mice after room-air or hypercapnic exposure. There were no obvious strain differences in the number of silver grains between C3 and B6 mice after room air. After hypercapnic challenge, both strains demonstrated an increase in the number of silver grains; however, the increase in the number of silver grains was greater in B6 relative to C3 mice.

DISCUSSION

The present study demonstrates that response variation in HCVS between C3 and B6 inbred parental strains is inherited by first- and second-generation offspring. The inheritance pattern of HCVS phenotypes among backcross and intercross progeny suggests that as few as two genes determine a major proportion of the variance between the parental strains. The results further suggest that CO$_2$-induced...
neuronal activity in B6 mice (high HCVS phenotype) is significantly greater compared with that in C3 mice (low HCVS phenotype). This observation is demonstrated by a greater CO$_2$-induced c-fos gene expression in the caudal NTS, which is a neuroanatomic region of the mouse brain stem known to integrate afferent respiratory control signals. Taken together, these results suggest that the genetic bases for variation in HCVS between C3 and B6 mice likely involve determinants that regulate differential CO$_2$-induced afferent neuronal activity.

Previous studies from our laboratory have identified the C3 and B6 mice as inbred mouse strains that occupy the extremes of a strain distribution pattern (34). Because the between-strain variation is markedly greater than the within-strain variation for C3 and B6 mice (Fig. 1), the genetic effect regulating HCVS (for normoxic inspirates from 3 to 8% CO$_2$) far exceeds the environmental component. Recent progress using this genetic model of ventilatory control has pursued patterns of inheritance among different offspring classes of C3 and B6 progenitors (33). For example, the hypercapnic VE response (during normoxic inspirate of 3% CO$_2$) of the first-generation offspring (i.e., B6C3F1, F1) is similar to that of the low-responsive C3 progenitor; however, the hypercapnic f response of the F1 offspring is similar to that of the B6 progenitor. Thus the attenuated CO$_2$-induced Vt response of F1 mice is the ventilatory trait that determines low-HCVS phenotype in first-generation offspring. When the pattern of inheritance is expanded to include backcross and intercross progeny, CO$_2$-induced Vt responses were shown to be genetically determined by two unlinked genes. Moreover, when CO$_2$-induced Vt responses were standardized for Tl (i.e., mean inspiratory flow), there was a significant increase in the probability that the results fit an inheritance pattern consistent with a two-unlinked-gene model. Because mean inspiratory flow is recognized as an index of the neural “drive” to breathe (22), the results from the present and previous studies (32, 33) support the hypothesis that at least one genetic determinant between C3 and B6 mice controls variation in HCVS via differences in central neuronal activity.

In the present study, a similar profile of differential hypercapnic ventilatory phenotypes between C3 and B6 strains is observed by using an inspirate challenge of 15% CO$_2$. Although VE responses at baseline are comparable between strains, the CO$_2$-induced VE response is significantly (P < 0.01) greater in B6 compared with C3 mice during the first 3 min of the exposure (Fig. 2). Whereas the VE response of B6 mice remains relatively higher than that of C3 mice throughout the 30-min exposure period, the average CO$_2$-induced VE response was not statistically different between strains after the first 3 min, indicating the potential involvement of the peripheral chemoreceptors (15). Nonetheless, the variation in breathing pattern between strains is significantly (P < 0.01) different at baseline and during the 30-min hypercapnic
challenge; that is, C3 mice demonstrate a slow, deep-breathing pattern relative to the rapid, shallow pattern of B6 mice (Fig. 3). Whereas both strains demonstrate a progressive decay in f and VT during the 30-min hypercapnic exposure, there appears to be a precipitous decline in the VT response of B6 mice between 3 and 7 min. Because the VT response is a temperature-sensitive measurement, we measured the body temperature in both strains with telemetry (exploratory data not shown). Indeed, a progressive decline in body temperature reaching $\theta < 11$ °C occurred in both strains after 30 min of hypercapnia, which may explain the decay in VT and VE observed in Figs. 2 and 3. Other sources of time-dependent alterations in hypercapnic ventilatory characteristics may include the activation of GABAergic pathways (1), CO$_2$-induced narcosis (21), and fatigue.

A number of studies have been devoted to characterizing the components of the brain stem respiratory network (13). Several techniques have been used to trace neuroanatomic pathways that are involved in regulating ventilation. These techniques demonstrate that peripheral and central afferent inputs are concentrated within the NTS of the medulla and modulate ventilatory responses during hypercapnia. In particular, immunohistochemical identification of Fos, the protein product of the immediate-early gene c-fos, has been used as a cellular marker to identify activated neurons within the central nervous system. This gene is rapidly and transiently expressed within the cell nucleus after cell activation induced by different stimuli, including hypercapnia. Furthermore, this technique has been repeatedly used as a marker of neuronal activation induced by hypercapnia in the NTS of the adult rat (4, 5, 7, 12, 29). We, therefore, employed this technique to determine whether the phenotypic difference in HCVS between C3 and B6 mice would also coincide with differences in neuronal activation within a central integrated nucleus of respiratory afferents, commissural nucleus of the NTS. To accomplish this objective, a higher level of CO$_2$ was used (29) because the ventilatory response is more sensitive than the CO$_2$-induced expression of c-fos. With the use of a semiquantitative in situ hybridization technique, the present results demonstrate that c-fos gene expression is induced by a CO$_2$ inspirate level of 15%. A similar level of hypercapnic stimulus has been used in the adult rat to localize c-Fos protein expression to the neurons in the ventrolateral medullary surface and NTS (29), as well as to characterize the chemosensory properties of locus coeruleus neurons (3). Furthermore, high levels of CO$_2$ have been used in other physiological experiments aimed at defining chemosensory characteristics of brain stem...
neurons (3). Assessing c-fos gene expression rather than c-Fos protein is favorable because it serves to determine not only the number of cells in which c-fos is induced but also the level of gene expression in each neuron. Furthermore, in situ hybridization techniques that use radioactive ribonucleotide probes are quite sensitive and have the capacity to detect neurons with very low levels of gene expression, whereas immunocytochemical detection of c-Fos protein expression underestimates the level of c-Fos protein expression in cells with low levels of protein. Therefore, we believe that the relative differences in CO₂-induced c-fos gene expression in caudal NTS neurons between B6 and C3 mice represent phenotypic variation in centrally integrated afferent neuronal input.

It is unknown, however, whether the neurons in the NTS that express the c-fos gene in C3 and B6 mice are primarily chemosensory units or are activated as second-order neurons. Sato et al. (29) demonstrated an increase in c-Fos protein expression in adult rats after similar hypercapnic exposure as in the present study. These investigators suggested that increased neuronal activity in response to hypercapnia occurred in the ventral medullary surface and in neurons of the NTS. In conclusion, using c-fos gene expression as a marker of neuronal activation is limited to the neurons that induce c-fos as an immediate-early gene transcription factor and do not include neurons that employ other regulatory factors. Therefore, assaying for c-fos gene expression in response to hypercapnia may underestimate the level of neuronal activation in respiratory- and nonrespiratory-related neurons. The results from the present study are consistent with the hypothesis that differences in HCVS between C3 and B6 progenitors are inherited by first- and second-generation offspring and are likely determined by as few as two genes. The results also demonstrate variation between C3 and B6 mice in the number of activated neurons in the NTS after hypercapnic challenge. These observations suggest that a genetic difference between C3 and B6 mice controls the development or response network that regulates hypercapnic ventilation. Alternative explanations suggest that the strain difference is attributable to variation in molecular markers of neuronal activation. Whereas the B6 mice strain might preferentially use c-fos as a transcription factor, the C3 mice strain might use another immediate-early gene as a transcription factor. In addition, differences in upstream signaling events as well as differences in neurotransmitters, which modulate the cellular response to CO₂, may account for the differences in c-fos expression observed between the two strains. Given certain limitations in interpretation of c-fos gene induction, the results of the present study suggest that a genomic
basis for varied hypercapnic chemoreception or transduction in high-responsive B6 mice compared with low-responsive C3 mice is associated with greater affrent neuronal activity in the NTs.

The present study also demonstrates results that are critically important in understanding the dynamics of responses to hypercapnic challenge. The differences in ventilatory and c-fos gene expression between C3 and B6 strains, even close to the saturation point, indicate that genes involved in regulating variation in ventilatory responses to CO2 also regulate the differential CO2-induced expression of c-fos transcriptional factor. The subsequent transcriptional regulation likely plays a pleiotropic role in orchestrating multiple intracellular signaling pathways in response to hypercapnic challenge.

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