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Fos-Tau-LacZ Mice Reveal Sex Differences in Brainstem *c-fos* Activation in Response to Mild Carbon Dioxide Exposure

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Abstract

There are sex differences in the neurochemistry of brainstem nuclei that participate in the control of breathing, as well as sex differences in respiratory responses to hypoxia. Central chemoreception refers to the detection within the brain of minute changes in carbon dioxide (CO₂) levels and the subsequent modulation of breathing. Putative central chemoreceptor sites are widespread and include cells located near the ventral surface of the brainstem in the retrotrapezoid nucleus (RTN), in the medullary midline raphe nuclei, and, more dorsally in the medulla, in the nucleus of the solitary tract and in the locus coeruleus at the pontomedullary junction, as well as in the fastigial nucleus of the cerebellum. In this study, we ask if the cells that respond to CO₂ differ between the sexes. We used a transgenic mouse with a *c-fos* promoter driven *tau-lacZ* reporter construct (FTL) to map the locations of cells in the mouse brainstem and cerebellum that responded to exposure of mice of both sexes to 5% CO₂ or room air (control). X-gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside) histochemical staining to detect the beta-galactosidase enzyme produced staining in the brains of mice of both sexes in all of the previously identified putative chemoreceptor sites, with the exception of the fastigial nucleus. Notably, the male RTN region contained significantly more x-gal labeled cells than the female RTN region. In addition to new observations regarding potential sex differences in the retrotrapezoid region, we found the FTL mouse to be a useful tool for identifying cells that respond to the exposure of the whole animal to relatively low concentrations of CO₂.

Keywords

ventilation; hypercapnia; chemosensation; *c-fos*; retrotrapezoid nucleus; sudden infant death syndrome

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1. Introduction

Numerous regions of the vertebrate brain have been shown to exhibit sexual dimorphism, ranging from large-scale anatomical differences between the sexes, such as differences in cortical thickness (Sowell et al., 2007), to more subtle neurochemical differences, such as the levels of neurotransmitters or neurotransmitter receptors present in certain nuclei (McCarthy et al., 1997, for review). Even though many of the anatomical and neurochemical sex differences in the brain are associated with reproductive behavioral differences between the sexes, such as the larger vocal control nuclei in male songbirds, who sing to attract their mates (Nottebohm and Arnold, 1976), and the pronounced neurochemical differences among these nuclei (Bottjer et al., 1997), there is no reason to assume that sex differences in the brain are limited to neural substrates that underlie mating behaviors (Cahill, 2006). Sex differences could exist in the neural circuitry underlying a behavior as fundamental as breathing. In fact, a number of studies have reported neurochemical sex differences in respiratory-related nuclei, including the phrenic nucleus (Behan et al., 2003), the hypoglossal nucleus (Behan et al., 2003; Schlenker and Hansen, 2007; Barker et al., 2009), the nucleus of the solitary tract (Schlenker et al., 2007), and the brainstem noradrenergic cell groups (Pequignot et al., 1997). In addition, sex differences in the respiratory responses to hypoxic challenges (Tatsumi et al., 1991; Mortola and Saiki, 1996), to hypercapnic challenges in serotonin transporter knockout mice (Li and Nattie, 2008), and age-related sex differences in responses to hypoxic and hypercapnic challenges in rats (Schlenker and Goldman, 1985; Wenninger et al., 2009) have been reported. All of these data suggest that sex may be a very important, although frequently overlooked, component of respiratory control and modulation. The data related to the hypercapnic response and the nuclei that participate in that response are especially interesting given the disproportionate number of males affected by the sudden infant death syndrome and sleep apnea, two disorders thought to be related to deficits in central chemoreceptor function.

Central chemoreceptors are specialized cells in the brainstem that sense and respond to very small changes in carbon dioxide (CO₂) levels via direct or indirect projections to respiratory control nuclei. The central chemoreceptors are very important because they help ensure that the amount of breathing is sufficient to eliminate the CO₂ generated by cellular metabolism. Studies in rats (Fukuda and Loeschcke, 1977) and cats (Mitchell et al., 1963) have shown that the central chemoreceptor cells are located along the ventral surface of the brainstem, in the retrotrapezoid nucleus (RTN; Nattie and Li, 1990), in the medullary midline raphe nuclei (Richerson, 1995), and more dorsally in the medulla in the nucleus of the solitary tract (Dean et al., 1989; see Nattie, 1999; Ballantyne and Scheid, 2001, for review). Central chemoreceptor cells also have been found in the locus coeruleus at the pontomedullary junction (Pineda and Aghajanian, 1997) and in the fastigial nucleus of the cerebellum (Xu et al., 2001). Additional data suggest that central chemoreceptors in the RTN and the region surrounding it may be identified based on their patterns of gene expression, specifically the expression of galanin, the vGlut2 glutamate transporter, and the Phox2b transcription factor (Stornetta et al., 2006; 2009). In fact, people with a rare disorder called congenital central hypoventilation syndrome, who lack central as well as peripheral chemoreceptor function, have been found to have mutations in the Phox2b gene (Amiel et al., 2003).

One way to identify central chemoreceptor cells is through detection of *c-fos* expression in the brain following exposure of the whole animal to higher than normal levels of CO₂. *c-fos* is an immediate early gene, also known as an inducible transcription factor, that is activated via a number of different potential pathways when a neuron is stimulated. *c-fos* activation can occur due to the binding of growth factors to receptor tyrosine kinases, ligand binding to G-protein coupled receptors, or activation of neurotransmitter receptors and the resulting change in intracellular ion concentrations. Once activated, Fos dimerizes with one of the Jun proteins to form activator protein-1, which in turn activates so-called late genes, resulting in both short-

and long-term changes in protein expression. Because basal levels of *c-fos* mRNA and the Fos protein product are very low, and because the mRNA and protein have short half-lives, *c-fos* expression can be a reliable marker of neuronal activation in response to a specific stimulus (Morgan and Curran, 1991; Herrera and Robertson, 1996; Chaudhuri, 1997; Herdegen and Leah, 1998). In addition to its use to identify cells that respond to CO₂ (Sato et al., 1992; Larnicol et al., 1994; Teppema et al., 1994; Miura et al., 1994; Teppema et al., 1995; Haxhiu et al., 1996; Teppema et al., 1997; Sica et al., 1999; Belegu et al., 1999; Berquin et al., 2000; Okada et al., 2002), the *c-fos* technique has been used to study a number of different stimuli, including somatosensory stimulation (Staiger et al., 2002), changes in sleep states (Sherin et al., 1996), exposure to odors of the opposite sex (Muroi et al., 2006), and sound (Yang et al., 2005). This informative technique even has exposed subtle species differences in the time course of developmental changes in the tonotopic arrangement of cells in the auditory pathway (Friauf, 1992). It is important to note that the *c-fos* approach cannot distinguish between cells that are directly activated (intrinsically sensitive to a stimulus such as CO₂) and those that are synaptically driven.

Newer genetic techniques allow *c-fos* activation to be detected using *c-fos* promoter mediated reporter gene expression (Smeyne et al., 1992; Wilson et al., 2002; Barth et al., 2004). In the present study, a transgenic mouse with a *c-fos* promoter driven *tau-lacZ* reporter construct (Wilson et al., 2002; Murphy et al., 2004) was used to map the locations of all cells, presumably including central chemoreceptor cells, in the mouse brainstem that responded to exposure of mice to 5% CO₂ in room air. Mice of both sexes were used in our study to examine potential differences between the sexes in brainstem-level processing in response to elevated environmental CO₂ levels. In this study, we report new observations regarding potential sex differences in the brainstem cells that respond to CO₂ by upregulating *c-fos*.

2. Results

Utility of the FTL Mouse

The FTL mouse is a very useful tool for identifying cells that respond to exposure of the whole animal to CO₂. In the FTL mouse, x-gal labeled or simply “labeled” cells indicate cells that were sufficiently stimulated during the gas exposure procedure to activate expression of the *fos-tau-lacZ* reporter (Figure 1A). We compared cell labeling in animals exposed to CO₂ to labeling in animals exposed to room air to eliminate regions or nuclei that were involved in the animal's response to other aspects of our experimental protocol, such as the stress of being handled, the novel environment, or the sound of the gas entering the box. There were significantly more x-gal labeled cells at most levels of the brainstem following exposure to 5% CO₂ than there were following exposure to room air (Figure 1B). Labeled cells were counted in all regions of brainstem sections extending from 1200 microns caudal to 800 microns rostral to the caudal pole of the facial nucleus. In female mice, the mean total was 951 ± 103 (standard error of the mean, SEM) for 5% CO₂ and 514 ± 80 (SEM) for room air. In male mice, the mean total was 1007 ± 88 (SEM) for 5% CO₂ and 596 ± 92 (SEM) for room air. Two-way ANOVA showed a significant effect of gas exposure ($p=0.0002$), but not sex ($p=0.35$) for total cell number (Table 1).

Distribution of Labeled Cells

While the mean total number of labeled cells did not differ significantly between male and female mice exposed to 5% CO₂, the *distribution* of labeled cells appeared different in female and male mice. In both three-dimensional reconstructions (Figure 2) and graphical representations (Figure 3) of the data, female mice exposed to 5% CO₂ appeared to have more labeled cells in the rostral regions of the brainstem, while male mice appeared to have more labeled cells in the caudal regions of the brainstem. When the average numbers of labeled cells

per section were compared level by level by two-way ANOVA, the statistically significant differences between 5% CO₂ and room air in males were between 600 μ m caudal and 400 μ m rostral to the caudal pole of the facial nucleus ($p < 0.01$), while in females the differences were at 1200 μ m caudal ($p < 0.01$), 800 μ m caudal ($p < 0.05$), and between 200 and 800 μ m rostral to the caudal pole of the facial nucleus ($p < 0.01$, except 400 rostral, $p < 0.05$; Figures 3 and 4). In other words, the differences in labeled cell numbers in males were concentrated around the caudal pole of the facial nucleus, while the differences in females were predominantly in the more rostral regions of the medulla (Figure 4). Because we wondered what nuclei or regions might account for the different distributions, we compared cell counts within several regions of interest thought to be involved in central chemoreception (Figure 5). With the exception of the fastigial nucleus, we observed an increase in the number of labeled cells after CO₂ inhalation in each of these putative chemosensitive regions, as well as in several unexpected regions and nuclei.

Sex Difference in the Number of Labeled Cells in the RTN Region

Male FTL mice exposed to 5% CO₂ had significantly more labeled cells in the RTN region of the ventrolateral medulla (20 ± 3) than female mice (5 ± 2) exposed to 5% CO₂ or mice of either sex exposed to room air (Table 2). The RTN region was defined as a triangular region extending from the middle of the dorsal border of the facial nucleus to the ventrolateral edges of the brainstem bilaterally, so this region contained the RTN proper as well as cells immediately lateral or medial to the RTN (Figure 5). Cells within the borders of the facial nucleus as defined by tracing of the adjacent Nissl-stained section were not included in the RTN region counts. The total numbers of cells in the left and right RTN regions were pooled for each section.

Labeled Cells in Other Putative CCR Regions

The raphe region was defined as a triangular region extending from the dorsal border of the raphe obscurus to the lateral edges of the pyramids on either side, as identified in Nissl-stained sections (Figure 5). The delineated region therefore included the raphe obscurus, raphe pallidus, and caudal raphe magnus. The inter-raphe-RTN region was defined as the inverted triangular region between the raphe region and RTN region triangles (Figure 5). The inter-raphe-RTN region was comprised of the nucleus paragigantocellularis and the ventral aspect of the nucleus gigantocellularis. The NTS was defined by its nuclear boundaries in Nissl-stained sections. Counts were pooled for the IRR on each side and the NTS on each side. Although the raphe region and the NTS in male and female mice contained significantly more labeled cells in mice exposed to 5% CO₂ versus room air ($p < 0.05$; Figure 6, Table 2), there was no difference between the sexes in these nuclei. The average number of labeled cells in the IRR region in females was similar between animals exposed to 5% CO₂ and those exposed to room air (Figure 6, Table 2), and did not differ significantly from males exposed to 5% CO₂ ($p > 0.05$). In the male IRR there was a significant difference, however, between the average number of labeled cells following 5% CO₂ exposure versus room air exposure.

Lack of Labeling in the Fastigial Nucleus

There were virtually no labeled cells in the fastigial nucleus following exposure to 5% CO₂ (Table 2). The fastigial nucleus does contain labeled cells following exposure to 12% oxygen (hypoxia; unpublished observations) or 10% CO₂ (unpublished observations), suggesting that the fastigial nucleus in the FTL mouse does have the potential to express the reporter in response to changes in blood gases, but simply does not express the reporter in response to lower levels of CO₂.

Labeling in Other Brainstem Nuclei

In addition to labeled cells in putative chemosensory nuclei and regions, several other nuclei regularly contained labeled cells in brainstems of both sexes exposed to either 5% CO₂ or room air. These nuclei included the hypoglossal nucleus (Figure 5), the cochlear nucleus, the nucleus ambiguus, the facial nucleus (Figure 5), the spinal trigeminal nucleus, and the dorsal motor nucleus of the vagus. Labeled cells in these nuclei were not quantified, but, in general, there appeared to be fewer labeled cells in the nucleus ambiguus, dorsal motor nucleus of the vagus, and facial nucleus in controls than in animals exposed to 5% CO₂. None of these regions appeared to single-handedly (or independently) account for the more rostral distribution of labeled cells in the female.

3. Discussion

One of the most important conclusions of our study is the fact that the FTL mouse is a useful tool for identifying cells in the brainstem that respond to the exposure of the whole animal to relatively low concentrations of CO₂. Many previous studies have used *c-fos* activation to identify cells that respond to CO₂, so this technique is not new, but the use of *c-fos* promoter mediated reporter gene expression rather than a secondary detection technique is new. Few studies have been able to detect *c-fos* mRNA or Fos immunoreactivity at levels of CO₂ as low as we used here (5%). Typically previous studies using other methods of *c-fos* detection have used nothing less than 7% CO₂. Therefore, we think that *c-fos* promoter mediated reporter gene expression has built upon the foundation of previous *c-fos* studies and enhanced the resolution of this technique. In addition, in combination with detection of proteins, such as serotonin or Phox2b, known to be present in certain cell types in the brainstem that participate in the animal's response to CO₂, this model will be especially useful for studies of the anatomy and morphology of activated cells due to the inclusion of the *tau* gene and resulting distribution of the reporter in dendrites and axons.

The most surprising finding of our study is that there are differences in the distribution of labeled cells in male and female FTL mice exposed to 5% CO₂. Males have more labeled cells in the RTN region; females have more labeled cells in the rostral medulla, at levels 200-800 μ m rostral to the caudal pole of the facial nucleus. This suggests the possibility that, for the fundamental tasks of sensing and responding to changes in levels of blood gases, males and females may (a) rely on different cell populations or (b) have different sensitivities at different chemoresponsive sites. Why would the RTN region, a site known to contain central chemoreceptors, differ between the sexes? And what downstream effect might the difference have?

It has long been recognized that sex steroids have an effect on respiration (White et al., 1983; Tatsumi et al., 1991). In the last ten years, several differences between the sexes, including variations correlated with the estrous cycle, actually have been reported in respiratory related nuclei (Behan et al., 2003; 2008 for review). For example, levels of serotonin vary in respiratory control nuclei, including the hypoglossal and phrenic nuclei, over the course of the estrous cycle in female rats (Barker et al., 2009). Male and female PASKIN knockout mice deficient in PASKIN, a putative oxygen sensor molecule homologous to the bacteria-derived FixL molecule, show differential ventilatory responses to acute hypoxia (Soliz et al., 2008). More recently, it has been shown that serotonin transporter knockout mice differ in their ventilatory responses to hypercapnia based on sex, with males having a more noticeably reduced response than females (Li and Nattie, 2008). In addition, it has been shown that there are sex differences in age-related changes in hypoxic and hypercapnic ventilatory responses in rats (Wenninger et al., 2009).

It is possible that males and females may have different thresholds in terms of chemosensitivity, with the threshold varying in females over the course of the four-five day estrous cycle. Since we did not ascertain the estrous period in the females that we used in our study and because *c-fos* activity may not vary reliably with neuronal firing, or the threshold for *c-fos* activation itself, rather than the threshold for chemosensitivity, may vary during the estrous cycle, we cannot know if chemosensitivity thresholds varied over the course of the estrous cycle in our study, thereby affecting the number of labeled cells we observed.

While the aforementioned sex differences could certainly be due to differences in the distribution of sex steroid hormone receptors in these nuclei and circulating levels of sex hormones, another, not mutually exclusive possibility, is that there are underlying sex differences in these nuclei that are not subject to change. In other words, there may be differences in the hardwiring of the respiratory control and modulatory systems that arise during early development and then are fine-tuned in postnatal and adult life as levels of hormones shift, causing more subtle and plastic anatomical and neurochemical changes in areas such as the expression levels, distributions, and locations of important neurochemicals, including transporters (such as the serotonin transporter SERT), neurotransmitters and their receptors, neuromodulators and their receptors, and even steroid hormones and their receptors. Of course, our evidence does not necessarily suggest hardwiring differences. The lack of labeling in the RTN region in females could also relate to expression levels of the aforementioned neurochemicals that affect the threshold of activation of cells in the RTN region in response to CO₂. Interestingly, some contend that the RTN is the exclusive site of central chemoreception (Guyenet et al., 2008). This contention is based to our knowledge almost entirely on data collected exclusively in male animals. It would be very interesting if the chemoreceptor function of the RTN varied by gender!

We must consider the possibility that the activated cells in the RTN region may not be the central chemoreceptor cells in this region. There are other types of cells in the RTN region. Because they differ significantly in number from the room air controls and the females exposed to 5% CO₂, however, the labeled RTN cells in the male do represent a population of cells that responds to CO₂ with an upregulation of *c-fos* only in males exposed to 5% CO₂. In fact, it is possible that the threshold for *c-fos* activation may be lower in these cells in males, rather than the threshold for chemosensitivity, which suggests a fundamental intrinsic sex difference at the cellular level. If the threshold for *c-fos* activation is different between males and females in some cells within this region, then these cells may function differently in the two sexes. As an immediate early gene, *c-fos* targets other genes and changes their expression, directly affecting the amount, distribution, and/or localization of proteins in the cells. For example, genes for a neuropeptide such as galanin (Jiang et al., 1998) or a secreted growth factor such as nerve growth factor (NGF; Cui et al., 1999) could be targeted for up- or down-regulation by *c-fos*, with an impact on nearby cells with receptors for these molecules, at least over the short term, and potentially over the long term.

The observed sex difference in the RTN region could have clinical relevance to the sudden infant death syndrome (SIDS), which is more prevalent in boys than in girls by a ratio of 5:3 (Centers for Disease Control). If boys are highly reliant on the RTN while girls are more reliant on other areas, then any defects in the anatomy or alteration of the neurochemistry that impacts the RTN or any of the nuclei that project to it could have a devastating effect in boys. In girls, these types of changes in the RTN itself or its afferent cell populations may be offset by their more substantial reliance on other chemoreceptor populations. Of course, if these other regions also are affected, girls would still be at significant risk for SIDS, but it is interesting that many of the defects observed in SIDS infants seem to be concentrated at the ventral aspect of the brainstem (Kinney et al., 2001, for review), where the RTN is located in other mammals and homologous cells are hypothesized to reside in the human.

One alternative explanation for the observed sex difference in the RTN region is that male and female mice react differently to the smell of the CO₂ and the observed difference is due to secondary or even more remote activation of the RTN by regions related to their differential behavioral responses. In fact, it was recently shown that at least male mice can smell CO₂ at near atmospheric concentrations (Hu et al., 2007). According to Hu et al., 2007, carbonic anhydrase II is expressed in a subset of olfactory neurons in mice and that CAII activity is required for the ability of mice to smell minute concentrations of CO₂ (CAII/Car2 ^{-/-} were unable to detect CO₂ below 1%). It would be interesting to CO₂-expose male and female mice that could not smell CO₂ and then examine *c-fos* expression in the RTN region.

Why might males and females react differently to the smell of CO₂? A male mouse might perceive CO₂ as indicative of a predator. A female mouse might tolerate more CO₂ because she perceives it to be from her pups who cuddle close to her and nurse in the nest, where there probably is a slightly higher than average CO₂ level due to nesting materials and her litter. A male mouse also might become excited more easily by CO₂ because it could signal to him that a predator is close by or that another male is in his territory. Males therefore might have a flight or fight reaction to the scent of CO₂, whereas females might stay more calm. Our knowledge of the connections of cells in the RTN region with other nuclei or brain regions is incomplete, so this possibility cannot be excluded at this time.

Our analysis of labeled cell numbers in the IRR produced somewhat surprising results at first glance. Although the mean number of labeled cells in males exposed to 5% CO₂ was twice the mean number of labeled cells in females exposed to 5% CO₂ or room air, these numbers were not significantly different in post-hoc comparisons ($p > 0.05$). In other words, the male IRR contained significantly more labeled cells following exposure to 5% CO₂ compared with exposure to room air, but that number of labeled cells was statistically the same as the number of labeled cells in females under both conditions. We think these somewhat perplexing results reflect the diverse functions subserved by the IRR's composite cells. As defined in our study, the IRR region contains cells between the midline raphe nuclei and the medial aspect of the RTN that are part of the nucleus paragigantocellularis and, to a lesser extent, the nucleus gigantocellularis. Both of these nuclei are known to contain serotonergic neurons and are involved in a variety of functions, including respiration, muscle tone during sleep, and pain processing. The nucleus paragigantocellularis receives projections from a wide variety of sources including the nucleus gigantocellularis, the spinal cord, the caudal lateral medulla, the contralateral paragigantocellularis, the NTS, the periaqueductal gray, the lateral hypothalamus, the paraventricular nucleus of the hypothalamus, the caudal raphe, the inferior colliculus, and the locus coeruleus (Van Bockstaele et al., 1989). The fact that both the control (room air) and experimental (5% CO₂) numbers of labeled cells in females were statistically indistinguishable from the number of labeled cells in control or experimental males suggests that in females this region has steady activity during our gas exposure procedure and is consistent with the diverse processing roles of this ventral reticular region.

There are conflicting results with regard to the potential of raphe neurons, particularly serotonergic neurons within the raphe, to respond to CO₂ *in vivo*. *In vitro* studies have shown that raphe neurons increase their firing in response to acidification (Wang et al., 1998) and additional studies have identified these neurons as serotonergic (Wang et al., 2001). Other studies, however, have shown that the serotonergic raphe neurons more likely participate in central chemoreception indirectly via activation of the RTN (Mulkey et al., 2007). Due to the nature of the *c-fos* approach and its inability to distinguish cells that respond directly to CO₂ versus those that are activated up- or down-stream of chemosensitive cells, we cannot comment on the nature of activation that we see in the raphe. We can say with certainty that raphe cells do respond with upregulation of *c-fos* in response to exposure of the whole animal to mild hypercapnia.

The lack of labeling in the fastigial nucleus of FTL mice exposed to 5% CO₂ is consistent with previous findings in the rat (Zhang et al., 1997) suggesting that the threshold for activation of *c-fos* in this nucleus is higher than in other putative central chemoreceptor regions. Since we believe the FTL mouse to be more sensitive than other techniques for detecting *fos* activation, we feel confident in our finding of virtually no labeling in the fastigial nucleus following exposure to 5% CO₂. We also have analyzed a limited number of cases of mice of both sexes exposed to 10% CO₂ or 12% oxygen (unpublished observations) and observed consistent labeling in the fastigial nucleus, suggesting that this nucleus has the potential to express the FTL reporter.

In addition to labeled cells in putative chemosensory nuclei and regions, the hypoglossal nucleus, cochlear nucleus, the nucleus ambiguus, the facial nucleus, the spinal trigeminal nucleus, and the dorsal motor nucleus of the vagus contained labeled cells in brainstems of both sexes exposed to either 5% CO₂ or room air. With the exception of the cochlear nucleus, these nuclei are related to motor control of the face or throat (sniffing and breathing), cardiorespiratory regulation, or sensory processing, so it is not surprising that they become activated during our whole animal gas exposure experiments. Other *c-fos* studies also have reported Fos protein or *c-fos* mRNA expression in the cochlear nucleus (Teppema et al., 1997; Berquin et al., 2000). We speculate that in our study, activation of the cochlear nucleus in control and 5% CO₂ conditions is due to the hissing sound of the gases as they enter the box. Seventy minutes of listening to the gas' hissing should be enough to stimulate the auditory pathway, and in fact, we observed labeling all along the auditory pathway, including labeling in the superior olivary complex, medial nucleus of the trapezoid body, nucleus of the lateral lemniscus, and the inferior colliculus.

Our analysis of central chemoreceptor-related regions of interest did not reveal a single nucleus that could account for the significant rostral distribution of labeled cells in the female. In fact, even in the RTN region, the small magnitude of the number of cells cannot solely account for the significant caudal distribution of cells in the male. Even though the rostral cells in the female appear to lie outside of the previously identified central chemoreceptor nuclei, it would be very interesting to identify their source(s). Knowledge of the source(s) of these cells could provide clues about the role(s) of these cells in the female's response to CO₂ and potentially give us more insight into the male's and female's differential responses.

In summary, we think we have identified a useful transgenic model for the further study of central chemoreceptors and other cells that respond to relatively low levels of CO₂. The fact that this model will allow us to trace the axonal projections and describe the dendritic morphology of these important cells is even more exciting. The findings that activated cells are differentially distributed in males and females and that there are more activated cells in the male RTN region than the female RTN region add to the growing body of literature suggesting that sex may matter in studies of central chemoreception and the regulation of breathing.

4. Experimental Procedure

Fos-Tau-LacZ Mouse Colony

Fos-Tau-LacZ (FTL) transgenic mouse colony founders were generously provided by Adam Puche at the University of Maryland by the kind permission of Mark Murphy at the University of Melbourne, Australia. FTL mice at weanling stage (21 days) were phenotyped using a tail snip assay. The tail snip assay consisted of placing the fresh tail snip in a solution of 40 mg/ml x-gal in phosphate buffered saline (PBS) with 2mM magnesium chloride, 5mM potassium ferrocyanide, and 5mM potassium ferricyanide at 37°C for 30 minutes. The differentiating cells in the hair follicles of mice that carried the FTL allele turned blue in this solution. FTL mice were maintained in the Dartmouth Medical School and Dickinson College animal

facilities on a 12:12 light dark cycle with free access to food and water. The experimental procedures were approved by the Dartmouth Medical School and Dickinson College Institutional Animal Care and Use Committees.

Gas Exposure

Following at least one hour of acclimation to the laboratory environment, adult (>8 weeks old) male and female FTL mice were exposed to room air (control condition) or 5% carbon dioxide (CO₂) in air for 75 minutes at room temperature in a vented plexiglass box (7×9×9 inches). Mice were exposed in groups of 3-5, with mice (typically littermates) that they had been housed with since weaning. After gas exposure, the mice were returned to their homecages for 3 hours. At the end of the 3 hour waiting period, which was experimentally determined to be the optimal time for transcription, translation, and transport of the reporter, the mice were overdosed with sodium pentobarbital and perfused through the heart with 10 mL normal saline at 37°C followed by 25 mL 2% paraformaldehyde in PBS (pH 7.4) at 4°C.

Tissue Processing

Brains were removed and immersed overnight in 30% sucrose in PBS at 4°C. The next day, brains were blocked and embedded in OCT, and stored at -70°C. Brains were sectioned in the coronal plane on a cryostat (Leica 3060 or Leica 2800N) at 25 microns, and sections were collected in 4 adjacent series on gelatin-coated glass slides and stored at -70°C until staining.

X-gal Histochemistry

Beta-galactosidase expression was detected using x-gal histochemistry. For each brain, one series of thawed slides was fixed in 2% paraformaldehyde in PBS (pH 7.4) at 4°C for 5 minutes, and then washed twice for 10 minutes in PBS with 2mM MgCl₂ at 4°C. Slides were incubated in x-gal staining solution (1 mg/ml x-gal in PBS with 2 mM MgCl₂, 5mM potassium ferrocyanide, and 5mM potassium ferricyanide) for 24 hours at 37°C in the dark. Slides were then washed in PBS, fixed in 4% paraformaldehyde in PBS, washed in PBS, dehydrated through a graded series of alcohols (70, 90, 100% ETOH), cleared in xylenes, and coverslipped with Permount (Fisher Scientific).

Nissl staining

For each brain, a series of sections adjacent to those that were x-gal stained was stained with cresyl violet. Thawed slides were stained in a 2% solution of cresyl violet for up to 2 hours, washed briefly in distilled water with 2 drops of glacial acetic acid, dehydrated through a graded series of alcohols (70, 90, and 100% ETOH), cleared with xylenes, and coverslipped with Permount (Fisher Scientific).

Neuroanatomical Mapping

Mapping of x-gal labeled cells was performed using a Nikon E800 or Nikon 80i microscope equipped with a Neurolucida system (Microbrightfield, Burlington, VT). During the mapping, slides were coded so that the counter was blind to the experimental condition and sex. For each x-gal stained section that was mapped, the nearest adjacent Nissl-stained section was used to create a neuroanatomical tracing by outlining the brainstem contour and major brainstem landmarks and nuclei (including the nucleus of the solitary tract, facial nucleus, dorsal motor nucleus of the vagus, hypoglossal nucleus, spinal trigeminal nucleus, raphe nuclei, superior olive, locus coeruleus, and facial nerve) at low power (2X or 4X objective). The neuroanatomical tracing was then aligned with the adjacent x-gal stained section so that staining could be mapped in relation to major landmarks and nuclei. X-gal labeled cells were marked on the entire section using the 10X objective. Criteria for marking an x-gal labeled cell were: (1) a dark blue precipitate filling the cell body, (2) a cell nucleus identifiable based on

the relatively sparse or absent blue staining, and (3) a characteristic neuronal morphology. X-gal labeled cells were mapped at 200 micron intervals extending from 1200 microns caudal to the caudal pole of the facial nucleus to 800 microns rostral to the caudal pole of the facial nucleus (over a total length of 2000 microns). After mapping, sections for each individual brain were stacked to create a 3-dimensional reconstruction. Using the serial section manager, additional contours were added to each section to represent the putative chemosensitive regions. These regions of interest included: the expanded raphe (defined as a triangle with its apex aligned at the dorsal aspect of the midline raphe, and its base corners at the lateral aspect of the pyramidal tracts on either side), the RTN region (defined as the region below the facial nucleus extending to the ventral surface or a comparable region in sections just caudal to the facial nucleus), and the inter-raphe-RTN region (defined as the triangular region between the expanded raphe and RTN regions). Whole section and regional counts of mapped cells were computed using NeuroLucida and NeuroExplorer software.

Data Analysis

Raw counts of x-gal labeled cells mapped on whole sections and within defined regions were entered separately for each case in an Excel spreadsheet. Whole section and regional cell counts for each case were aligned based on the caudal pole of the facial nucleus. For each case, the average number of cells per section, region, or nucleus was calculated. Means were compared by two-way ANOVA (treatment group and sex).

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A **B**

Figure 1. Labeled cells in the Fos-Tau-LacZ mouse brainstem following exposure to 5% CO₂ or room air

(A) Histochemical detection of the tau-beta-galactosidase reporter produced a blue reaction product in the axons, dendrites, and cell bodies of activated neurons, but the dark blue reaction product was relatively sparse in their nuclei. These two cells are examples from the inter-raphe-retrotrapezoid region (scale bar = 10 μ m). (B) This graph illustrates the mean number of labeled cells at each level of the brainstem in animals exposed to 5% CO₂ (black) or room air (gray). There were significantly more labeled cells at most levels of the brainstem following exposure to 5% carbon dioxide than there were following exposure to room air (* p <0.05, ** p <0.01, *b* = borderline significance; p =0.059 for -400 and p = 0.056 for -200; bars are standard errors).

Figure 2. Three-dimensional reconstructions and accompanying graphs for two sample cases
 In three-dimensional reconstructions of the data, there appeared to be a different distribution of labeled cells in male and female mice exposed to 5% carbon dioxide. Lateral views of sample three-dimensional reconstructions of a 5% CO₂-exposed female (A Top, case 615) and a 5% CO₂-exposed male (B Top, case 6111) are shown side by side. The arrow points to the level of the caudal pole of the facial nucleus in each reconstruction. Raw data for the reconstructions are illustrated in graph form below each reconstruction (A Bottom, case 615 and B Bottom, case 6111).

Female

Male

Figure 3. Graphs illustrating labeled cells along the rostrocaudal axis

In graphical representations of the data, the different distributions of labeled cells along the rostrocaudal axis in females (top) and males (bottom) is clear (black = 5% CO₂, gray = room air; * $p < 0.05$, ** $p < 0.01$; bars are standard errors).

Figure 4. Graph showing rostrocaudal mean number of labeled cells following 5% CO₂ exposure as percent control by level for each sex

This graph represents the average amount of CO₂-activation over baseline activation in the experimental exposure box at each rostrocaudal level. When viewed side-by-side as percent control values rather than as absolute values, the sex difference in the rostrocaudal distribution of labeled cells is striking (black = female, gray = male; *p<0.05, **p<0.01 for within sex, between exposure groups).

Figure 5. Regions of interest as defined for this study and photos of labeled cells in different regions of the FTL mouse brainstem following exposure to 5% CO₂

(A) The regions of interest as defined for our study are shown in cross-section at three representative levels of the brainstem (shown caudal to rostral from the left to the right). Regions of interest included the raphe region [raphe obscurus (ROb), raphe pallidus (RPa), raphe magnus (RMg)], the inter-raphé-retrotrapezoid region (IRR), the retrotrapezoid region (RTN), the nucleus of the tractus solitarius (NTS), and the fastigial nucleus (FN). The facial nucleus (7) served as a landmark. Other landmarks and prominent nuclei include the spinal trigeminal nucleus (Sp5), inferior olivary nucleus (ION), hypoglossal nucleus (HG), pyramidal tract (Pyr), and the cochlear nucleus (8). (B) Labeled cells were observed in several regions of

interest related to central chemoreception, including the NTS and the raphe, RTN, and IRR regions. Labeled cells also were observed in other nuclei or regions, including the facial nucleus and the hypoglossal nucleus. Dotted lines delineate the nucleus (NTS, facial, HG) or the ventral surface of the brainstem (raphe region, RTN region). Bar = 25 μ m.

Figure 6. Graphs illustrating mean counts of labeled cells for each region of interest following exposure to 5% CO₂ or room air

In the RTN region (A) there was a significant difference between males and females, while in the the raphe region (B), the inter-raphe-retrotrapezoid region (IRR, C), and the nucleus of the tractus solitarius (NTS, D) there were no apparent sex differences but there was a significant difference between the experimental and control groups for each sex, except in the IRR, where there was no effect of gas in the female (* $p < 0.05$, ** $p < 0.01$; bars are standard errors).

Table 1
Mean numbers of cells activated in response to 5% carbon dioxide or room air in male and female mice

Counts are rounded to the nearest whole cell.

	5% Carbon Dioxide (mean $\pm$ SEM)	Room Air (mean $\pm$ SEM)
Female	951 $\pm$ 103 *	514 $\pm$ 80
Male	1007 $\pm$ 88 *	596 $\pm$ 92

* Significant effect of CO₂, p<0.01

Table 2
Mean numbers of cells activated in response to 5% carbon dioxide or room air in defined regions of interest
Counts are rounded to the nearest whole cell.

	RTN Region (mean ± SEM)	Raphe Region (mean ± SEM)	IRR Region (mean ± SEM)	NTS (mean ± SEM)	Fastigial Nucleus (mean ± SEM)
Male 5% CO ₂	20 ± 3**	18 ± 4*	19 ± 5*	165 ± 34*	6 ± 3
Female 5% CO ₂	5 ± 2	26 ± 8*	9 ± 1	143 ± 33*	2 ± 2
Male RA	3 ± 1	7 ± 1	4 ± 1	93 ± 24	0
Female RA	7 ± 2	10 ± 3	10 ± 3	54 ± 21	0

* Significant effect of CO₂, p<0.05
** significant effect of sex and CO₂, p<0.01