A search for genes that may confer divergent morphology and function in the carotid body between two strains of mice

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Submitted 27 September 2006; accepted in final form 9 November 2006

Balbir A, Lee H, Okumura M, Biswal S, Fitzgerald RS, Shirahata M. A search for genes that may confer divergent morphology and function in the carotid body between two strains of mice. Am J Physiol Lung Cell Mol Physiol 292: L704–L715, 2007. First published November 10, 2006; doi:10.1152/ajplung.00383.2006.—The carotid body (CB) is the primary hypoxic chemosensory organ. Its hypoxic response appears to be genetically controlled. We have hypothesized that: 1) genes related to CB function are expressed less in the A/J mice (low responder to hypoxia) compared with DBA/2J mice (high responder to hypoxia); and 2) gene expression levels of morphogenic and trophic factors in the CB are significantly lower in the A/J mice than DBA/2J mice. This study utilizes microarray analysis to test these hypotheses. Three sets of CBs were harvested from both strains. RNA was isolated and used for global gene expression profiling (Affymetrix Mouse 430 v2.0 array). Statistically significant gene expression was determined as a minimum six counts of nine pairwise comparisons, a minimum 1.5-fold change, and $P \leq 0.05$. Our results demonstrated that 793 genes were expressed less and that 568 genes were expressed more in the A/J strain vs. the DBA/2J strain. Analysis of individual genes indicates that genes encoding ion channels are differentially expressed between the two strains. Genes related to neurotransmitter metabolism, synaptic vesicles, and the development of neural crest-derived cells are expressed less in the A/J CB vs. the DBA/2J CB. Through pathway analysis, we have constructed a model that shows gene interactions and offers a roadmap to investigate CB development and hypoxic chemosensing/chemotransduction processes. Particularly, Gdnf, Bmp2, Kcnmb2, Tph1, Hifi1a, and Arnt2 may contribute to the functional differences in the CB between the two strains. Bmp2, Phox2b, Dlx2, and Mx2 may be important for the morphological differences.

hypoxia; microarray; development; glomus cell

ABNORMALITIES IN THE VENTILATORY response to hypoxia and hypercapnia during sleep are hallmark features in disorders such as congenital central hypoventilation syndrome, sudden infant death syndrome, and obstructive sleep apnea. The role of the carotid body (CB) in the autonomic reflex response to hypoxia is very well defined (35). Dysfunction, as well as the size of the CB, may contribute to the aforementioned disorders (19, 23, 48). Hypoxia increases ventilation, but this response is variable among individuals (28, 106, 109). Genetics may play a critical role in explaining this variability. Indeed, longitudinal studies in the same individuals and studies in twins demonstrate the role of genetics in the hypoxic ventilatory response (20, 53). Studies utilizing inbred strains of mice have also demonstrated the effect of genetics on the response to hypoxia (102). In particular, two strains of mice, DBA/2J and A/J, were identified as high and low responders to hypoxia, respectively. To further elucidate phenotypic differences between DBA/2J and A/J mice, Rubin et al. (93) examined these two strains in a model of sleep-induced hypoxia. The DBA/2J strain demonstrated a higher sensitivity to hypoxia during sleep, as evidenced by shorter time to arousal, compared with that of the A/J strain. Many factors may contribute to this phenotype of depressed hypoxic sensitivity in the A/J strain. However, a prominent contributor to this phenotype appears to be the CB. We (114) have shown that the volume of the A/J CB is significantly smaller than that of the DBA/2J CB. Furthermore, the A/J CB contains significantly fewer chemosensory cells (glomerus cells) compared with its DBA/2J counterpart (114). In addition to these morphological differences, the glomus cell sensitivity to acetylcholine (ACh) is lower in the A/J mice than that in the DBA/2J mice (114). Therefore, both morphological and functional abnormalities in the CB may correlate to decreased hypoxic sensitivity in the A/J strain.

Exposure to environmental stressors plays a critical role in CB structure and function. Studies have indicated that exposing animals to chronic hypoxia results in complex morphological, genetic, and functional changes (13, 59, 85). Some of the changes include enlargement of the CB (46), increased or decreased CB response to acute hypoxia (59), and modified neurotransmitter responses (13, 43, 44). From a developmental standpoint, exposure to hyperoxia at early neonatal periods induces hypoplasia of the CB and decreases systemic response to hypoxia during the adult stage (38). Also, postnatal exposure to hypoxia decreases glomus cell sensitivity to hypoxia (98). However, no environmental stressors were imposed on the DBA/2J and A/J mice preceding the studies described above. Therefore, we can presume that differences in the CB structure, as well as hypoxic sensitivity of the CB, between these two strains are a direct result of genetics. However, the genes responsible for the phenotypes described above are relatively unknown. We hypothesize that gene expression in the CBs of these two strains of mice are divergent, thus contributing to the disparity in the phenotypes. More specifically, 1) genes related to CB function are expressed less in the A/J mice compared with the DBA/2J mice, and 2) gene expression levels of morphogenic and trophic factors of the CB are significantly lower in the A/J mice compared with the DBA/2J mice. To test these hypotheses, we utilized microarray analysis to examine transcriptional differences between CBs of both strains of mice.
mice. This is the first attempt in the field to demonstrate differences in basal gene expression in the CBs between the hypoxic sensitive mice (DBA/2J) and the hypoxic insensitive mice (A/J). These genes may regulate components related to hypoxic sensitivity in the CBs of these mice.

METHODS

Animals. Male DBA/2J and A/J strains were initially purchased from Jackson Laboratories (Bar Harbor, ME), later bred in the animal facility of the Johns Hopkins Bloomberg School of Public Health (Baltimore, MD), and housed with proper temperature (∼22°C) and regulated light cycle (12:12-h light-dark cycle). Food (Agway Pro-Lab RMH 1000) and water were available ad libitum before tissue harvesting. All animal experiments were conducted in accordance with the Guiding Principles of the American Physiological Society for the Care and Use of Animals. Protocols for animal experimentation were approved by the Johns Hopkins University Animal Care and Use Committee.

Tissue preparation. Due to the low amount of RNA per CB, it was necessary to harvest large quantities of CBs to perform microarray analysis. For both strains, three sets of CBs were established (Table 1) to perform adequate statistical analyses (see Microarray data analysis; 3 × 3 comparison). Mice were anesthetized with urethane by peritoneal injection (1.25 g/kg). Bilateral carotid sinuses were excised via a midline incision of the neck, thus permitting visualization of CB region. The heart was excised to prevent excessive bleeding into the CB region. Immediately following heart excision, carotid bifurcations were harvested and digested into chilled phosphate-buffered saline. CBs were cleaned of extraneous tissue, deposited in ice-cold TRIzol reagent (Invitrogen), and frozen at −80°C until use. CBs were pooled into appropriate sets for subsequent RNA isolation.

RNA isolation for microarray analysis. RNA was isolated utilizing the TRIzol method (Invitrogen). Briefly, each set of CBs was homogenized in 0.8 ml of TRIzol reagent using a pestle homogenizer. Insoluble tissue was removed via centrifugation of the sample tube at 12,000 rpm for 10 min at 4°C. Clear homogenate solution was then transferred to a fresh microcentrifuge tube. The homogenate solution was dissociated via nucleoprotein units. Chloroform (0.16 ml) was added to the homogenate, agitated, and allowed to sit at room temperature for 2–3 min. The sample was again centrifuged at 12,000 rpm for 15 min at 4°C. The RNA-containing, aqueous phase was placed in a fresh microcentrifuge tube. Two microliters of glycogen (5 μg/μl) was added to facilitate RNA precipitation, followed by an addition of isopropanol (0.4 μl). The sample was incubated at room temperature for 10 min and centrifuged at 12,000 rpm for 10 min at 4°C. The RNA pellet was identified, and the overlying supernatant was discarded. The RNA pellet was washed with 0.8 ml of 75% ethanol, vortexed for mixing, and centrifuged at 7,600 rpm for 10 min at 4°C. The ethanol was discarded, and the RNA pellet was air-dried for 5–10 min. RNA was eluted in 10 μl of TE buffer and incubated for 10 min at 55°C.

Amplification, labeling, fragmentation, and hybridization for microarray. Transcriptional profiling via microarray has been previously described (89, 103). Briefly, total RNA for each set was analyzed for purity and concentration (Agilent 2100 Bioanalyzer) (Table 1). Microarray experiments were performed with the Affymetrix Mouse Genome 430 v2.0 GeneChip Array. Hybridization method was adapted from previous work (18, 89, 103). Briefly, the Two-Cycle Target Labeling protocol for GeneChip Expression Analysis (Affymetrix) was used. Beginning with 100 ng of total RNA for the first cycle of amplification, the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) was used to convert RNA into first cycle, first and second strand cDNA. After purification with the GeneChip Sample Cleanup Module (Affymetrix), first cycle cDNA was converted to cRNA for amplification using the MEGascript T7 Kit (Ambion). The first cycle cRNA was cleaned with the RNeasy Mini Kit (Qiagen) and converted to second cycle, first and second strand cDNA in a similar manner to first cycle cDNA except that a T7-oligo(DT) promoter primer (Affymetrix) was incorporated. The second cycle cDNA was cleaned in a similar manner to the first cycle cDNA and then converted to biotin-labeled cRNA with the BioArray High Yield RNA Transcript Labeling Kit (ENZO). Labeled cRNA was cleaned with the GeneChip Sample Cleanup Module (Affymetrix). Fragmentation was achieved by addition of 5X Fragmentation Buffer (Affymetrix). External control was Control Oligo B2 (3 nM), Eukaryotic Hybridization control (Affymetrix). Hybridization protocol consisted of 200 μl of hybridization cocktail containing 10 μg of fragmented cRNA placed on a microarray chip. Hybridization occurred for 18 h in the GeneChip Hybridization Oven 640 (Affymetrix). After hybridization, the chip was washed and stained using two streptavidin stains and one antibody stain on the GeneChip Fluidics Station 400 (Affymetrix) for 1.5 h. Arrays were scanned with a GCS300 Scanner (Affymetrix) for images and intensities.

Microarray data analysis. Data extraction was processed by Affy GeneChip Operating Software v1.3 (Affymetrix). Global scaling was equal to 500. Normalization factor was equal to 1. Analysis of differentially expressed transcripts was accomplished using a pairwise comparative analysis (Affy Data Mining Tool v3.1; Affymetrix). Analysis compares the differences in values of perfect match to mismatch of each probe pair in the A/J strain array to its matching probe pair on the DBA/2J strain array. P values were determined by the Wilcoxon signed rank test and denoted as increase, decrease, or no change. Signal log ratio, obtained from Data Mining Tool, estimates the magnitude and direction of change in a transcript when two arrays are compared (A/J vs. DBA/2J). Signal log ratio output was converted to fold change using the following equation recommended by Affymetrix:

fold change = \begin{cases} 
2^{\text{signal log ratio}}, & \text{signal log ratio} > 0 \\
-2^{-\text{signal log ratio}}, & \text{signal log ratio} < 0 
\end{cases} 

The p value was determined using the Lin plot according to the number of significant probes per strain by performing Student’s t-test. In our current study, we examined nine pairwise comparisons (A/J, n = 3 sets vs. DBA/2J, n = 3 sets). Significant, differentially expressed genes were selected based on the following inclusion criteria: 1) minimum six counts out of nine confirming pairwise comparisons; 2) >1.5-fold change average; 3) Mann-Whitney pairwise comparison test: P ≤ 0.05 (test performed to rank the results by concordance as a calculation of significance of each identified change in gene expression) (103). The expressed sequence tags of the selected

Table 1. Mice used for microarray analysis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Experimental Set</th>
<th>A/J</th>
<th>DBA/2J</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Number of mice</td>
<td>19</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Number of CB</td>
<td>38</td>
<td>36</td>
<td>34</td>
</tr>
<tr>
<td>Age, days</td>
<td>28.2±0.1</td>
<td>30.0±0.0</td>
<td>29.0±0.2</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>12.9±0.4</td>
<td>13.8±0.4</td>
<td>14.6±0.4</td>
</tr>
<tr>
<td>RNA concentration, ng/μl</td>
<td>41.6</td>
<td>38.4</td>
<td>20.2</td>
</tr>
</tbody>
</table>

Age and body weight are shown as means ± SE. Three sets of carotid bodies (CBs) were prepared in each strain of mice for microarray analysis.
genes were deciphered for their annotation in the NetAffx Analysis Center of the Affymetrix site (http://www.affymetrix.com). Also, gene ontology (GO), and subsequent GO mining information, was obtained at the NetAffx Analysis Center. The microarray data was submitted to the GEO database (accession no. GSE5313).

Real-time RT-PCR analysis. RNA was isolated from DBA/2J and A/J CBs utilizing the same TRIzol method as described above. However, for this analysis, a pair of CBs was individually used for RNA isolation, and RNA was treated with DNase I (Invitrogen). Briefly, 3 µl of 10× DNase I Reaction Buffer and 3 µl of DNase I Amp Grade was added to the RNA solution for a total volume of 26 µl. The solution was incubated at room temperature for 15 min followed by enzyme inactivation for 10 min at 65°C. cDNA synthesis was accomplished with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). RNA-primer mixture was composed of 2 µl of primer (50 ng/µl random hexamers) and 2 µl of 10 mM deoxynucleotide triphosphate (dNTP) mix plus 16 µl of RNA sample for a total solution volume of 20 µl. The RNA-primer mixture was incubated for 5 min at 65°C followed by a 1-min ice bath treatment. The cDNA synthesis mix solution was composed of 4 µl of 10× RT buffer, 8 µl of 25 mM MgCl₂, 4 µl 0.1 M DTT, 2 µl of RNase OUT (40 U/µl), and 2 µl of SuperScript III RT (200 U/µl) for a total volume of 20 µl. The cDNA synthesis mix solution was added to 20 µl of the RNA-primer mixture for a total volume of 40 µl and incubated for 10 min at 25°C followed by 50 min at 50°C. The reaction was terminated at 85°C for 5 min. The reaction mixture was chilled in an ice bath for 1 min, followed by the addition of 2 µl of RNase H, and incubated for 20 min at 37°C. The final volume of cDNA available for real-time PCR assay was 42 µl.

TaqMan gene expression assays (Applied Biosystems), consisting of primer/probe solutions, were used to detect transcriptional levels of endogenous control and experimental genes. The endogenous control was mouse β-actin (Mouse ACTB). Experimental genes included glial cell line-derived neurotrophic factor (Gdnf; Mn00599849_m1), bone morphogenetic protein-2 (Bmp2; Mn01340178_m1), potassium large conductance calcium-activated channel, subfamily M, β-member-2 (Kcnmb2; Mn00511481_m1), tyrosine hydroxylase (Th; Mn0047546_m1), choline acetyltransferase (Chat; Mn01221876_m1), tryptophan hydroxylase-1 (Tph1; Mn00493794_m1), hypoxia-inducible factor-1, α-subunit (Hif1a; Mn00468869_m1), aryl hydrocarbon receptor nuclear translocator-2 (Arnt2; Mn00476009_m1), paired-like homeobox-2b (Phox2b; Mn00435872_m1), distal-less homeobox-2b (Dlx2; Mn00438427_m1), and homeobox msh-like-2 (Mmx2; Mn00442992_m1).

PCR amplification of cDNA was performed with a total volume of 25 µl, comprising 12.5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems), 1.25 µl of TaqMan primer/probe assay, 10.25 µl of diethyl pyrocarbonate (DEPC)-treated water (Quality Biological), and 1 µl of template cDNA. Real-time PCR amplification was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) according to the following protocol: 2 min at 50°C, 10 min at 95°C, and 50 cycles of 95°C for 15 s followed by 60°C for 1 min. Individual cDNA samples were run in triplicates for each gene of interest, including endogenous control. After completion of real-time amplification, threshold designation was determined on the amplification plot using ABI PRISM 7000 Sequence Detection System Software (Applied Biosystems). Reaction efficiencies were similar for all real-time assays due in part to adherence to manufacturers’ suggestions (8). Due to the small quantity of RNA per pair of CBs (~6 ng/µl), we utilized a “semiquantitative” approach for analysis. The threshold for all genes was established at the ~1/3 point of the linear phase of replication. Mean cycle threshold (Ct) was calculated for each gene transcript. Then the expression value of each gene from a pair of CBs was expressed as ΔCt, where ΔCt = (mean Ctgene) – (mean Ctβ-actin). A cycle difference of 1 corresponds to a twofold change in gene expression. The data from several animals were expressed as mean ΔCt ± SE, and the Mann-Whitney test was performed between the A/J and the DBA/2J. In addition, we used the Relative Expression Software Tool v1.9.12 (REST 2005, Corbett Life Science; http://test-2005.gene-quantification.info) to compare Ct values for genes of interest between two samples (A/J vs. DBA/2J). This semiquantitative analysis for real-time RT-PCR is based on a mathematical randomization model that has been previously described (83).

RESULTS

Animals. The age of mice (29.5 ± 0.18 days, mean ± SE, n = 54 in DBA/2J; 29.3 ± 0.06 days, mean ± SE, n = 60 in A/J) and body weight (13.7 ± 0.28 g, mean ± SE, in DBA/2J; 13.6 ± 0.23 g, mean ± SE, in A/J) were not significantly different (unpaired t-test, P > 0.05 for both variables). The ages and body weights among the three experimental sets were not statistically different (one-way ANOVA, P > 0.05; Table 1).

Microarray profile. Microarray quality and hybridization information for each experimental set can be found in Supplement 1. Normalized and summarized microarray data for the comparative CB analysis can be found in Supplement 2. Data supplements are available online at the AJP-Lung Cellular and Molecular Physiology web site.

Gene expression. A total of 10,261 probe sets were expressed less and 7,285 probe sets were expressed more in the A/J CB vs. the DBA/2J CB. Based on inclusion criteria for significance, 738 genes were identified as expressed less in the A/J strain vs. the DBA/2J strain, and 554 genes were expressed more in the A/J strain vs. the DBA/2J strain. Gene expression profiles, increased and decreased, were subsequently organized into their respective GO branch: Cellular Component, Molecular Function, and Biological Process. Within each GO branch, genes were further sorted according to functional annotation terms (Fig. 1). A large number of genes involved in signal transducer activity, catalytic activity, cell, and development are expressed less in the A/J CB. Genes associated with binding, catalytic activity, envelope, and cellular process are expressed more in the A/J CB. Furthermore, we examined individual genes and their relevance to the CB. Several genes from which products are confirmed or suggested to be involved in CB function were identified. These include genes involved in ion channels, the metabolism of neurotransmitters, neurotransmitter receptors, transporters, and putative chemosensors. Some of these genes were expressed less in the CB of the A/J mice than that of the DBA/2J mice (Table 2). Additionally, we examined other genes that may also be involved in ion movement, the metabolism of neurotransmitters, neurotransmitter receptors, synaptic vesicles, second messengers, and putative chemosensors. Table 3 lists these genes that were differentially expressed in the CBs of the two strains. These genes may play a role in CB function. The final genes identified were those which are considered to be involved in morphogenesis, differentiation, and degeneration in neural crest-derived tissues. Many genes were differentially expressed between the two strains of mice (Table 4). A brief description of each gene in Tables 2–4 is in Supplement 3. To understand the interactions among differentially expressed genes, pathway analysis (Ingenuity Pathway Analysis, Ingenuity Systems) was performed. Based on this analysis and our independent literature searches, we have constructed a simplified model that explains possible pathways contributing to the morphological and functional phenotypic differences in the CB between the A/J and DBA/2J mice (Fig. 2).
Previous studies, including ours, demonstrated that the DBA/2J mice have a larger, hypoxic-sensitive CB compared with the A/J mice (76, 102, 114). These differences are likely genetically regulated. Although genetic differences may also exist in the CB afferent system, the current study has focused on the CB. We have hypothesized that 1) genes related to CB function are expressed less in the A/J mice compared with the DBA/2J mice, and 2) gene expression levels of morphogenic and trophic factors of the CB are significantly lower in the A/J mice than the DBA/2J mice. Our microarray analysis, together with real-time RT-PCR, identified many genes that fit these categories and were differentially expressed between the CBs of the two strains. We believe that this work will lead to future studies to examine the role of each gene in CB function and morphology.

We demonstrated differences in multiple genes expressed in the CB between the A/J and DBA/2J strains at 4 wk of age (Fig. 1). GO classification and functional annotation terms are broad designations for genes. Many genes have multiple functions; therefore, significant redundancy is incorporated into this manner of gene expression sorting. In addition, the CB is heterogeneous in its cellular composition. It contains not only chemosensory glomus cells, but also supporting sheath cells, in addition to blood vessels, blood cells, nerve fibers, Schwann cells, and fibroblasts. Therefore, Fig. 1 may not represent the gene expression differences in components related to oxygen sensing. Thus we examined all significant, differentially expressed genes and categorized them based on our knowledge regarding the CB function and morphology (Tables 2–4). The adult A/J mice exhibits a smaller CB with fewer chemosensory cells (114), a decreased ventilatory (102) and cardiovascular response to hypoxia (16), as well as a decreased arousal response to hypoxia during sleep (93) compared with the DBA/2J mice. Therefore, one can suspect that transcriptional differences between CBs of the two strains may account for such phenotypic expression. We have particularly focused on the genes related to current theories of hypoxic chemotransduction to assign significance to the many differentially expressed genes in our study. Due to the large number of genes discovered, we have limited our discussion mainly to the genes that may well be involved in chemoreception/chemotransduction processes (Table 2), the genes involved in the development of neural crest-derived cells (Table 4), and the genes that are closely linked to each other (Fig. 2).

The presence of oxygen-sensitive potassium channels has been established in the CB (41). These include Ca2+-activated K+ channels (also called BK or maxi-K+ channels) (80) and background TASK-like K+ channels in the rat (111), Kv4.1 and Kv4.3 (Kv4.3–1 variant) in the rabbit (94), and Kv3 in the C57bl/6J mouse (82). Furthermore, oxygen-sensitive K+ channels in neuroepithelial bodies are Kv3.3 (Kcnq3) channels (107) and Kv1.2 (Kcn2a) channels in PC12 cells (21). Since hypoxic sensitivity of the CB is compromised in the A/J mice, the expression of these channel subunits might well have been less in these mice. In our comparative gene expression data, however, no significant transcriptional differences are seen in Kv channel subunits or TASK channels. However, a specific β-subunit of BK channels (β2: Kcnnb2) was expressed significantly less in the A/J CB (Table 2). The result is consistent with our previous report (76). Inhibition of K+ channels by hypoxia is believed to depolarize glomus cells, leading to
Table 2. Differentially expressed genes that pertain to CB function

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
<th>A/J &lt; DBA/2J</th>
<th>A/J &gt; DBA/2J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cacna1b</td>
<td>Calcium channel, voltage-dependent, N type, α1B subunit 3</td>
<td>9/9</td>
<td>6/9</td>
</tr>
<tr>
<td>Cacna2d3</td>
<td>Calcium channel, voltage-dependent, α3β subunit 3</td>
<td>9/9</td>
<td>6/9</td>
</tr>
<tr>
<td>Kcnmb2</td>
<td>Potassium large conductance calcium-activated channel, subfamily M, β member 2</td>
<td>9/9</td>
<td>6/9</td>
</tr>
<tr>
<td>Scnn1b</td>
<td>Sodium channel, nonvoltage-gated 1 β</td>
<td>9/9</td>
<td>6/9</td>
</tr>
<tr>
<td>Scn8a</td>
<td>Sodium channel, voltage-gated, type VIII, α</td>
<td>7/9</td>
<td>6/9</td>
</tr>
<tr>
<td>Trpc5</td>
<td>Transient receptor potential cation channel, subfamily C, member 5</td>
<td>7/9</td>
<td>6/9</td>
</tr>
<tr>
<td>Trpm2</td>
<td>Transient receptor potential cation channel, subfamily M, member 2</td>
<td>7/9</td>
<td>6/9</td>
</tr>
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</table>

Cacna1b, Cacna2d3, and Kcnmb2 were expressed significantly less in the A/J CB than in the DBA/2J CB. In contrast, Scnn1b, Scn8a, Trpc5, and Trpm2 were expressed significantly more in the A/J CB than in the DBA/2J CB.

Not all studies listed in the table directly address the involvement of a particular gene in the CB function. For example, ion channels are composed of a few different subunits, products of different genes. Physiological experiments may not have addressed the involvement of a particular subunit (gene). Rather, the experiments suggest the contribution of the subunit (gene) to the CB function. Pairwise refers to pair counts/total number of pairs in a 3 × 3 analysis (equal to 9 possible comparisons). Genes are either decreased (A/J < DBA/2J) or increased (A/J > DBA/2J). FC avg., fold change average; Ref., reference.
and Syt7) were expressed less in the CB of the A/J mice compared with the CB of the DBA/2J mice (Tables 2 and 3). Thus the variable expression levels of genes associated with neurotransmitters and neuromodulators in the CB indicate the complexity associated with different CB chemotransduction between these two strains of mice.

The differences described above may not directly address the issue of the actual hypoxic sensor of the CB. Current theories suggest sensor mechanisms such as NADPH-oxidase (4, 45) and AMP-kinase (AMPK) (30, 31). NADPH-oxidase is thought to couple changes in oxygen tension to K+ channel activity via reactive oxygen species production (45). AMPK may function to link mitochondrial oxidative phosphorylation to calcium-mediated signaling in the CB (30). Another current theory suggests that heme oxygenase-2, with NADPH-cytochrome P-450 reductase, functions to alter BK channel dynamics under different oxygen tensions (112). However, we found no significant differences in the expression of these genes between the A/J CB and the DBA/2J CB. These findings suggest that other genes may regulate the differences in hypoxic sensitivity of the CB between the two mice. For example, Hif1a is expressed less in the A/J CB vs. the DBA/2J CB (Tables 2 and 5). Although precise mechanisms are not well understood, this gene is a candidate in the early steps of hypoxic chemotransduction or perhaps functions in the ability of the CB to adapt to long-term hypoxia or long-term intermittent hypoxia (54, 92).

An important step in the hypoxic chemotransduction paradigm is the integration of the CB output to the central nervous system via chemosensory afferent neurons of the petrosal ganglion. Gdnf and brain-derived neurotrophic factor (Bdnf) have been shown to play critical roles in the survival of chemosensory afferent neurons (29) as well as in the development of medullary neurons responsible for respiratory control.
Fig. 2. Interactions of genes. Most of the gene interactions were identified and described by Ingenuity Pathway Analysis (Ingenuity Systems). Gene interaction references can be found in Supplement 4. Genes: glial cell-derived neurotrophic factor (Gdnf); bone morphogenetic protein-2 (Bmp2); brain-derived neurotrophic factor (Bdnf); potassium large conductance calcium-activated channel, subfamily M, H9252-member-2 (Kcnmb2); Gdnf signaling receptor tyrosine kinase c-RET (Ret); Gdnf family receptor-H9251 (Gfra1); tyrosine hydroxylase (Th); nitric oxide synthase-1 (neuronal NO synthase; Nos1); choline acetyltransferase (Chat); Tachykinin-1 (Tac1); tryptophan hydroxylase-1 (tryptophan 5-monooxygenase; Tph1); single-minded homolog-1 (Sim1); hypoxia-inducible factor-1, H9251-subunit (Hif1a); aryl-hydrocarbon receptor nuclear translocator-2 (Arnt2); paired-like homeobox-2a (Phox2a); achaete-scute complex-like-1 (Ascl1); paired-like homeobox-2b (Phox2b); distal-less homeobox-2 (Dlx2); and msh homeobox homolog-2 (Msx2).

Table 4. Novel, differentially expressed genes that may be related to CB morphology

<table>
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<tbody>
<tr>
<td>Bmp2</td>
<td>Bone morphogenetic protein 2</td>
<td>8/9</td>
<td>1.89</td>
<td>(66, 110)</td>
<td>Dhh</td>
<td>Desert hedgehog</td>
<td>8/9</td>
<td>1.61</td>
<td>(77)</td>
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<td>Chemokine (C-X-C motif) ligand 12</td>
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<td>2.71</td>
<td>(12, 17)</td>
<td>Reln</td>
<td>Reelin</td>
<td>9/9</td>
<td>1.74</td>
<td>(9)</td>
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<td>(7)</td>
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<td>(97)</td>
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<td>Fgf12</td>
<td>Fibroblast growth factor 12</td>
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<td>(11, 51, 52)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Gdnf</td>
<td>Glial cell line-derived neurotrophic factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Gfap</td>
<td>Glial fibrillary acidic protein</td>
<td>9/9</td>
<td>3.68</td>
<td>(90)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hoxa2</td>
<td>Homeobox A2</td>
<td>8/9</td>
<td>1.61</td>
<td>(25)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Msx2</td>
<td>Homeobox, msh-like 2</td>
<td>6/9</td>
<td>3.05</td>
<td>(88)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Nrg3</td>
<td>Neuregulin 3</td>
<td>7/9</td>
<td>2.27</td>
<td>(115)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Phox2b</td>
<td>Paired-like homeobox 2b</td>
<td>9/9</td>
<td>2.41</td>
<td>(24)</td>
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**Genes Responsible for Development/Differentiation**

- **Bmp2**: Bone morphogenetic protein 2
- **Cxcl12**: Chemokine (C-X-C motif) ligand 12
- **Dlx1**: Distal-less homeobox 1
- **Dlx2**: Distal-less homeobox 2
- **Fgf12**: Fibroblast growth factor 12
- **Gdnf**: Glial cell line-derived neurotrophic factor
- **Gfap**: Glial fibrillary acidic protein
- **Hoxa2**: Homeobox A2
- **Msx2**: Homeobox, msh-like 2
- **Nrg3**: Neuregulin 3
- **Phox2b**: Paired-like homeobox 2b

**Genes Related to Apoptosis/Necrosis/Inflammation/Degeneration**

- **Bmf**: Bcl2 modifying factor
- **Bcl2l11**: BCL2-like 11 (apoptosis facilitator)
- **Snca**: Synuclein, α
- **Tlr1**: Toll-like receptor 1
- **Tlr4**: Toll-like receptor 4

Phox2b is known to be responsible for CB development. Other genes, which are reported to be involved in neural development, differentiation, and degeneration, may contribute to morphological differences between the two strains. Pairwise refers to pair counts/total number of pairs in a 3 × 3 analysis (equal to 9 possible comparisons). Genes are either decreased (A/J < DBA/2J) or increased (A/J > DBA/2J).
### Table 5. Real-time RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Analysis Strain</th>
<th>DBA/2J</th>
<th>A/J</th>
<th>REST (Fold Change)</th>
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<tr>
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<td></td>
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<td>5.79</td>
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<td>5.44</td>
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<td>4.00</td>
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<td>3.80</td>
<td>5.29</td>
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<td>3.60</td>
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<td>4.73</td>
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For each gene of the newly differentially expressed genes in Table 4, gene expression was analyzed in the A/J and DBA/2J CBs. The statistical significance of the results was determined by the Mann-Whitney test (P < 0.05). Negative values indicate a decreased fold change when comparing A/J vs. DBA/2J. NS, not significant.
upregulatory effect of Bmp2 on Tph1 (33). Because Tph1 is the only significant, differentially expressed gene among genes that control known neurotransmitters in the CB, further studies would provide important information regarding Bmp2’s respective role in CB development and serotonin production.

In this study, we used the microarray technique to profile differential gene expression in the CBs of two strains of mice that were not exposed to any environmental stressors. Ganforina et al. (39) examined the CB gene expression profile in mice (female, C57BL/6J01aHsd) that were exposed to physiological hypoxia. They identified groups of genes that were either switched on or switched off under physiological hypoxia. We compared our results to theirs under the premise that the A/J mice are low responders to hypoxia and therefore may not express genes identified as being switched on during hypoxia in the above study. The switched on genes are: serine (or cysteine) proteinase inhibitor, clade A, member 3 N (Serpina3n); methyl-CpG binding domain protein-1 (Mbd1); a disintegrin and metalloproteinase domain 15 (metarginid; Adam15); cytochrome P-450, family 2, subfamily b, polypeptide 20 (Cyp2b20); heat shock protein 12B (Hsp12b); and protease, Ser19 (neuropsin; Prss19). We identified similar genes from our study that were expressed less in the CB of the A/J vs. the DBA/2J: Serpina1b, Serpina3c, Adam1a, Cyp1b1, Hspa2, and Prss12. However, Mbd1 was the only gene from our data set that matched with their data and it was expressed less in the A/J CB than in the DBA/2J CB. Cyp2b9, a cytochrome P-450 family member, was found to be expressed more in the A/J CB than in the DBA/2J CB. It may be premature to state that these genes are involved in the hypoxic response of the CB due to the strain and gender differences between these two studies. Future studies including both sexes, A/J and DBA/2J mice exposed to hypoxia, and other strains would shed light on the roles of these genes in the CB hypoxic chemotransduction process.

This study offers a comprehensive analysis of the differential gene expression in the CBs of the A/J and DBA/2J strains of mice. We consider this study a useful roadmap to investigate CB development and hypoxic chemosensing/chemotransduction processes because these two strains of mice show clear differences in CB morphology and hypoxic sensitivity. However, some cautions must be applied. For this study, we used 4-wk-old animals. In our previous study (10), we found that glomus cell density within the CB was similar in young animals of both strains. However, the glomus cell density begins to diverge at 4 wk of age (10). Thus genes differentially expressed at this age might be related to some morphological and functional differences of the CB between the two strains. In addition, other genes may well be responsible for the differences between the two strains at different ages, including sex-dependent genes. Some technical issues also must be considered for interpretation of our data. For example, the CB has heterogeneous cellular populations. Therefore, careful verification is required to determine whether a certain gene is specific for glomus cells. Also, due to the small size of the CB, possible contamination of other tissues cannot be totally ruled out. In the current study, the CB was dissected under a dissecting microscope (×30 magnification) with microsurgical instruments. It was technically impossible to identify the microstructures of the CB and eliminate all extraneous tissues. This is an inherent problem with use of the whole CB. Therefore, our gene expression data and the subsequent interpretation must be evaluated carefully for significance. Particularly, the relatively small fold changes in expression would have to be examined further to evaluate their significance in this model of gene expression. In future studies, additions of histological techniques (e.g., in situ hybridization and immunohistochemistry) and possibly gene expression profiling on the single-cell level would help us to determine the possible location of an mRNA transcript, its protein product, and its relative expression in the CBs between these two strains. Regarding the confirmation of microarray analysis, we interpreted real-time transcript expression relative to endogenous control (β-actin) because initial RNA of the CBs was not quantified due to small sample size. We believe that β-actin is justified as an endogenous control in this study for the following reasons. First, in our microarray analysis, in which the amount of RNA used was controlled, β-actin expression in the CB did not differ between the two strains. Second, β-actin expression per a pair of CBs in real-time RT-PCR analysis was similar between both strains (mean Ct ± SE: 27.04 ± 0.21 in DBA/2J, n = 28; and 27.07 ± 0.23 in A/J, n = 29). Third, β-actin expression in the brain (used as a representative neural tissue) was indistinguishable between the two strains when normalized with total RNA quantity (A. Balbir, unpublished observation). Fourth, the utilization of the REST 2005 software added additional statistical power by analyzing the relationship between Ct values of the target genes and the β-actin, endogenous control that was used for this study.

In summary, the transcriptional differences uncovered in this investigation greatly support our hypotheses. The results implicate many genes involved in the development of the CB and associated hypoxic chemotransduction. All of these genes could function in concert, or independently, during the early stages of life to establish functional CB chemotransduction processes. However, further detailed studies will be required to determine the relationship between the genes depicted in Fig. 2. Also, future studies will need to address how these genes actually influence the function and development of the CB and its affrent system.

GRANTS

This work was supported by National Institutes of Health Grants HL-72293, P30-ES-038819, and AHA-0255358N. A. Balbir, R. S. Fitzgerald, and S. Biswal are supported by T32-HL-07534, HL-50712, and HL-081205, respectively.

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AJP-Lung Cell Mol Physiol • VOL 292 • MARCH 2007 • www.ajplung.org


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