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## Gene expression analyses reveal metabolic specifications in acute O<sub>2</sub>-sensing chemoreceptor cells

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**Running title:** Signature metabolic profile of acute O<sub>2</sub>-sensing

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**Key points**

- \* Glomus cells in the carotid body (CB) and chromaffin cells in the adrenal medulla (AM) are essential for reflex cardiorespiratory adaptation to hypoxia. However, the mechanisms whereby these cells detect changes in O<sub>2</sub> tension are poorly understood.
- \* The metabolic properties of acute O<sub>2</sub>-sensing cells have been investigated by comparing the transcriptomes of CB and AM cells, which are O<sub>2</sub>-sensitive, with superior cervical ganglion neurons, which are practically O<sub>2</sub>-insensitive.
- \* In O<sub>2</sub>-sensitive cells, we found a characteristic prolyl hydroxylase 3 down-regulation and hypoxia inducible factor 2 $\alpha$  up-regulation, as well as overexpression of genes coding for three atypical mitochondrial electron transport subunits and pyruvate carboxylase, an enzyme that replenishes tricarboxylic acid cycle intermediates.
- \* In agreement with this observation, the inhibition of succinate dehydrogenase impairs CB acute O<sub>2</sub> sensing. The responsiveness of peripheral chemoreceptor cells to acute hypoxia depends on a "signature metabolic profile".

**Abstract**

Acute O<sub>2</sub> sensing is a fundamental property of cells in the peripheral chemoreceptors, e.g. glomus cells in the carotid body (CB) and chromaffin cells in the adrenal medulla (AM), and is necessary for adaptation to hypoxia. These cells contain O<sub>2</sub>-sensitive ion channels, which mediate membrane depolarization and transmitter release upon exposure to hypoxia. However, the mechanisms underlying the detection of changes in O<sub>2</sub> tension by cells are still poorly understood. Recently, we suggested that CB glomus cells have specific metabolic features that favour the accumulation of

reduced quinone and the production of mitochondrial NADH and reactive oxygen species during hypoxia. These signals alter membrane ion channel activity. To investigate the metabolic profile characteristic of acute O<sub>2</sub>-sensing cells, we used adult mice to compare the transcriptomes of three cell types derived from common sympathoadrenal progenitors, but exhibiting variable responsiveness to acute hypoxia: CB and AM cells which are O<sub>2</sub>-sensitive (glomus cells > chromaffin cells) and superior cervical ganglion (SCG) neurons which are practically O<sub>2</sub>-insensitive. In the O<sub>2</sub>-sensitive cells, we found a characteristic mRNA expression pattern of prolyl hydroxylase 3/hypoxia inducible factor 2 $\alpha$  and up-regulation of several genes, in particular three atypical mitochondrial electron transport subunits and some ion channels. In addition, we found that pyruvate carboxylase, an enzyme fundamental to tricarboxylic acid cycle anaplerosis, is overexpressed in CB glomus cells. We also observed that the inhibition of succinate dehydrogenase impairs CB acute O<sub>2</sub> sensing. Our data suggest that responsiveness to acute hypoxia depends on a “signature metabolic profile” in chemoreceptor cells.

#### Abbreviations

**7-AAD**, 7-aminoactinomycin D; **Acacb**, acetyl-CoA carboxylase b; **Acly**, ATP citrate lyase; **AM**, adrenal medulla; **Cacna1d/Cav1.3**, calcium channel, voltage-dependent, L type, alpha 1D subunit; **Cacna1h/Cav3.2**, calcium channel, voltage-dependent, T type, alpha 1H subunit; **CB**, carotid body; **Chga**, chromogranin A; **Cox4i2**, cytochrome c oxidase subunit IV isoform 2; **Cox8b**, cytochrome c oxidase subunit VIIIb; **DAPI**, 4',6'-diamidino-2-phenylindole; **DMEM**, Dulbecco's modified Eagle's medium; **DMM**, dimethyl malonate; **ETC**, electron transport chain; **ETF**, electron transport flavin/quinone oxidoreductase; **FFA**, free fatty acid; **Gdf10**, growth differentiation factor 10; **Gdnf**, glial cell line derived neurotrophic factor; **GFP**, green fluorescent protein positive; **Gls**, glutaminase; **Hif1 $\beta$ /Arnt**, aryl hydrocarbon receptor nuclear translocator; **Hif2 $\alpha$ /Epas1**, hypoxia inducible factor

2 $\alpha$ /endothelial PAS domain protein 1; **Hif2 $\beta$ /Arnt2**, aryl hydrocarbon receptor nuclear translocator 2; **HVR**, hypoxic ventilatory response; **Idh1**, isocitrate dehydrogenase 1 (NADP+), soluble; **Idh3**, isocitrate dehydrogenase 3 (NAD+); **Igfbd3**, insulin-like growth factor binding protein 3; **Kcnh5**, potassium voltage-gated channel, subfamily H (eag-related), member 5; **Kcnh7**, potassium voltage-gated channel, subfamily H (eag-related), member 7; **Kcnip3**, Kv channel interacting protein 3, calsenilin; **Kcnj3**, potassium inwardly rectifying channel, subfamily J, member 3; **Kcnma1**, potassium large conductance calcium-activated channel, subfamily M, alpha member 1; **Kcnmb1**, potassium large conductance calcium-activated channel, subfamily M, beta member 1; **Kcnmb2**, potassium large conductance calcium-activated channel, subfamily M, beta member 2; **Kcnn2**, potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2; **Kcnq3**, potassium voltage-gated channel, subfamily Q, member 3; **Kcnq5**, potassium voltage-gated channel, subfamily Q, member 5; **Kcnt2**, potassium channel, subfamily T, member 2; **Kv/Kcn**, potassium voltage gated channel; **Kv3/Kcnc**, potassium voltage gated channel, Shaw-related subfamily; **Kv4/Kcnd**, potassium voltage-gated channel, Shal-related family; **L-15**, Leibowitz medium; **Ldh**, lactate dehydrogenase; **MCI**, **MCII**, **MCIII**, **MCIV**, mitochondrial complex I, II, III, IV, respectively; **Ndufa4l2**, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2; **Ntrk1**, neurotrophic tyrosine kinase receptor; **O<sub>2</sub>**, oxygen; **Olf78**, olfactory receptor 78; **Pcx**, pyruvate carboxylase; **Pdha1**, pyruvate dehydrogenase E1 alpha 1; **Pdk4**; pyruvate dehydrogenase kinase, isoenzyme 4; **pFDR**, p values adjusted with the false discovery rate; **Phd3/Egl3**, prolyl hydroxylase 3/egl-9 family prolyl hydroxylase 3; **Pnmt**, phenylethanolamine-N-methyltransferase; **PO<sub>2</sub>**, O<sub>2</sub> tension; **QH<sub>2</sub>**, ubiquinol/reduced ubiquinone; **Rgs5**, regulator of g-protein signaling 5; **RIN**, RNA integrity number; **ROS**, reactive oxygen species; **SCG**, superior cervical ganglion; **Scn7a**, sodium channel, voltage-gated, type VII, alpha; **Scn9a/Nav1.7**, sodium channel, voltage-gated, type IX, alpha; **SDHD**, succinate dehydrogenase complex, subunit D, integral membrane protein; **Slc1a5**, solute carrier family 1

(neutral amino acid transporter), member 5; **Slc18a1**, solute carrier family 18 (vesicular monoamine), member 1; **Slc7a5**, solute carrier family 7 (cationic amino acid transporter, y+ system), member 5; **SST-RMA**, signal space transformation-robust multiarray average; **Task1/Kcnk3**, potassium channel, subfamily K, member 3; **Task3/Kcnk9**, potassium channel, subfamily K, member 9; **TCA**, tri-carboxylic acid; **TH**, tyrosine hydroxylase; **Trpc5**, transient receptor potential cation channel, subfamily C, member 5; **Ucp2**, uncoupling protein 2; **Vegfa**, vascular endothelial growth factor A; **Vegfc**, vascular endothelial growth factor C.

## Introduction

Acute oxygen (O<sub>2</sub>) sensing is essential for individuals to survive in environmental or pathological conditions that result in low O<sub>2</sub> tension (PO<sub>2</sub>) in the blood (hypoxemia). The carotid body (CB), strategically located at the carotid bifurcation, is the main arterial chemoreceptor that responds to hypoxia by triggering fast (in seconds) adaptive cardiorespiratory reflexes (hyperventilation and sympathetic activation) to compensate for the decrease in PO<sub>2</sub> (for a recent review see López-Barneo et al., 2016a). Together with other O<sub>2</sub>-sensitive organs, the CB forms part of the homeostatic acute O<sub>2</sub> sensing system (Weir et al., 2005). It has close developmental and functional links with the adrenal medulla (AM), which is innervated by sympathetic nerves and, although less potently than the CB, also has, particularly in the neonate, intrinsic, non-neurogenic O<sub>2</sub> sensitivity (Adams et al., 1996; Mochizuki-Oda et al., 1997; Mojet et al., 1997; Thompson et al., 1997, 2002; García-Fernández et al., 2007a). Recently, the physiology of the CB-AM axis has attracted medical interest due to the fact that its over-activation can contribute to the exaggerated sympathetic outflow underlying hypertension and other comorbidities associated with highly prevalent human diseases (McBryde et al., 2013; Ribeiro et al., 2013; Marcus et al., 2014; del Río et al., 2016).

The mechanisms of acute O<sub>2</sub> sensing have been studied in greatest detail in the CB, which is composed of clusters of O<sub>2</sub>-sensitive glomus cells. These neurosecretory, presynaptic-like, elements release transmitters that activate sensory fibers, which impinge upon brainstem neurons involved in the control of respiration and autonomic function. Glomus cells contain a variety of K<sup>+</sup> channels, which are inhibited during hypoxia to produce depolarization and Ca<sup>2+</sup>-dependent secretory vesicle exocytosis (see Lopez-Barneo et al., 2016a). O<sub>2</sub>-regulated K<sup>+</sup> channels have also been described in AM cells as well as in other cells of the acute O<sub>2</sub>-sensing system (for reviews see Nurse et al., 2009; Weir et al., 2005). However, the precise molecular processes underlying the detection of changes in O<sub>2</sub> by chemoreceptor cells, and the nature of the signals that link O<sub>2</sub> sensing to membrane ion channels have remained unclear and a matter of debate (Peers, 2015; for an updated review see López-Barneo et al., 2016b). Recently, we have shown that ablation of the mouse *Ndufs2* gene, which encodes a component of the ubiquinone binding site in mitochondrial complex I (MCI) (see Baradaran et al., 2013), results in selective abolition of both the hypoxic ventilatory response (HVR) and sensitivity to hypoxia in single glomus and AM chromaffin cells. The data suggest that normoxic peripheral chemoreceptor cells possess special metabolic features, which result in the accumulation of reduced ubiquinone (QH<sub>2</sub>). Slow-down of the mitochondrial electron transport chain (ETC) during hypoxia may further increase the QH<sub>2</sub> pool, thereby enhancing the production of reactive oxygen species (ROS) and reduced pyridine nucleotides in MCI to signal membrane ion channels (Fernández-Agüera et al., 2015; Gao et al., 2017).

To advance our knowledge of the metabolic specifications characteristic to acute O<sub>2</sub>-sensing cells, we performed a comparative analysis of the gene expression profile in the mouse CB, AM and superior cervical ganglion (SCG). A previous gene expression study, carried out on the CB and AM from mice subjected to either normoxia or sustained hypoxia, focused on ion channels and considered the AM as an O<sub>2</sub>-insensitive tissue, the gene expression profile of which was used for

background subtraction (Ganformina et al., 2005). In another study, the gene expression profile of the CB from two different mouse strains with variable responsiveness to hypoxia was analyzed. This work reported differences in genes encoding ion channels or related to neurotransmitter metabolism, synaptic vesicles and the development of neural crest-derived cells (Balbir et al., 2007). More recently, the human CB transcriptome has been studied with attention to the expression of channels and receptors relevant for anesthesia and the up-regulation of CB genes involved in the inflammatory response (Fagerlund et al, 2010; Mkrtchian et al., 2012). While the current investigation was in progress, a transcriptomic analysis, comparing mouse CB with olfactory or vomeronasal sensory neurons using single-cell RNA sequencing, was reported (Zhou et al., 2016). This study identified abundant G protein-coupled receptor signaling, various types of ion channels, and hypoxia inducible factor  $2\alpha$  (Hif2 $\alpha$ ) in neonatal (P4-P5) glomus cells. In addition, two atypical mitochondrial ETC subunits were among the most specifically expressed genes identified in CB cells (Zhou et al., 2016). In the current study, we used adult mice to compare the transcriptomes of three cell types with the same embryological origin (neural crest-derived sympathoadrenal progenitors) but variable responsiveness to acute hypoxia: CB glomus cells and AM chromaffin cells which are O<sub>2</sub>-sensitive (glomus cells > chromaffin cells) and SCG neurons which are O<sub>2</sub>-insensitive. We expected this experimental approach to facilitate the identification of genes relevant to acute O<sub>2</sub> sensing in comparison with genes related to other cellular functions, developmental specifications, or age. Our results reveal a characteristic mRNA expression pattern of prolyl hydroxylase 3 (Phd3)/Hif2 $\alpha$  in O<sub>2</sub>-sensitive cells and expand previous studies regarding the abundance of atypical mitochondrial ETC subunits in these cells. In addition, we found that several metabolic enzymes, in particular pyruvate carboxylase (Pcx), which is fundamental to tricarboxylic acid (TCA) cycle anaplerosis (see Owen et al., 2002), are differentially expressed in CB chemoreceptor cells compared to the SCG. Finally, we show that pharmacological or genetic inhibition of succinate dehydrogenase impairs CB acute O<sub>2</sub> sensing.



Our data support the concept that responsiveness to acute hypoxia depends on a “signature metabolic profile” in peripheral chemoreceptor cells.

## Methods

### Ethical approval

All procedures were approved by the Institutional Committee of the University of Seville for Animal Care and Use (2012PI/LB02 and 22-09-15-332). Handling of the animals was conducted in accordance with the European Community Council directives 86/609/EEC, and 2010/63/EU for the Care and Use of Laboratory Animals. The experiments comply with the principles of animal research established by the Journal of Physiology (Grundy, 2015).

### Animals

TH-GFP transgenic mice were originally obtained from GENSAT (RRID: MMRRRC\_000292-UNC) on a mixed background (Gong et al., 2003) and back-crossed to C57/B6 background in our laboratory. This line was generated by random insertion of a bacterial artificial chromosome containing regulatory sequences of tyrosine hydroxylase (TH) expression followed by EGFP reporter gene. TH-SDHD mice, in which the mitochondrial complex II (MCII) subunit D (*Sdhd*) was deleted in TH<sup>+</sup> cells, were generated previously in our laboratory (Díaz-Castro et al., 2012; Platero-Luengo et al., 2014). Transgenic and wild-type (C57/B6) mice were housed at regulated temperature ( $22 \pm 1$  °C) in a 12 h light/dark cycle with *ad libitum* access to food and drink. Both male and female mice were used in the current study. Mice were sacrificed with intraperitoneal administration of a lethal dose of sodium thiopental (120-150 mg/kg) before tissue dissection. Dissected tissues were either fast-frozen with liquid N<sub>2</sub> and stored at -80°C for RNA isolation, or processed for immunohistochemical analysis, cell sorting, or functional analyses, as described below.

### Microarray analysis

Total RNA was isolated from CB, AM, and SCG of wild-type adult (~2 month old) mice using RNeasy Micro kit (Qiagen). Due to the small tissue size, each CB replicate was pooled from 10 mice, whereas each AM and SCG replicate was pooled from 3 mice to obtain enough amount of RNA. The RNA quality was determined using Agilent 2100 Bioanalyzer (Agilent). RNA samples with RNA integrity number (RIN)  $\geq 7.8$  were further processed for microarray analysis.

RNA was amplified and labeled using the GeneChip WT PLUS Reagent Kit (Affymetrix). Amplification was performed with 50 ng of total RNA input following procedures described in the WT PLUS Reagent Kit user manual. The amplified cDNA was quantified, fragmented, and labeled in preparation for hybridization to GeneChip Mouse Transcriptome 1.0 Array (Affymetrix) using 5.5  $\mu$ g of single-stranded cDNA product and following protocols outlined in the user manual. Washing, staining (GeneChip Fluidics Station 450, Affymetrix), and scanning (GeneChip Scanner 3000, Affymetrix) were performed following protocols outlined in the user manual for cartridge arrays. Data were processed for gene-level background subtraction, normalization and signal summarization (SST-RMA, signal space transformation-robust multiarray average) using Affymetrix Expression Console. Gene level differential expression analysis was then performed using Transcriptome Analysis Console 3.0 (Affymetrix). One-way between-subject (unpaired) ANOVA was used and p values adjusted with the false discovery rate (pFDR) were calculated. Gene expression was considered different between groups with pFDR < 0.05 and fold change > 2 or < -2. In addition, after the SST-RMA normalization, data were analyzed with Bioconductor to determine the similarity of gene expression profiles among samples using hierarchical clustering analysis, to evaluate the difference of gene expression patterns among the samples using principal component analysis, and to visualize differentially expressed genes using volcano plot (Huber et al. 2015). Microarray raw data are deposited in NCBI GEO database (accession number: GSE99593).

### Flow cytometry

Freshly dissected CB and AM from TH-GFP mice were quickly placed in ice-cooled modified Tyrode solution (in mM: 148 NaCl, 2 KCl, 3 MgCl<sub>2</sub>, 10 Hepes, 10 glucose, pH 7.4) for enzymatic dispersion. Dispersion of CB glomus cells and AM chromaffin cells were performed following the procedures described by our laboratory (Piruat, et al., 2004, Muñoz-Cabello et al., 2005, Levitsky and López-Barneo 2009). SCG dissected from TH-GFP mice were collected in ice-cooled Leibowitz medium (L-15), followed by enzymatic cell dispersion as described previously (Alberola-Die et al., 2013). Dispersed cells from each tissue were incubated with 7-AAD (7-Aminoactinomycin D, 1:200 dilution, BD Biosciences) in FACS buffer (L-15 supplemented with 1% penicillin/streptomycin, 0.2% bovine serum albumin, 10 mM Hepes, 5mM EDTA) to label the non-viable cells. Green fluorescent protein positive (GFP+) cells were sorted using FACSJazz cell sorter (BD Biosciences). GFP+ cells were either collected in PBS for immunocytochemical analysis or in Buffer RLT (Qiagen) and immediately frozen at -80 °C for RNA isolation.

### Real-time quantitative PCR

To validate the results of microarray analysis, total RNA was isolated using RNeasy Micro kit (Qiagen) from CB, AM, and SCG of wild-type adult mice, which were different from those used for microarray analysis. Each CB replicate was pooled from 15 mice, whereas each AM and SCG replicate was pooled from 4 mice. In addition, total RNA was also isolated from GFP+ cells of each tissue from TH-GFP mice, which were sorted by flow cytometry as mentioned above. The RNA quality was determined using Agilent 2100 Bioanalyzer (Agilent) and cRNA was amplified using either GeneChip WT PLUS Reagent Kit (Affymetrix) from whole tissues or GeneChip WT Pico kit (Affymetrix) from GFP+ cells.

Five hundred ng of cRNA was copied to cDNA using QuantiTect Reverse Transcription Kit (Qiagen) in a final volume of 20  $\mu$ l. Taqman mouse Endogenous Control Array (Applied Biosystem) was used in a Vii7 Real-Time PCR system (Applied Biosystem) in order to select a house-keeping gene among three tissues. Real-time quantitative PCR reactions were performed in a 7500 Fast Real Time PCR System (Life Technologies). PCR reactions were performed in duplicates in a total volume of 20  $\mu$ l containing 1-4  $\mu$ l of cDNA solution and 1  $\mu$ l of Taqman probe of the specific gene (ThermoFisher Scientific). *Ppia* was also estimated in each sample to normalize the amount of cRNA input in order to perform relative quantifications.

### Immunohistological analysis

GFP+ cells sorted by flow cytometry were plated on poly-L-lysine treated coverslips and incubated for 2 hours in either Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, and 84 mU/ml insulin for CB and SCG, or DMEM supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1% penicillin/streptomycin for AM. For immunohistochemical studies, mice were perfused first with PBS and then with 4% paraformaldehyde in PBS before scarification and tissue dissection. Carotid bifurcation and adrenal gland were fixed with 4% paraformaldehyde in PBS for 2 hours, cryoprotected overnight with 30% sucrose in PBS and embedded in OCT (Tissue-Tek). Tissue sections of 10  $\mu$ m were cut with cryostat (Leica). Cells and tissue sections were incubated with primary antibodies overnight at 4°C: Cox4i2 (1:100 dilution, 11463-1-1AP, Proteintech); Ndufa4i2 (1:50 dilution, 16480-1-AP, Proteintech); Pcx (1:100 dilution, ab115579, Abcam); TH (1:5000 dilution, NB300-109, Novus Biological); or TH (1:100 dilution, AB1542, Millipore). This was followed by the

incubation with fluorescent secondary antibodies: Alexa 568 or Alexa 488 (1:500 dilution, A11057, A11011, A11008, A11015, Invitrogen). Nuclei were labeled with 4',6'-diamidino-2-phenylindole (DAPI) staining. Immuno-fluorescent images were obtained using Nikon A1R+ confocal microscopy (Nikon).

### **Amperometric recording of catecholamine secretion**

Dissected CBs were placed in ice-cooled modified Tyrode solution and CB slices were prepared as described previously (Ortega-Sáenz et al., 2010). Briefly, CB slices (150  $\mu\text{m}$ ) were sectioned using vibratome (VT1000, Leica). After a brief enzymatic digestion, the slices were incubated in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, 1.2 U/ml erythropoietin and 84 mU/ml insulin at 37°C in 5% CO<sub>2</sub> for 24 hours. In some experiments, 2 mM dimethyl malonate (DMM) was added 12 h before recording. Catecholamine secretion was recorded perfusing CB slices continuously with a recording solution (in mM: 117 NaCl, 4.5 KCl, 23 NaHCO<sub>3</sub>, 1 MgCl<sub>2</sub>, 2.5 CaCl<sub>2</sub>, 5 glucose and 5 sucrose, pH 7.4) using an upright microscope (Axioscope, Zeiss). The 'normoxic' solution was bubbled with 5% CO<sub>2</sub>, 20% O<sub>2</sub>, and 75 % N<sub>2</sub> (PO<sub>2</sub> = 150 mmHg). The 'hypoxic' solution was bubbled with 5% CO<sub>2</sub> and 95% N<sub>2</sub> (PO<sub>2</sub> ~10-20 mmHg). In the high K<sup>+</sup> solution, NaCl was replaced by KCl equimolarly. DMM (2 mM) was added to the recording solution when necessary. All experiments were carried out at ~36°C. Secretory events were recorded with a 10  $\mu\text{m}$  polarized carbon fiber electrode. Amperometric currents were recorded with an EPC-8 patch-clamp amplifier (HEKA Electronics, Lambrecht/Pfaltz). Data acquisition and analysis were carried out with an ITC-16 interface (Instrutech Corporation) and PULSE/PULSEFIT software (HEKA Electronics). The secretion rate (fC/min) was calculated as the amount of charge transferred to the recording electrode during a given period of time and cumulative secretion was calculated as the integral on time of the amperometric recording.

### **Cytosolic Ca<sup>2+</sup> measurements by microfluorimetry**

CB glomus cells were dispersed as described previously (Piruat, et al., 2004), seeded on glass poly-L-lysine-treated glass coverslips, and kept in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, and 84 mU/ml insulin. To measure changes in cytosolic Ca<sup>2+</sup> concentration, glomus cells were first incubated with 4  $\mu\text{M}$  2-AM Fura (F1225, Thermo Scientific) in DMEM for 30 minutes at 37°C in a 5% CO<sub>2</sub> incubator. Loaded cells were then recorded using an inverted microscope (Eclipse Ti, Nikon) equipped with epifluorescence and photometry

(Fernández-Agüera et al. 2015). Alternating excitation wavelengths of 340 and 380 nm were used, and background fluorescence was subtracted before calculating the ratio of fluorescence intensity between 340 and 380 nm. Data were digitized at a sampling interval of 1 s. All the experiments were performed at 36 °C.

### **Statistics**

Data were presented as mean  $\pm$  SEM with the number (n) of experiments indicated and analyzed using Sigmaplot v12.0. Normality was tested with the Shapiro-Wilk test. When necessary, a log transformation was performed to normalize the data distribution prior to parametric analyses using t test. A p value  $< 0.05$  was considered statistically significant. The statistical analysis of the microarray data is described in the “Microarray analysis” section.

### **Results**

#### **Gene expression profiles in the CB, AM and SCG**

Gene expression profiles were compared among cells in the CB, AM, and SCG from adult mice using Affymetrix GeneChip Mouse Transcriptome Assay 1.0 Array, which includes >23000 protein coding genes and >55000 non-protein coding genes, such as non-coding RNA, pseudogenes, or rRNA. Hierarchical clustering analysis was first performed to analyze the similarity of the gene expression profiles between the samples. As shown in Fig. 1A, replicates from the same tissue were clustered together and appeared distant from replicates of other tissues. Principal component analysis was performed to determine the difference in gene expression patterns among the samples, revealing that the three tissues were clearly separated with two principal components (Fig. 1B). These data demonstrate the existence of a global difference in gene expression among CB, AM, and SCG cells.

### *Differential gene expression between the CB and SCG*

Gene expression in the CB compared to the SCG was studied based on cutoff values of fold change  $> 2$  and  $pFDR < 0.05$ . Up- and down-regulated genes can be visualized in Fig. 1C (light red). The genes with the largest changes in expression level (top 20 up-regulated and top 20 down-regulated genes) are listed in Table 1. Among the most differentially expressed genes, a number related to G-protein signaling (e.g. regulator of g-protein signaling 5 [*Rgs5*]) were up-regulated in the CB. Genes related to trophic factors were also up-regulated in the CB (growth differentiation factor 10 [*Gdf10*] and insulin-like growth factor binding protein [*Igfbd3*]) or the SCG (neurotrophic tyrosine kinase receptor [*Ntrk1*]). Notably, several cyto/chemokines and extracellular matrix proteins were overexpressed in the CB, which may be related to the abundance of immune cells in this organ (Mkrtchian et al., 2012) and the organization of various cell types in the CB glomeruli. In this unbiased general analysis, three genes putatively involved in  $O_2$  sensing were among the most highly expressed in the CB: NADH dehydrogenase 1 alpha subcomplex, 4-like 2 (*Ndufa4l2*), endothelial PAS domain protein 1 (*Hif2 $\alpha$* ) and the Kcnk9  $K^+$  channel (*Task3*) (see below).

We focused our analysis on genes implicated in the  $O_2$ -sensing pathway or related to cellular functions that could be relevant to acute responsiveness to hypoxia. In addition to the up-regulation of *Hif2 $\alpha$* , we found significantly increased expression of the constitutively active Hif $\beta$  subunit (*Arnt2*) and Hif-dependent angiogenic genes (*Vegfa* and *Vegfc*). Among the Phd enzymes, which hydroxylate Hif $\alpha$  protein isoforms for degradation by the proteasome, we found selective mRNA down-regulation of *Phd3* (*Egln3*) (Table 2). As MCI integrity seems to be essential for acute  $O_2$  sensing by peripheral chemoreceptors (Fernández-Agüera et al., 2015), we also studied the expression of genes encoding ETC subunits. The level of mRNA expression of most of the ETC subunits was similar between the CB and SCG. However, several genes, which code for subunits of MCI to MCIV were

slightly down-regulated in the CB compared to the SCG (Table 2). In contrast, the mRNAs of three ETC subunits were markedly overexpressed in CB cells: the *Ndufa4l2* subunit and the genes encoding for cytochrome c oxidase subunit IV isoform 2 (*Cox4i2*) and cytochrome c oxidase subunit VIIIb (*Cox8b*).

CB glomus cells (and, to a lesser degree, AM chromaffin cells) contain large amounts of biotin, a coenzyme of carboxylases (Ortega-Sáenz et al., 2016). In addition, CB cells contain high levels of succinate which could be involved in acute O<sub>2</sub> sensing (Fernández-Agüera et al., 2015). These facts led us to explore the status of genes involved in pyruvate metabolism and the TCA cycle. Notably, our analysis showed a clear induction of *Pcx* with decreased expression of a pyruvate dehydrogenase subunit (*Pdha1*), suggestive of pyruvate-mediated TCA anaplerosis (see Owen et al., 2002). We also found increased mRNA expression of pyruvate dehydrogenase kinase (Pdk4 isoform), which phosphorylates *Pdha1* and inhibits the conversion of pyruvate to acetyl-CoA (Table 2). However, Pdk4 overexpression was not confirmed in real-time quantitative PCR analyses of sorted tyrosine hydroxylase-positive (TH+) cells (see below). In contrast, the mRNA level of citrate lyase (*Acly*), a classical cataplerotic enzyme that converts cytosolic citrate to acetyl-CoA and oxaloacetate (see Owen et al., 2002), was down-regulated in sorted CB cells (see below). Acetyl-CoA carboxylase b (*Acacb*) was slightly up-regulated in CB cells, whereas several mitochondrial and cytosolic isoforms of isocitrate dehydrogenase (*Idh1* and *Idh3*) were down-regulated in CB samples. In parallel, we observed a decrease in the mRNA expression of the cationic amino acid transporter (*Slc7a5*), which could be related to decreased glutamate uptake and cytosolic production of  $\alpha$ -ketoglutarate (Table 2). Among other relevant genes, we found that the neurotrophic factor *Gdnf*, which is necessary for the maintenance of glomus cells (Villadiego et al., 2005; Pascual et al., 2008), was significantly overexpressed in the CB (Table 2).

Peripheral chemoreceptor cells contain several subtypes of O<sub>2</sub>-regulated K<sup>+</sup> channels, which play a central role in cellular responsiveness to hypoxia. In addition, other channel types mediate the cell excitability and Ca<sup>2+</sup> influx that are necessary for neurotransmitter release. The ion channels which were differentially expressed in the CB relative to the SCG are listed in Table 3. *Task3* (*Kcnk9*, see above) was the most highly up-regulated K<sup>+</sup> channel gene in CB cells, although *Task1* (*Kcnk3*) and maxi-K<sup>+</sup> β-subunits (*Kcnmb1* and *Kcnmb2*) were also induced. CB cells expressed a broad variety of voltage-gated K<sup>+</sup> channel α-subunits, including members of the Kv3 (*Kcnc*) and Kv4 (*Kcnd*) families, which are regulated by hypoxia in mouse glomus cells (Pérez-García et al., 2004). However, several subclasses of Kv (Kcn) α-subunits were markedly down-regulated in the CB (see below). The robust up-regulation of a Na<sup>+</sup>-activated K<sup>+</sup> channel (*Kcnt2*) seen in the microarray analysis was not validated by quantitative PCR studies (see below). Interestingly, calsenilin (*Kcnip3*), a K<sup>+</sup> channel-interacting Ca<sup>2+</sup>-binding protein involved in the regulation of gene expression and cell excitability (Spreafico et al., 2001), was up-regulated in CB tissue. *Kcnq3* channels, which mediate the acetyl choline-activated M-current characteristic of sympathetic neurons (see Brown and Passmore, 2009), were markedly up-regulated in SCG tissue (Table 3), further supporting the proposition that the differences in the microarray expression profile reflect genes that are differentially expressed in CB versus SCG cells. Similar to Kv channels, most Na<sup>+</sup> and Ca<sup>2+</sup> channel subunits were up-regulated in the SCG, which is compatible with the need for a high density of voltage-gated ion channels to support the electrical excitability of large SCG sympathetic neurons in comparison with small CB glomus cells (Table 3). Notable exceptions to this general trend were the CB expression of *Cacna1h* (T-type Ca<sup>2+</sup> channel α-1H subunit), which is induced by hypoxia in a HIF2α-dependent manner (del Toro et al., 2003; Carabelli et al., 2007), and *Scn7a*, an atypical Na<sup>+</sup> channel activated by extracellular Na<sup>+</sup> that is involved in the regulation of salt intake behavior (Hiyama et al., 2002). CB overexpression of *Scn7a*, however, was not confirmed in our PCR validation studies. Interestingly, the *Scn9a* gene, which



encodes the Na<sup>+</sup> channel  $\alpha$ -subunit (Nav1.7) that is involved in transmission of pain sensation (Cox et al., 2006), was markedly down-regulated in the CB relative to the SCG. In agreement with the single-cell sequencing study of Zhou et al. (2016), the gene encoding the cation-permeable transient receptor potential channel *Trpc5* was among the most highly expressed in CB cells.

#### *Differential gene expression between the AM and SCG*

Up- and down-regulated AM genes can be visualized in Fig. 1C (light red). The genes with the largest changes in expression level (top 20 up-regulated and down-regulated genes) are listed in Table 4. Among the most differentially up-regulated genes in the AM were some related to steroid metabolism, which suggests contamination from adrenocortical cells that in the mouse can be embedded in the AM region. However, within this group we also found several highly induced genes, such as phenylethanolamine-N-methyltransferase (*Pnmt*), vesicle monoamine transporter 1 (*Slc18a1*) and chromogranin A (*Chga*), which are characteristic of AM chromaffin cells. In addition, this unbiased analysis revealed genes relevant to CB acute O<sub>2</sub> sensing (mitochondrial subunit *Ndufa4l2* and *Task3* and *Task1* K<sup>+</sup> channels; see Table 1) which were also highly expressed in AM cells (Table 4). Moreover, a number of genes that were more highly expressed in the SCG than the CB, such as *Htr3a*, *Htr3b*, *Napb*, *Nefl*, *Tubb2a*, *Nrip3* (see Table 1), appeared in the list of the top 20 down-regulated genes in AM cells (Table 4).

When we focused our analysis on genes related to O<sub>2</sub> sensing or potentially relevant to acute responsiveness to hypoxia, we found a qualitative profile similar to that previously revealed in the comparison between CB and SCG cells. We observed high expression of *Hif2 $\alpha$*  and *Hif2 $\beta$*  (*Arnt*) isoforms as well as down-regulation of *Phd3* in the AM versus SCG. Moreover, in parallel with a slight decrease in the expression of some subunits of mitochondrial ETC complexes, the three atypical mitochondrial subunits up-regulated in the CB (*Ndufa4l2*, *Cox4i2* and *Cox8b*) were also

enriched in AM cells (Table 5). In addition, our analysis identified a clear induction of *Pcx*, with down-regulation of *Pdha1* and decreased expression of the neutral amino acid transporter (*Slc1a5*), which can also transport glutamine (van Geldermalsen et al., 2016). The data further revealed a clear decrease in lactate dehydrogenase (*Ldh*) and glutaminase (*Gls*) expression in AM cells, which was not detected in CB tissue.

The general pattern of ion channel gene expression in the AM versus SCG was, despite some notable exceptions, qualitatively similar to that observed in the comparison between the CB and SCG. The K<sup>+</sup> channels with more pronounced differential expression were *Task3* (*Kcnk9*) and *Task1* (*Kcnk3*). As in the CB, maxi-K<sup>+</sup> channel subunits (*Kcnma1* and *Kcnmb2*) and calsenilin (*Kcnip3*) were up-regulated in AM cells (Table 6). A member of the small/intermediate conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel family (*Kcnn2*), which could mediate part of the O<sub>2</sub>-sensitive K<sup>+</sup> current (Keating et al., 2001) was also slightly overexpressed in AM cells. Interestingly, the level of expression of the *Kcnj11* subunit, which encodes a K<sub>ATP</sub> channel (Kir 6.2) highly relevant to the developmental decrease of hypoxia sensitivity in chromaffin cells (Buttigieg et al., 2009; Salman et al., 2014) was practically the same in AM in comparison with CB or SCG cells (1.09 fold change AM vs. SCG and 1.06 fold change CB vs. SCG). As in the CB, we also observed a generalized decrease in the expression of voltage-gated K<sup>+</sup> channels subunits in the AM, particularly in the case of the genes encoding eag-related (*Kcnh5* and *Kcnh7*) and G protein-regulated inward rectifier (*Kcnj3*) channels. K<sup>+</sup> channels mediating the M-current (*Kcnq3* and *Kcnq5*) were down-regulated in AM cells. Similar to the CB, several subunits of voltage-gated Ca<sup>2+</sup> and Na<sup>+</sup> channels were down-regulated in the AM in comparison with SCG cells. The largest difference was seen in the *Scn9a* gene, which encodes the Na<sup>+</sup> channel  $\alpha$ -subunit (Nav1.7) that mediates pain sensation in the peripheral nervous system (Cox et al., 2006). In contrast, the *Cacna1d* and *Cacna1h* genes which encode, respectively, the  $\alpha$ -subunit of low voltage-activated L-type Ca<sup>2+</sup> channels (Cav1.3) (Vandael et al., 2015) and the O<sub>2</sub>-sensitive component of the

T-type current (del Toro et al., 2003; Carabelli et al., 2007) were overexpressed in chromaffin cells. As in the CB, the *Trpc5* gene was also highly induced in AM cells.

### Expression of selected genes in tissues and sorted tyrosine hydroxylase-positive cells

To validate our microarray results, real-time quantitative PCR was performed using a new set of biologically independent samples. As shown in Table 7, similar results were obtained for most of the genes tested in the two comparative analyses (CB vs. SCG and AM vs. SCG), thereby supporting the results of the microarray study. A limitation of using whole tissues is the contamination from non-neuronal cells that occurs, particularly in the case of the CB, which, in addition to TH<sup>+</sup> glomus cells, contains numerous capillaries and other cell types. To circumvent this issue, we used TH-GFP mice in which the expression of GFP was under the control of the TH promoter. This allowed us to isolate TH<sup>+</sup> (GFP<sup>+</sup>) cells from each tissue (Fig. 2A). We confirmed that the sorted cells were TH<sup>+</sup> by immunofluorescence staining (Fig. 2B). The percentage of TH<sup>+</sup>/GFP<sup>+</sup> cells counted in random samples was 87% (377/434), 95% (124/131), and 83% (80/97) from the CB, SCG, and AM, respectively. Genes analyzed by real-time PCR using the sorted TH<sup>+</sup> cells showed similar relative expression to that seen in the analysis of whole tissues (Table 7). The up-regulation of *Hif2 $\alpha$*  and the ETC subunits *Ndufa4l2*, *Cox4i2* and *Cox8b*, and the down-regulation of *Phd3* in the CB and, less potently, in the AM versus SCG were validated with high significance in the PCR analyses on isolated TH<sup>+</sup> cells. Overexpression of *Pcx* and down-regulation of *Acly*, *Slc7a5* and *Idh1* were also confirmed in the comparison between TH<sup>+</sup> CB and SCG cells. With the exception of *Pcx* and *Cox4i2*, this metabolic gene profile was also clearly seen in AM cells (Table 7). All the ion channel-related genes overexpressed in the CB and AM were also validated with high significance by quantitative PCR of sorted TH<sup>+</sup> cells, with the exception of *Trpc5* in the AM. In general, high variability among the replicates of sorted TH<sup>+</sup> cells from each tissue was observed compared to that of whole tissue

analysis, which could explain the lack of statistical significance obtained using TH<sup>+</sup> cells. Among other genes tested, uncoupling protein 2 (*Ucp2*) was up-regulated in TH<sup>+</sup> CB and AM cells which also validates the microarray data. In addition, the quantitative PCR analysis of whole tissue or sorted TH<sup>+</sup> cells showed that the *Olfir78* gene, which encodes an atypical olfactory receptor expressed in several tissues outside the nasal epithelium, and particularly in the ganglia of the autonomic nervous system (Weber et al., 2002), was up-regulated in CB glomus cells relative to sympathetic neurons, but markedly down-regulated in AM chromaffin cells (Table 7).

To investigate whether the differential gene expression observed in our microarray analysis was reflected at the protein level, we performed immunofluorescence experiments on carotid bifurcations and adrenal glands. We focused this analysis on the mitochondrial ETC subunits for which antibodies are available (*Ndufa4l2* and *Cox4i2*) and *Pcx*. As shown in Fig. 3A, the immunoreactive signal against *Ndufa4l2* was higher in CB glomus cells than SCG neurons, as demonstrated by *Ndufa4l2*/TH co-localization. A similar result was observed when comparing the AM with the adrenal cortex (Fig. 3B). Strong immunostaining against the *Cox4i2* subunit was observed in both CB and AM TH<sup>+</sup> cells (Fig. 4). *Pcx* was more highly expressed in the CB than the SCG (Fig. 5). However, the immunostaining signal against this protein in the AM was indistinguishable from that in the adrenal cortex (data not shown). This is in agreement with our quantitative PCR data, in which no up-regulation of *Pcx* mRNA was observed in sorted AM chromaffin cells (Table 7).

### **Impairment of acute O<sub>2</sub> sensing by genetic and pharmacological inhibition of succinate dehydrogenase**

In a previous study, we suggested that CB glomus cells contain high levels of QH<sub>2</sub> due to succinate-dependent metabolism, and that a further increase in the QH<sub>2</sub> pool during hypoxia leads to the generation of ROS and reduced pyridine nucleotides to signal membrane ion channels

(Fernández-Agüera et al., 2015). This proposal, which is compatible with the up-regulation of Pcx in glomus cells described here (see Discussion), predicts that inhibition of succinate dehydrogenase should decrease acute responsiveness to low PO<sub>2</sub>. We have generated a MCII-null mouse (TH-SDHD) carrying a floxed *Sdh* allele which encodes the membrane anchoring subunit D of succinate dehydrogenase. This allele was deleted in CB glomus cells and other catecholaminergic cells by the transgenic expression of a Cre recombinase under the control of the TH promoter (Díaz-Castro et al., 2012). The analysis of responsiveness to hypoxia in the glomus cells of TH-SDHD mice is not straightforward because these cells enter a degenerative process that leads to their death (Díaz-Castro et al., 2012, Platero-Luengo et al., 2014). However, we were able to demonstrate that responsiveness to hypoxia disappears in *Sdh*-deficient glomus cells before they stop responding to high extracellular K<sup>+</sup> or hypoglycemia (Fig. 6A & B). These data, which suggest that succinate dehydrogenase activity is required for normal acute O<sub>2</sub> sensing, were confirmed by experiments using glomus cells incubated overnight with dimethyl malonate (DMM), a membrane-permeant competitive inhibitor of succinate dehydrogenase (see Gutman, 1978). DMM-treated (for ~12 h) glomus cells showed a drastic decrease in hypoxia-induced catecholamine secretion, which was partially recovered during washout of malonate from the extracellular solution. Incubation with DMM did not significantly affect the secretory response to high extracellular K<sup>+</sup> (Fig. 6C-E).

## Discussion

### Differential gene expression profiles in the CB or AM versus SCG

In this study, we analyzed the gene expression pattern in three sympathoadrenal tissues (CB, AM and SCG) with variable sensitivity to hypoxia to identify genes which are relevant to acute O<sub>2</sub> sensing in peripheral chemoreceptors. Our results reveal several genes potentially related to acute O<sub>2</sub> sensing that are modulated (induced or repressed) in the CB (and, to a lesser extent, in the AM) in comparison with the SCG. We then focused on genes that encode proteins related to the Phd/Hif

pathway, mitochondrial ETC, metabolic enzymes and transporters, and ion channels. Some of the genes described here were also investigated in previous microarray analyses of CB tissue (Ganfornina et al., 2005; Balbir et al., 2007; Fagerlund et al., 2010; Mkrtchian et al., 2012) and particularly in a recent work that used single CB cell RNA sequencing (Zhou et al., 2016). However, our experimental approach, based on the comparison of adult tissues of the same developmental origin but variable sensitivity to hypoxia, allowed us to focus on genes with a similar qualitative expression pattern in O<sub>2</sub>-sensitive tissues (CB and AM) in comparison with O<sub>2</sub>-insensitive SCG cells.

### *Phd3 and Hif2 $\alpha$*

*Hif2 $\alpha$*  is one of the most highly expressed genes in CB cells versus SCG neurons, whereas *Hif1 $\alpha$*  is only slightly up-regulated. This finding confirms previous reports of the constitutive expression of *Hif2 $\alpha$*  in adult mouse CB tissue (Tian et al., 1998) and the large number of *Hif2 $\alpha$*  transcripts in neonatal mouse CB cells (Zhou et al., 2016). In contrast, we found a robust and previously undetected down-regulation of *Phd3* mRNA in CB versus SCG cells. Indeed, *Phd3* mRNA expression was negligible in sorted glomus cells. Phd3 has relatively higher influence on Hif2 $\alpha$  hydroxylation leading to its degradation (Appelhoff et al., 2004). Therefore, in addition to other functions (see below), down-regulation of Phd3 probably contributes to the maintenance of a high Hif2 $\alpha$  protein level in normoxic CB glomus cells. *Hif2 $\alpha$*  mRNA overexpression and *Phd3* down-regulation were also clearly observed in AM cells. Hif2 $\alpha$  has an important role in CB homeostasis (Peng et al., 2011), and is necessary for a normal HVR and CB growth in response to chronic hypoxia (Hodson et al., 2016). On the other hand, the Phd3/Hif2 $\alpha$  pathway is involved in the regulation of sympathoadrenal development (Bishop et al., 2008; Macías et al., 2014). The CB and AM are slightly hypertrophied in Phd3-null animals, and glomus cells exhibit a robust responsiveness to hypoxia (Bishop et al., 2008; Macias et al., 2014; our own unpublished observations). Therefore, our findings

suggest that the pattern “*Phd3* down-regulation/*Hif2 $\alpha$*  overexpression” is essential for the metabolic specialization that confers acute O<sub>2</sub> sensitivity upon CB and AM cells (see below).

#### *Atypical mitochondrial subunits*

We found three atypical mitochondrial ETC subunits, *Ndufa4l2*, *Cox4i2* and *Cox8b*, the mRNA of which was highly up-regulated in CB and AM versus SCG cells. The protein levels of *Ndufa4l2* and *Cox4i2* were also higher in CB and AM chemoreceptor cells in comparison with SCG neurons. High levels of *Ndufa4l2* and *Cox4i2* transcripts have also been observed in neonatal CB glomus cells (Zhou et al., 2016). It has previously been reported that *Ndufa4l2* and *Cox4i2* mRNAs are strongly up-regulated by hypoxia (Fukuda et al., 2007; Brown et al., 2010; Tello et al., 2011; Aras et al., 2013), but, to our knowledge, the regulation of *Cox8b* expression by hypoxia has not been documented. However, the mouse *Cox8b* gene contains two putative Hif binding sites in the promoter, one of them near the transcription initiation site (data not shown). Therefore, it is possible that the three atypical mitochondrial subunits are overexpressed in glomus and chromaffin cells due to the constitutively high *Hif2 $\alpha$*  expression in these cells. However, the function of these atypical ETC subunits is unclear and their role in CB or AM cell physiology remains to be determined. *Ndufa4l2* is a paralog of the more ubiquitous subunit *Ndufa4*, which was thought to be a component of MCI (Carroll et al., 2006), although recent studies have suggested that it is associated with MCIV (Balsa et al., 2012; Kadenbach and Hüttemann, 2015). *Ndufa4l2* expression seems to decrease O<sub>2</sub> consumption and ROS production by mitochondria (Tello et al., 2011). On the other hand, *Cox4i2*, the expression of which is restricted to lung and other highly oxygenated tissues, is an isoform of the broadly expressed subunit *Cox4i1*. In some mammalian cells exposed to hypoxia, *Cox4i2* is induced to replace *Cox4i1* thereby increasing the efficiency with which MCIV facilitates the transfer of electrons to O<sub>2</sub> and decreasing oxidative stress (Fukuda et al., 2007). In lung, *Cox4i2* knockout

reduces MCIV activity to 50%, despite the presence of the *Cox4i1* isoform (Hüttemann et al., 2012). There are three tissue-specific Cox8 isoforms (a, b and c), although to date no specific function has been identified for none of these (Kadenbach and Hüttemann, 2015). In several studies, however, Cox8b has been associated with thermogenic differentiation (“browning”) of the white adipose tissue in rodents (Fisher et al., 2012; García et al., 2016). How the combination of the three subunits (Ndufa4l2, Cox4i2 and Cox8b) could render glomus cell mitochondria highly O<sub>2</sub>-sensitive is unknown. Cox4i2 and Cox8b are integral proteins with parallel single transmembrane  $\alpha$ -helices that run in close proximity (see Tsukihara et al., 1996). One could therefore speculate that these subunits interact to regulate O<sub>2</sub> diffusion through the inner membrane, in order to reach heme a<sub>3</sub>, which is buried inside MCIV (Michel et al., 1998). Low O<sub>2</sub> accessibility to the catalytic site would make the rate of cytochrome a<sub>3</sub> oxidation highly sensitive to decreases in PO<sub>2</sub>. Together, the data discussed here suggest the existence of mitochondrial specializations that are characteristic of acute O<sub>2</sub>-sensing cells. Several decades ago a low affinity cytochrome C oxidase was proposed to exist in the CB, although located in type II rather than O<sub>2</sub>-sensitive glomus (type I) cells (Mills and Jöbsis, 1970). More recently, the involvement of mitochondria in acute O<sub>2</sub> sensing has been suggested by numerous studies of CB and AM cells (Duchen and Biscoe, 1992a and b; Ortega-Sáenz et al., 2003; Wyatt and Buckler, 2004; Buttigieg et al., 2008; Buckler and Turner, 2013, Fernández-Agüera et al., 2015). It is also worth noting that the existence of a mitochondrial based redox acute O<sub>2</sub> sensor is a long-standing hypothesis, which was postulated to explain acute hypoxic pulmonary artery vasoconstriction (Archer et al., 1993; Waypa et al., 2001).

#### *Pyruvate carboxylase and other metabolic enzymes and transporters*

Our results demonstrate that Pcx is highly expressed in CB glomus cells (at the level of mRNA and protein) in comparison with SCG neurons. This occurs in parallel with a smaller decrease in pyruvate



dehydrogenase (*Pdha1*) mRNA levels. Therefore, these data suggest that pyruvate is preferentially used by Pcx in glomus cells mitochondria to generate oxaloacetate, which represents a classical anaplerotic reaction that results in replenishment of the pool of TCA cycle intermediates (see Owen et al., 2002). These observations explain the extraordinarily high levels of biotin (a cofactor of carboxylases) recently found in glomus cells (Ortega-Sáenz et al., 2016) and the need for  $\text{HCO}_3^-/\text{CO}_2$  buffers (which are the source of the carbon atoms required by carboxylases) to maintain a robust chemosensory function in CB *in vitro* preparations (Iturriaga and Lahiri, 1991; our own unpublished observations). Replenishment of TCA cycle intermediates is also compatible with the high levels of succinate in the CB and the postulated accumulation of  $\text{QH}_2$  in acutely responding  $\text{O}_2$ -sensitive cells (Fernández-Agüera et al., 2015). If pyruvate is preferentially used to generate oxaloacetate in glomus cell mitochondria, the main source of acetyl-CoA necessary for the TCA cycle could be free fatty acid (FFA)  $\beta$ -oxidation. Although we do not have any direct evidence that FFA catabolism is activated in chemoreceptor cells, this idea is compatible with the existence of abundant adipose tissue in the CB (Ortega-Sáenz et al., 2013) and the low *Phd3* level characteristic of glomus cells, as it is known that *Phd3*-dependent hydroxylation of *Acacb* activates the synthesis of malonyl-CoA, a potent inhibitor of mitochondrial FFA uptake (German et al., 2016). Enhanced FFA catabolism (facilitated by *Phd3* down-regulation) would also significantly contribute to increasing the  $\text{QH}_2$  pool in chemoreceptor cells, as each  $\beta$ -oxidation cycle produces  $\text{FADH}_2$ , which is directly converted to  $\text{QH}_2$  by the electron transport flavin/quinone oxidoreductase (ETF). Numerous cell types (in particular proliferating cells) rely on glutamine oxidation for TCA cycle anaplerosis. Glutamine is deaminated to glutamate, which is converted into  $\alpha$ -ketoglutarate, a TCA cycle intermediate (Yang et al., 2014) and a substrate of the  $\text{O}_2$ -regulated prolyl hydroxylases (Epstein et al., 2001). Interestingly, in glomus cells, we observed a significant decrease not only in the expression of the cationic amino acid transporter *Slc7a5*, but also in the mRNA levels of the soluble isoform of isocitrate dehydrogenase (*Idh1*), which is necessary for

the synthesis of  $\alpha$ -ketoglutarate in the cytosol. These metabolic adaptations, which help to maintain low levels of  $\alpha$ -ketoglutarate, are probably required to ensure low Phd3 activity, as it is known that under conditions in which Phd3 is inhibited (e.g. during hypoxia) the enzyme can be reactivated when cells are exposed to daily  $\alpha$ -ketoglutarate administration (Tennant and Gottlieb, 2010). The metabolic specializations of CB glomus cells, which may be relevant to acute O<sub>2</sub> sensing, were also qualitatively present in AM cells with the exception of *Pcx* which was not up-regulated in our sorted TH<sup>+</sup> AM cells. However, immunocytochemical analyses have directly shown high levels of biotin in the AM, which suggests the presence of high carboxylase activity in chromaffin cells (Ortega-Sáenz et al., 2016).

#### *Ion channels and other genes*

Our comparative microarray analysis has shown that mouse CB and AM cells express a broad variety of calcium- and/or voltage-gated ion channel genes, including some of the subunits which have been proposed to form the O<sub>2</sub>-sensitive voltage-gated K<sup>+</sup> channels (Wyatt and Peers, 1995; Pérez-García et al., 2004). However, we also found that numerous voltage-dependent K<sup>+</sup>, Na<sup>+</sup> and Ca<sup>2+</sup> channel subunits were down-regulated in the CB or AM in comparison with the SCG. This is probably a consequence of the high density of voltage-gated ion channels that is needed to sustain the electrical excitability of sympathetic neurons, which have a large somatodendritic arbor and profuse axonal branching. *Task3* (*Kcnk9*) was the most highly up-regulated K<sup>+</sup> channel gene in CB and AM cells, although *Task1* (*Kcnk3*) was also overexpressed. High expression of *Task1* has previously been reported in the CB; however, *Task3* was not detected in either a human CB microarray study (Mkrtchian et al., 2012) or single neonatal mouse CB cell transcriptomes (Zhou et al., 2016). This suggests that the expression of Task subunits may differ between species or during development. Task-like channels appear to be the major channels responsible for the hypoxia-induced

depolarization of CB chemoreceptor cells (Buckler et al., 2000; Kim et al., 2009; Kobayashi and Yamamoto 2010). In addition, Task1/Task3 heteromers have been proposed to be the channels that mediate the O<sub>2</sub>-sensitive background current in adult rat (Kim et al., 2009) and mouse (Turner and Buckler, 2013) glomus cells. Whereas Task1-deficient glomus cells have normal electrical parameters, cells from double *Task1/Task3* knockout mice exhibit a clear depolarization, thereby supporting the role of Task3 channels in setting the resting potential of glomus cells (Ortega-Sáenz et al., 2010). However, it must be noted that responsiveness to hypoxia is fully maintained in cells from Task1- or Task1/Task3-null mice. Therefore, Task1 and Task3 channels do not seem to be indispensable for acute O<sub>2</sub> sensing, with other channels appearing to mediate sensitivity to changes in PO<sub>2</sub> in their absence (Ortega-Sáenz et al., 2010). Two additional ion channel genes potentially related to acute responsiveness to hypoxia were overexpressed in glomus and chromaffin cells: *Cacna1h* (T-type Cav3.2) and *Trpc5*. Ca<sup>2+</sup> currents mediated by T-type channels have previously been recorded from mouse CB cells (Ortega-Sáenz et al., 2010), and it has recently been suggested that the Cav3.2 channel subtype mediates the CB over-activation induced by chronic intermittent hypoxia (Makarenko et al., 2016). On the other hand, Cav3.2 mRNA is up-regulated by chronic hypoxia in a Hif2 $\alpha$ -dependent manner in PC12 and chromaffin cells (del Toro et al., 2003; Carabelli et al., 2007). Cav3.2 expression is high in neonatal AM chromaffin cells but, as this occurs with acute sensitivity to hypoxia, it decreases with postnatal maturation. However, adult chromaffin cell sensitivity to hypoxia increases after AM denervation in parallel with the re-appearance of T-type Ca<sup>2+</sup> channels (Levitsky and López-Barneo, 2009). Together, these data suggest that Cav3.2 channels may play a fundamental role in facilitating the responsiveness of peripheral chemoreceptor cells to hypoxia. Similarly, the strong mRNA up-regulation of *Trpc5* channels in CB and AM cells suggests that they may also have an important role in the physiology of peripheral chemoreceptors. The existence of a significant background cationic conductance has been proposed to explain the relatively depolarized

resting potential of glomus cells with respect to the  $K^+$  equilibrium potential (Carpenter and Peers, 2001; García-Fernández et al., 2007b). In addition, it has been suggested that cationic currents participate in hypoxic activation of glomus (Kang et al., 2014) and chromaffin (Inoue et al., 1998) cells. Moreover, a cationic conductance, possibly mediated by Trp channels is activated by hypoglycemia in glomus cells (García-Fernández et al., 2007b). As there are several subtypes of Trp channels in the CB (Buniel et al., 2003), Trpc homo- or heteromers could mediate the cationic currents mentioned above. Trpc channels are promiscuously activated by stretch, phospholipids and other variables (Beech, 2007). Interestingly, they are also inhibited by anesthetics such as chloroform, propofol or halothane (Bahnasi et al., 2008), which could help to explain the strong respiratory depression induced by these drugs (Teppema et al., 2002).

Among other genes studied, we found that *Ucp2* was systematically up-regulated in CB and AM cells. *Ucp2* and other uncoupling proteins are associated with thermogenesis and appear to be co-expressed with *Cox8b* mRNA in some studies of adipose tissue thermogenic differentiation (Wu et al., 1999; Fisher et al., 2012). However, the significance of these observations in the context of CB physiology is currently unknown. Although it has been reported that *Ucp2* knockout mice present pseudohypoxic pulmonary vascular remodeling and hypertension (Dromparis et al., 2013), preliminary experiments performed in this mouse model indicate that CB responsiveness to hypoxia is not significantly affected (our own unpublished observations). *Olf78* is an atypical olfactory receptor that is expressed outside the nasal mucosa and particularly in CB glomus cells (Chang et al., 2015; Zhou et al., 2016). In our analyses, *Olf78* mRNA was up-regulated in the CB relative to the SCG but markedly down-regulated in the AM. It has been reported that *Olf78* is a lactate receptor that is required for acute  $O_2$  sensing, given that *Olf78*-deficient mice lose the HVR and glomus cell responsiveness to hypoxia. Based on these data, an endocrine model of acute  $O_2$  sensing has been proposed in which lactate released from tissues during hypoxia activates glomus cells to produce

hyperventilation (Chang et al., 2015). However, this model is incompatible with abundant data indicating that acute O<sub>2</sub> sensitivity is a cell-autonomous phenomenon that can be observed in isolated cells bathed in lactate-free solutions (see López-Barneo et al., 2016 a and b). In addition, two independent groups have found that, in contrast to the findings of Chang et al. (2015), Olfr78 is a poor lactate receptor (Aisenberg et al., 2016; Zhou et al., 2016). The fact that *Olfr78* mRNA expression is much higher in the SCG than in the AM argues against any direct involvement of this receptor in acute O<sub>2</sub> sensing. However, ongoing experiments in several laboratories using various strains of Olfr78-null mice should clarify the role of Olfr78 in CB physiology.

### **Signature gene-expression profile in chemoreceptor cells and the mechanism of acute O<sub>2</sub> sensing**

Together, the data available on the gene expression profile of chemoreceptor cells versus O<sub>2</sub>-insensitive SCG neurons suggest that a mix of genes encoding mitochondrial subunits, metabolic enzymes/transporters and ion channels is characteristic of cells that are acutely responding to decreases in PO<sub>2</sub>. Our study suggests that down-regulation of *Phd3* and up-regulation of *Hif2α*, *Ndufa4l2*, *Cox4i2*, *Cox8b*, and *Pcx* confer CB glomus cells with their special sensitivity to hypoxia. Other genes that may also contribute to the “acute O<sub>2</sub>-sensing signature metabolic profile” of chemoreceptor cells are *Pdha1*, *Idh1*, *Acacb* and *Slc7a5*. The absence of frank *Pcx* overexpression is probably one of the reasons why AM cells are less O<sub>2</sub>-sensitive than glomus cells. In addition to these ten “metabolic” genes, four other genes which encode ion channels (*Task3*, *Task1*, *Trp5*, and *Cacna1h*) are characteristic of CB and AM O<sub>2</sub>-sensitive cells. Although it is possible that none of these genes is absolutely required for acute O<sub>2</sub> sensing, their concerted action could result in a metabolic status that renders the cells sensitive to hypoxia. These data fit quite well with the MCI signaling model of acute O<sub>2</sub> sensing which is based on high succinate content and QH<sub>2</sub> accumulation in glomus cells (Fernández-Agüera et al., 2015), as well as the abundant evidence suggesting that special

mitochondrial properties could contribute to hypoxia responsiveness in glomus cells (Mill and Jöbsis, 1970; Duchen and Biscoe, 1992a and b; Ortega-Sáenz et al., 2003; Wyatt and Buckler, 2004; Buckler and Turner, 2013, Fernández-Agüera et al., 2015).

The metabolic features of chemoreceptor cells, in particular glomus cells, are schematically summarized in Figure 7. Phd3 has a central position in this scheme, as its low level of expression probably permits Hif2 $\alpha$  stabilization and the subsequent induction of the genes that encode the three atypical mitochondrial subunits (Ndufa4l2, Cox4i2, and possibly Cox8b). The suppression of Phd3 activity, which is favored by maintaining a low level of cytosolic  $\alpha$ -ketoglutarate, also decreases malonyl-CoA synthesis and therefore enables FFA-dependent mitochondrial metabolism and the generation of both acetyl-CoA for the TCA cycle and large amounts of QH<sub>2</sub>. In parallel, the high levels of Pcx and relative down-regulation of Pdh provide abundant oxaloacetate to replenish TCA cycle intermediates and to further increase the QH<sub>2</sub> pool through the activity of MCI and MCII. The presence of Cox4i2, Cox8b and Ndufa4l2 could make cytochrome c oxidase activity highly sensitive to decreases in PO<sub>2</sub>, such that even relatively mild hypoxia causing accumulation of an extra amount of QH<sub>2</sub> leads to the slow-down or even reversal of MCI and the production of molecules (reduced pyridine nucleotides and ROS) that signal via ion channels in the plasma membrane (see Fernández-Agüera et al., 2015).

For the past three decades, the identification of the “O<sub>2</sub> sensor” in glomus cells has been a matter of much discussion and investigation. However, numerous studies, in many cases using genetically modified animals with selective ablation of specific genes, have suggested that, in line with the model proposed here, O<sub>2</sub> sensing is a multifactorial process that depends on the biophysical and metabolic properties of chemoreceptor cells rather than on the function of a specific O<sub>2</sub>-sensing molecule (for recent reviews see Lopez-Barneo et al., 2016a & b). Interestingly, recent work on mice

with ablation of MCI genes (Fernandez-Agüera et al., 2015), and the gene expression analyses discussed here suggest the compatibility of the two classical “models” of CB acute O<sub>2</sub> sensing: the “membrane model”, based on the modulation of ion channels by hypoxia and the “metabolic hypothesis” which claims a fundamental role for mitochondria in this process. A major advantage of the scheme in Fig. 7 is that it allows predictions that can be experimentally tested. It has already been shown that pharmacological or genetic abolition of MCI selectively abolishes responsiveness to hypoxia in glomus and chromaffin cells (Ortega-Saenz et al., 2003; Thompson et al., 2007; Fernández–Agüera et al., 2015). Similarly, it is also known that CB cells are highly dependent on MCII for survival (Díaz-Castro et al., 2012; Platero-Luengo et al., 2014) and that genetic or pharmacological MCII dysfunction causes inhibition of responsiveness to hypoxia in pulmonary myocytes (this paper; see also Paddenberg et al., 2012). It can therefore be expected that future experiments, focused on the genes discussed here, will provide further understanding of the mechanisms of acute O<sub>2</sub> sensing by chemoreceptor cells, and their modifications in variable pathophysiological or developmental conditions.

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**Competing interests**

The authors have no conflicts of interest to declare

**Author contributions**

L.G., V.B.-H., P.G.-F., I.A.-M. and P.O.-S. performed the experiments and participated in the interpretation of data. L. G., P.O.-S and J.L.-B. designed the figures and wrote the first draft of the paper. J.L.B. wrote the final draft of the paper and supervised the project. All authors read and approved this manuscript.

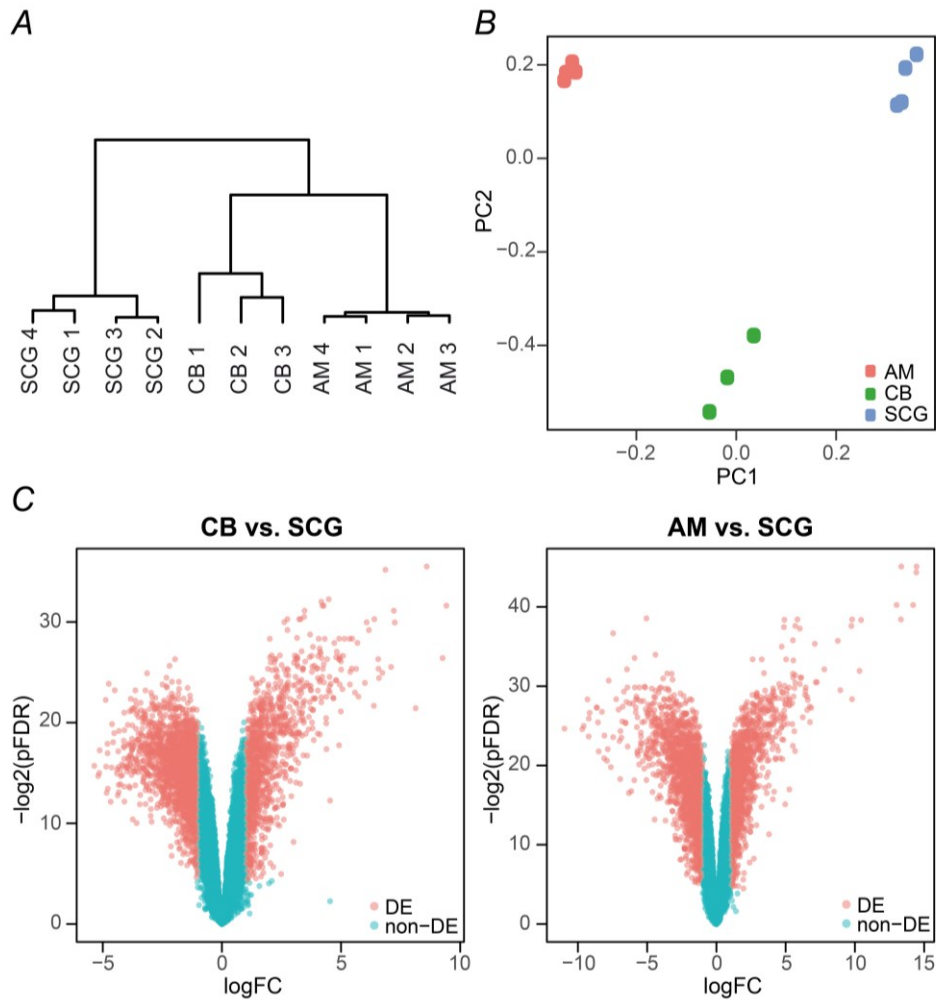
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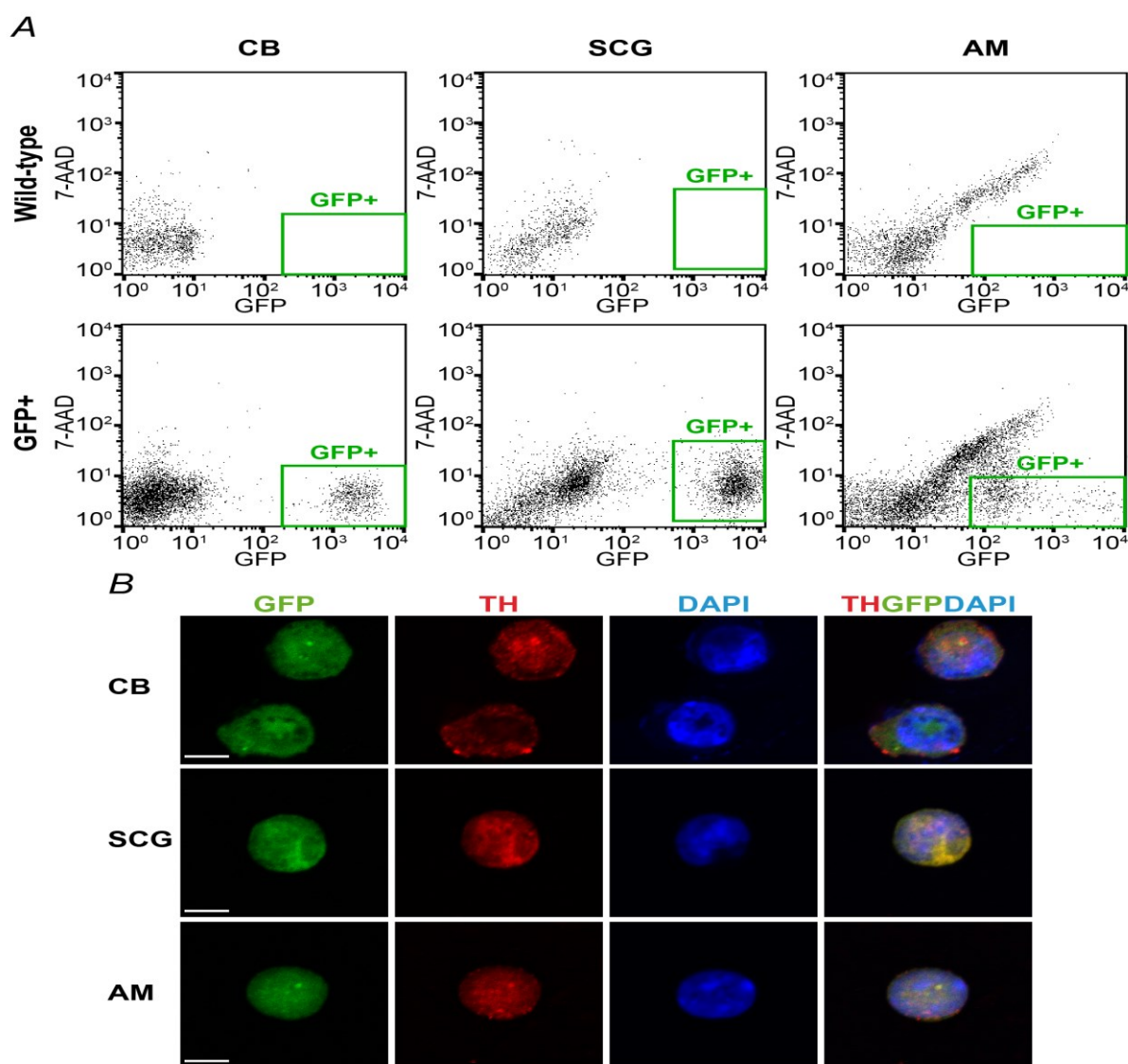
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## Figure legends



**Figure 1. Microarray analysis of transcriptomes of the carotid body (CB), adrenal medulla (AM), and superior cervical ganglion (SCG) from adult mice.** *A.* Hierarchical clustering analysis. Numbers indicate separate tissue samples. *B.* Principal component (PC) analysis. *C.* Volcano plots demonstrating differentially expressed genes (DE, light red, fold change > 2, pFDR < 0.05) comparing to genes without differential expression (non-DE, light blue). Left, CB versus SCG; right, AM versus SCG.



**Figure 2. Sorting of tyrosine hydroxylase (TH) positive cells from adult wild-type and TH-GFP mice by flow cytometry.** A. Isolation of green fluorescent protein positive (GFP+) cells from the carotid body (CB), superior cervical ganglion (SCG), and adrenal medulla (AM) of TH-GFP mice by flow cytometry. 7-AAD, 7-aminoactinomycin D. Plots from wild-type animals are shown as control. B. Immunofluorescent staining demonstrating that the GFP+ cells express TH. Scale bar, 5  $\mu$ m.

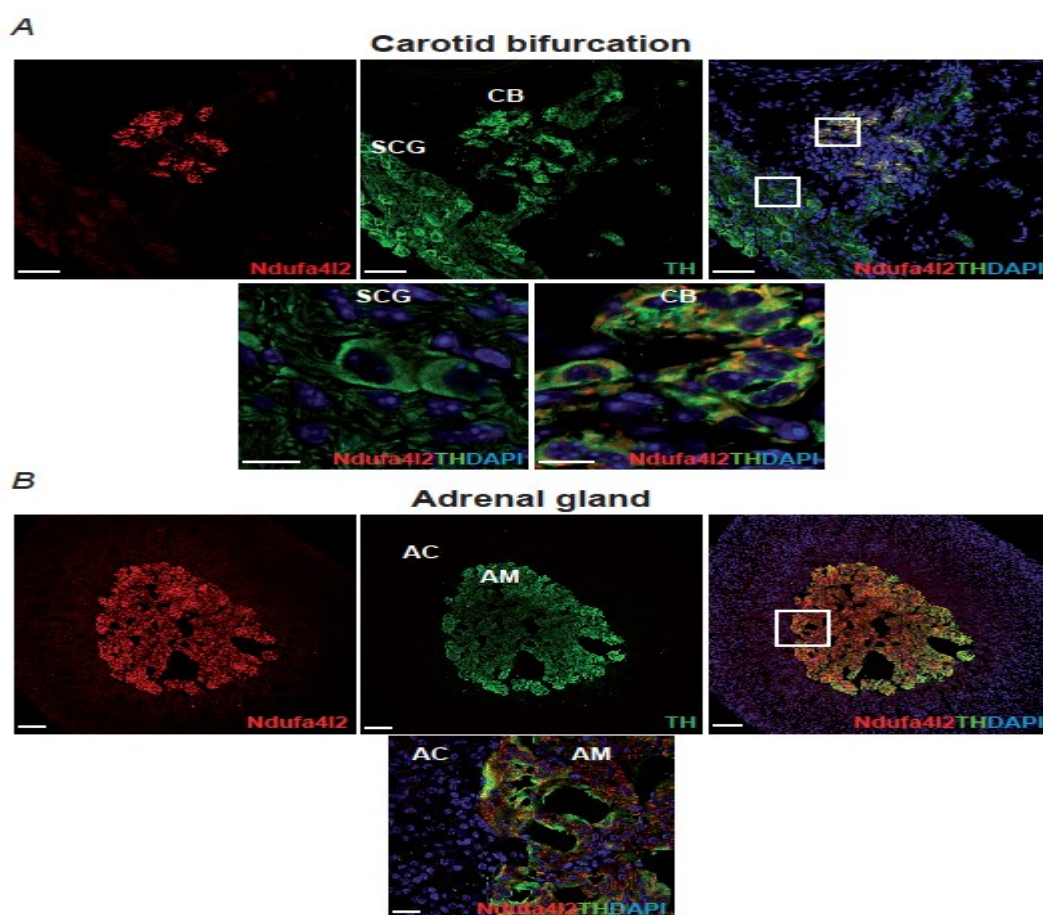
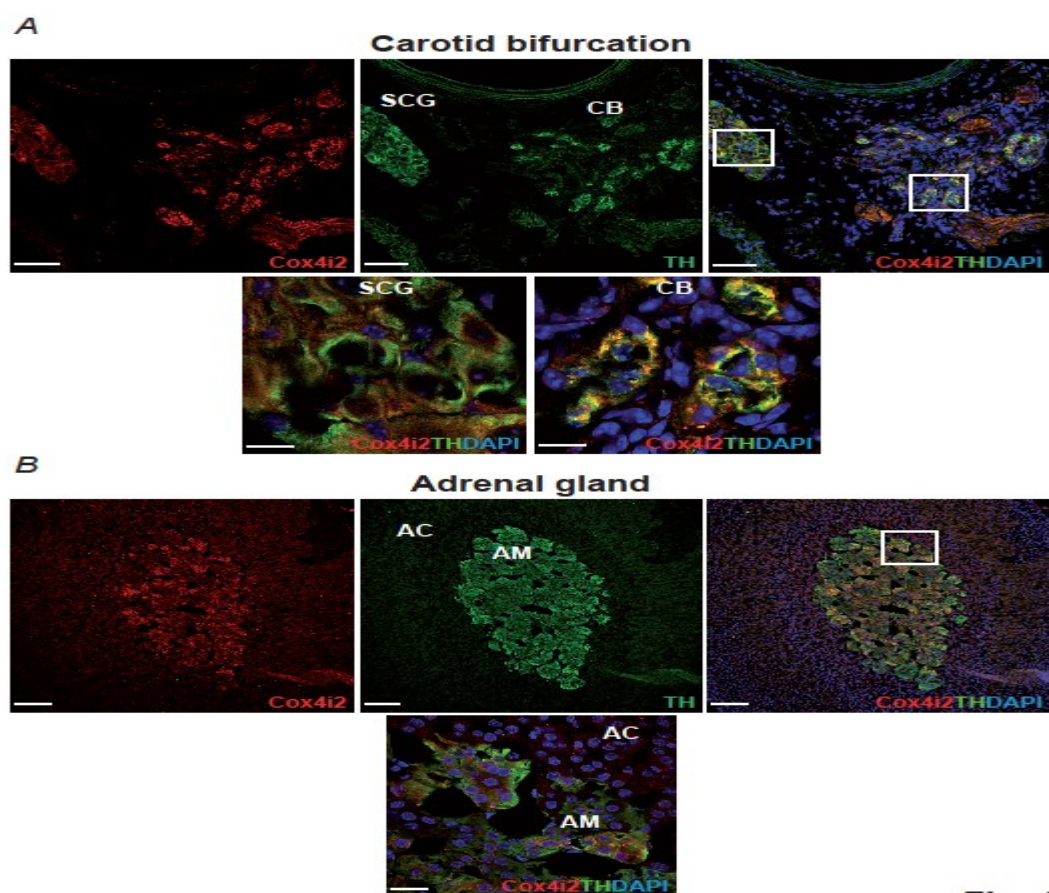
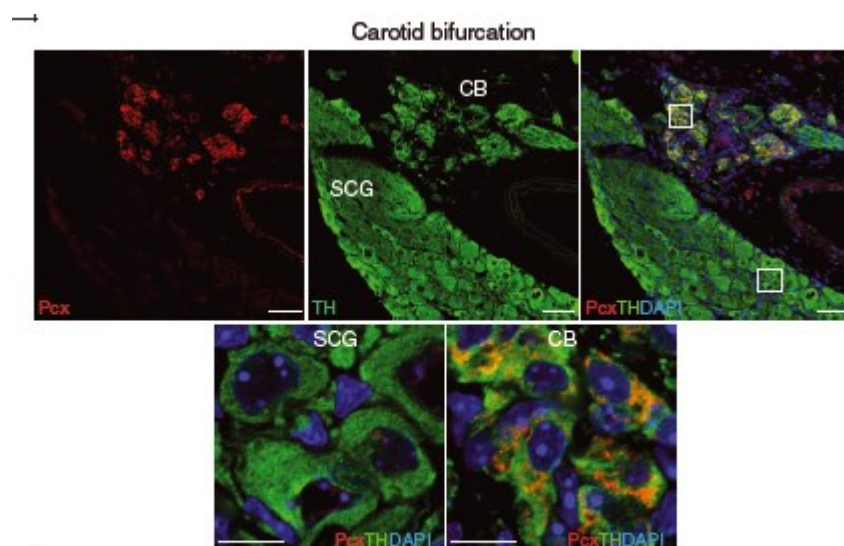


Fig. 3

**Figure 3. Immunohistochemical analysis of NADH dehydrogenase 1 alpha subcomplex 4-like 2 (Ndufa4l2) expression in adult mice.** A. Upper panels. Representative section of the carotid bifurcation demonstrating high Ndufa4l2 immunoreactivity in the carotid body (CB) compared to superior cervical ganglion (SCG) and the co-localization of Ndufa4l2 (red) and tyrosine hydroxylase (TH, green). Scale bar, 50  $\mu$ m. Lower panels. Magnification of SCG and CB regions indicated in the upper right panel. Scale bar, 10  $\mu$ m. B. Upper panels. Representative section of the adrenal gland demonstrating high Ndufa4l2 immunostaining in the adrenal medulla (AM) compared to adrenal cortex (AC) and the co-localization of Ndufa4l2 (red) and TH (green). Scale bar, 100  $\mu$ m. Lower panel. Magnification of AC and AM regions indicated in the upper right panel. Scale bar, 20  $\mu$ m.

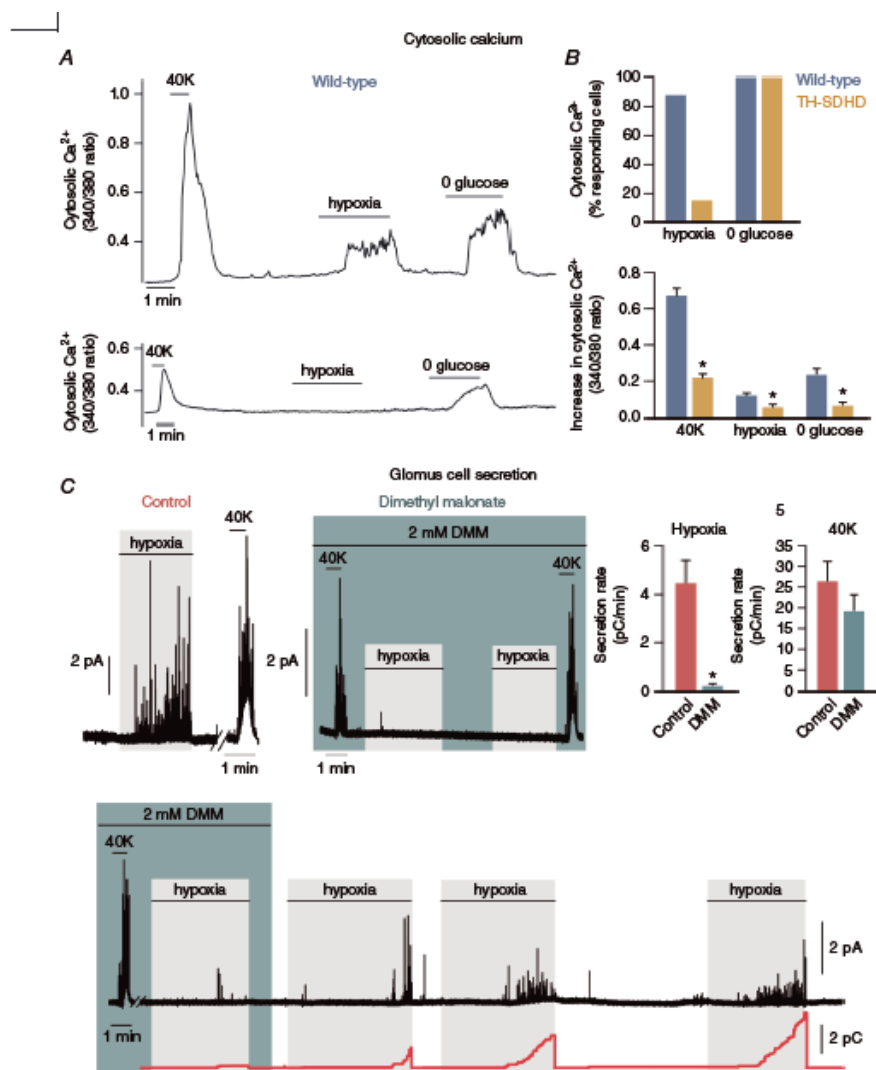
**Fig. 4**

**Figure 4. Immunohistochemical analysis of cytochrome c oxidase subunit IV isoform 2 (Cox4i2) expression in adult mice.** A. Upper panels. Representative section of the carotid bifurcation demonstrating high Cox4i2 immunoreactivity in the carotid body (CB) compared to superior cervical ganglion (SCG) and the co-localization of Cox4i2 (red) and tyrosine hydroxylase (TH, green). Scale bar, 50  $\mu\text{m}$ . Lower panels. Magnification of SCG and CB regions indicated in the upper right panel. Scale bar, 10  $\mu\text{m}$ . B. Upper panels. Representative section of the adrenal gland demonstrating high Cox4i2 immunostaining in the adrenal medulla (AM) compared to adrenal cortex (AC) and the co-localization of Cox4i2 (red) and TH (green). Scale bar, 100  $\mu\text{m}$ . Lower panel. Magnification of AC and AM regions indicated in the upper right panel. Scale bar, 20  $\mu\text{m}$ .



**Figure 5. Immunohistochemical analysis of pyruvate carboxylase (Pcx) expression in adult mice.**

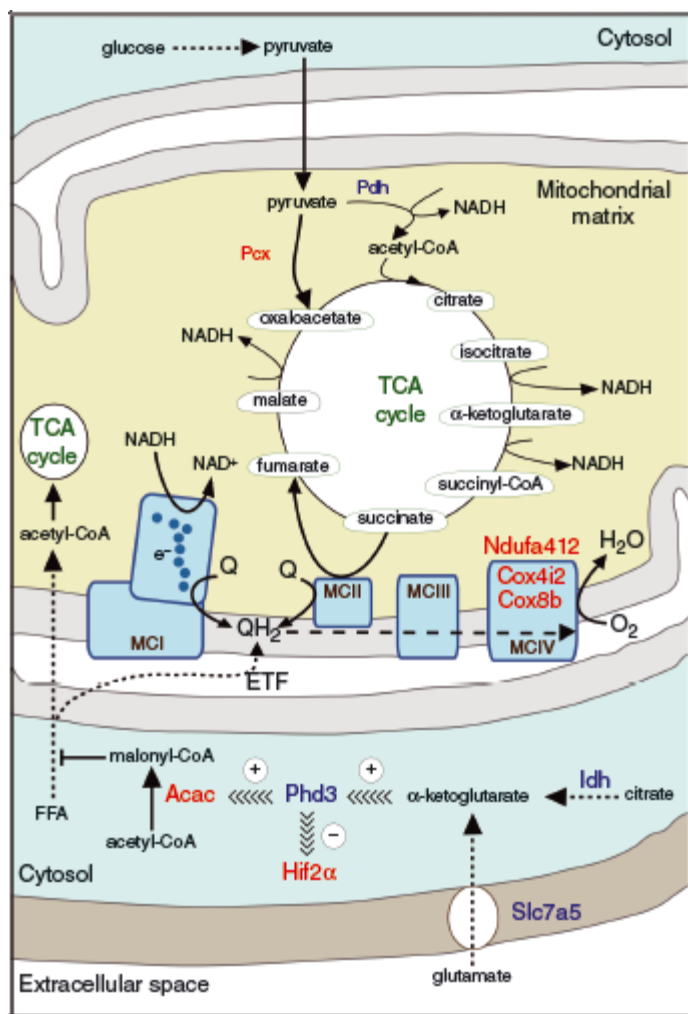
Upper panels. Representative section of the carotid bifurcation demonstrating high Pcx immunoreactivity in the carotid body (CB) compared to superior cervical ganglion (SCG) and the co-localization of Pcx (red) and tyrosine hydroxylase (TH, green). Scale bar, 50  $\mu$ m. Lower panels. Magnification of SCG and CB regions indicated in the upper right panel. Scale bar, 10  $\mu$ m.



**Figure 6. Impairment of acute O<sub>2</sub> sensing by genetic and pharmacological inhibition of succinate dehydrogenase.** A. Representative recordings of the ratiometric increase in cytosolic [Ca<sup>2+</sup>] elicited in Fura-2 loaded dispersed glomus cells from wild-type and TH-SDHD animals in response to hypoxia, 0 glucose and 40 mM K<sup>+</sup>. B. Top. Percentage of the number of cells that responded to a specific stimulus compared to the number of cells that responded to high potassium (Hypoxia: wild-type, n = 69 from 7 mice; TH-SDHD, n = 32 from 9 animals). 0 glucose: wild-type, n = 19 from 3 mice; TH-SDHD, n = 14 from 6 animals). Bottom. Quantification of the increase in cytosolic [Ca<sup>2+</sup>] in glomus cells from wild-type and TH-SDHD animals that responded to the specific stimulus. (40 mM K<sup>+</sup>: wild-type, n = 69

from 7 mice; TH-SDHD, n = 32 from 9 mice. Hypoxia: wild-type, n = 61 from 7 mice; TH-SDHD, n = 5 from 2 mice. 0 glucose: wild-type, n = 19 from 3 mice; TH-SDHD, n = 14 from 6 mice). *C.* Representative amperometric recordings of responses to hypoxia and 40 mM K<sup>+</sup> of glomus cells in wild-type mouse CB slices incubated overnight with or without 2 mM dimethyl malonate (DMM). *D.* Quantification of the secretion rate elicited by hypoxia (left) and 40 mM K<sup>+</sup> (right) in control and DMM-treated CB glomus cells from wild-type animals. (Hypoxia: control, n = 8 from 6 mice; DMM, n = 7 from 5 animals. 40 mM K<sup>+</sup>: control, n = 7 from 5 mice; DMM, n = 6 from 4 animals.) *E.* Representative recording demonstrating the recovery of hypoxia-induced secretory activity of glomus cells in wild-type mouse slices after the washout of DMM. Cumulative secretion rate (red line) is represented in the bottom. \*, p < 0.05 compared to wild-type or control.





**Figure 7. Model of the metabolic features of chemoreceptor glomus cells relevant to  $O_2$  sensing based on their gene expression profile.** Proteins (or subunits of an enzyme complex) highly expressed in the CB in comparison to the SCG, as suggested by the microarray analysis, are highlighted in red, whereas proteins (or subunits of an enzyme complex) with relatively low expression in the CB are represented in dark blue. Arrows attached to continuous lines indicate one-step chemical reactions, whereas arrows attached to discontinuous lines represent multistep chemical modifications. Acac, acetyl-coenzyme A carboxylase; Cox4i2, cytochrome c oxidase subunit IV isoform 2; Cox8b, cytochrome c oxidase subunit VIIIb; ETF, electron transport flavin/quinone oxidoreductase; FFA, free fatty acid; Hif2 $\alpha$ , endothelial PAS domain protein 1 (Epas1); Idh, isocitrate

dehydrogenase; MCI, MCII, MCIII, MCIV, mitochondrial complex I, II, III, IV, respectively; Ndufa4l2, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2; Pcx, pyruvate carboxylase; Pdh, pyruvate dehydrogenase; Phd3, egl-9 family prolyl hydroxylase 3 (Egln3); Q, ubiquinone; QH<sub>2</sub>, ubiquinol/reduced ubiquinone; Slc7a5, solute carrier family 7 (cationic amino acid transporter,  $\gamma$ + system), member 5; TCA, tri-carboxylic acid. See text for detailed explanation.

**Table 1.** Top 20 up- and down-regulated genes in the CB versus the SCG of adult mice by microarray analysis.

Gene symbol	Description	Fold change (linear)	pFDR
<b>Up-regulated</b>			
<i>Gdf10</i>	growth differentiation factor 10	760	1.49E-03
<i>Ndufa4l2</i>	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	721	3.90E-04
<i>Mgp</i>	matrix Gla protein	485	4.55E-03
<i>Acta2</i>	actin, alpha 2, smooth muscle, aorta	388	2.20E-04
<i>Dpt</i>	dermatopontin	164	1.26E-03
<i>Igfbp3</i>	insulin-like growth factor binding protein 3	160	2.29E-04
<i>Rgs5</i>	regulator of G-protein signaling 5	142	8.02E-04
<i>Cfh</i>	complement component factor h	119	2.29E-04
<i>Ccl21a</i>	chemokine (C-C motif) ligand 21A (serine)	104	1.39E-03
<i>Epas1(Hif2a)</i>	endothelial PAS domain protein 1	102	2.49E-03
<i>Cpa3</i>	carboxypeptidase A3, mast cell	100	1.39E-03
<i>Myh11</i>	myosin, heavy polypeptide 11, smooth muscle	98	6.71E-04
<i>Itga8</i>	integrin alpha 8	96	2.74E-03

<i>Cyt11</i>	cytokine-like 1	85	4.35E-03
<i>Kcnk9(Task3)</i>	potassium channel, subfamily K, member 9	83	2.19E-03
<i>Ccl21a/Ccl21b/Ccl21c</i>	chemokine (C-C motif) ligand, 21A (serine), 21B (leucine), 21C (leucine)	81	1.49E-03
<i>Slc9a2</i>	solute carrier family 9 (sodium/hydrogen exchanger), member 2	71	8.22E-04
<i>Adm</i>	adrenomedullin	64	1.99E-03
<i>My19</i>	myosin, light polypeptide 9, regulatory	62	2.23E-03
<i>Mcpt4</i>	mast cell protease 4	62	3.36E-03
<b>Down-regulated</b>			
<i>Cxcr4</i>	chemokine (C-X-C motif) receptor 4	-41	7.12E-03
<i>Napb</i>	N-ethylmaleimide sensitive fusion protein attachment protein beta	-39	2.34E-02
<i>Tubb4a</i>	tubulin, beta 4A class IVA	-38	4.66E-02
<i>Mapk11</i>	mitogen-activated protein kinase 11	-36	1.29E-02
<i>Tmem179</i>	transmembrane protein 179	-35	1.06E-02
<i>Htr3a</i>	5-hydroxytryptamine (serotonin) receptor 3A	-35	2.19E-02
<i>Ntrk1</i>	neurotrophic tyrosine kinase, receptor, type 1	-30	3.42E-02
<i>Nrip3</i>	nuclear receptor interacting protein 3	-29	4.13E-03
<i>Sult4a1</i>	sulfotransferase family 4A, member 1	-29	1.03E-02

<i>Htr3b</i>	5-hydroxytryptamine (serotonin) receptor 3B	-28	1.83E-02
<i>Nefl</i>	neurofilament, light polypeptide	-27	3.10E-02
<i>Vwc2l</i>	von Willebrand factor C domain-containing protein 2-like	-27	3.00E-03
<i>Maob</i>	monoamine oxidase B	-27	1.71E-02
<i>Gpr158</i>	G protein-coupled receptor 158	-25	1.64E-02
<i>Ehd3</i>	EH-domain containing 3	-25	3.06E-02
<i>Tll1</i>	tubulin tyrosine ligase-like 1	-24	2.16E-02
<i>Cyp2j12</i>	cytochrome P450, family 2, subfamily j, polypeptide 12	-24	1.01E-02
<i>Stk32a</i>	serine/threonine kinase 32A	-23	9.87E-03
<i>Slc6a15</i>	solute carrier family 6 (neurotransmitter transporter), member 15	-22	7.08E-03
<i>Maoa</i>	monoamine oxidase A	-21	1.92E-02

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**Table 2.** Hypoxia-related differential gene expression in the CB compared to the SCG of adult mice by microarray analysis\*.

Gene Symbol	Description	Fold change (linear)	pFDR
<b>Phd/Hif pathway and targets</b>			
<i>Epas1(Hif2a)</i>	endothelial PAS domain protein 1	102.0	2.49E-03
<i>Arnt2</i>	aryl hydrocarbon receptor nuclear translocator 2	2.9	1.47E-02
<i>Egl-9(Phd3)</i>	egl-9 family hypoxia-inducible factor 3	-4.1	1.89E-02
<i>Vegfa</i>	vascular endothelial growth factor A	4.2	1.86E-02
<i>Vegfc</i>	vascular endothelial growth factor C	3.9	7.34E-03
<b>Mitochondria</b>			
<i>Ndufa4l2</i>	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	720.9	3.90E-04
<i>Ndufa8</i>	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8	-2.1	4.01E-02
<i>Ndufa9</i>	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9	-3.4	5.17E-03
<i>Ndufa10</i>	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10	-2.4	1.28E-02
<i>Ndufaf7</i>	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex assembly factor 7	-3.1	1.20E-02
<i>Ndufb6</i>	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6	-2.1	3.16E-02
<i>Ndufs8</i>	NADH dehydrogenase (ubiquinone) Fe-S protein 8	-3.9	1.09E-02

<i>Sdhd</i>	succinate dehydrogenase complex, subunit D, integral membrane protein	-4.0	1.03E-02
<i>Uqcrc1</i>	ubiquinol-cytochrome c reductase core protein 1	-2.6	1.07E-02
<i>Cyb561</i>	cytochrome b-561	-5.0	2.78E-02
<i>Cyb561d2</i>	cytochrome b-561 domain containing 2	-2.7	3.33E-03
<i>Cyb5b</i>	cytochrome b5 type B	-5.0	6.07E-03
<i>Cyb5d1</i>	cytochrome b5 domain containing 1	-3.6	3.94E-02
<i>Cyba</i>	cytochrome b-245, alpha polypeptide	3.2	1.49E-02
<i>Cox4i2</i>	cytochrome c oxidase subunit IV isoform 2	10.1	6.03E-03
<i>Cox8b</i>	cytochrome c oxidase subunit VIIIb	11.8	1.30E-02
<i>Cox5a</i>	cytochrome c oxidase subunit Va	-2.2	4.54E-02
<i>Coa3</i>	cytochrome C oxidase assembly factor 3	-3.0	2.21E-02
<i>Cox15</i>	cytochrome c oxidase assembly protein 15	-3.6	1.79E-02
<i>Slc25a27</i>	solute carrier family 25, member 27	-2.8	2.33E-03
<b>TCA cycle/anaplerosis/biotin-related</b>			
<i>Pcx</i>	pyruvate carboxylase	4.9	1.31E-02
<i>Pdha1</i>	pyruvate dehydrogenase E1 alpha 1	-2.5	1.90E-02
<i>Pdk4</i>	pyruvate dehydrogenase kinase, isoenzyme 4	9.1	3.90E-03

<i>Clybl</i>	citrate lyase beta like	-2.7	9.23E-03
<i>Acacb</i>	acetyl-Coenzyme A carboxylase beta	2.7	9.57E-03
<i>Slc7a5</i>	solute carrier family 7 (cationic amino acid transporter, $\gamma$ + system), member 5	-3.6	2.24E-02
<i>Idh1</i>	isocitrate dehydrogenase 1 (NADP+), soluble	-3.5	1.34E-02
<i>Idh3a</i>	isocitrate dehydrogenase 3 (NAD+) alpha	-3.1	5.50E-03
<i>Idh3b</i>	isocitrate dehydrogenase 3 (NAD+) beta	-4.6	3.83E-02
<i>Ogdhl</i>	oxoglutarate dehydrogenase-like	-2.6	2.09E-02
<i>Ogfod1</i>	2-oxoglutarate and iron-dependent oxygenase domain containing 1	-3.2	9.12E-03
<i>Mdh1</i>	malate dehydrogenase 1, NAD (soluble)	-2.4	8.89E-03
<b>Others</b>			
<i>Gdnf</i>	glial cell line derived neurotrophic factor	2.1	9.36E-03
<i>Pparg</i>	peroxisome proliferator activated receptor gamma	2.4	8.63E-03

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\*pFDR < 0.05 and fold change >2 or < -2.



**Table 3.** Differential expression of ion channel genes in the CB compared to the SCG of adult mice by microarray analysis\*.

Gene Symbol	Description	Fold change (linear)	pFDR
<b>Potassium channels</b>			
<i>Kcnab1</i>	potassium voltage-gated channel, shaker-related subfamily, beta member 1	-5.0	1.09E-02
<i>Kcnab2</i>	potassium voltage-gated channel, shaker-related subfamily, beta member 2	-5.3	6.56E-03
<i>Kcnb2</i>	potassium voltage gated channel, Shab-related subfamily, member 2	-4.0	6.99E-03
<i>Kcnc4</i>	potassium voltage gated channel, Shaw-related subfamily, member 4	-10.3	1.66E-02
<i>Kcnd1</i>	potassium voltage-gated channel, Shal-related family, member 1	-2.7	1.64E-02
<i>Kcnd2</i>	potassium voltage-gated channel, Shal-related family, member 2	-11.6	6.27E-03
<i>Kcne4</i>	potassium voltage-gated channel, Isk-related subfamily, gene 4	2.3	1.88E-02
<i>Kcnh1</i>	potassium voltage-gated channel, subfamily H (eag-related), member 1	-6.7	4.35E-03
<i>Kcnh5</i>	potassium voltage-gated channel, subfamily H (eag-related), member 5	-13.3	3.20E-03
<i>Kcnh6</i>	potassium voltage-gated channel, subfamily H (eag-related), member 6	-2.5	3.48E-02
<i>Kcnh7</i>	potassium voltage-gated channel, subfamily H (eag-related), member 7	-4.1	1.20E-02
<i>Kcnip3</i>	Kv channel interacting protein 3, calsenilin	5.7	1.54E-03
<i>Kcnip4</i>	Kv channel interacting protein 4	-4.8	3.64E-02
<i>Kcnj3</i>	potassium inwardly rectifying channel, subfamily J, member 3	-7.0	6.51E-

			03
<i>Kcnj10</i>	potassium inwardly rectifying channel, subfamily J, member 10	-2.9	2.30E-02
<i>Kcnk3(Tas k1)</i>	potassium channel, subfamily K, member 3	2.5	2.17E-02
<i>Kcnk9(Tas k3)</i>	potassium channel, subfamily K, member 9	83.5	2.19E-03
<i>Kcnk10</i>	potassium channel, subfamily K, member 10	-3.7	5.80E-03
<i>Kcnk18</i>	potassium channel, subfamily K, member 18	-6.3	9.87E-03
<i>Kcnma1</i>	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	-2.1	3.64E-02
<i>Kcnmb1</i>	potassium large conductance calcium-activated channel, subfamily M, beta member 1	4.6	5.68E-03
<i>Kcnmb2</i>	potassium large conductance calcium-activated channel, subfamily M, beta member 2	3.1	1.45E-02
<i>Kcnmb4</i>	potassium large conductance calcium-activated channel, subfamily M, beta member 4	-2.3	1.96E-02
<i>Kcnn3</i>	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3	-3.8	1.87E-02
<i>Kcnq2</i>	potassium voltage-gated channel, subfamily Q, member 2	-3.5	1.01E-02
<i>Kcnq3</i>	potassium voltage-gated channel, subfamily Q, member 3	-20.1	1.40E-02
<i>Kcnq5</i>	potassium voltage-gated channel, subfamily Q, member 5	-5.6	1.75E-02
<i>Kcnt1</i>	potassium channel, subfamily T, member 1	-4.7	4.81E-03
<i>Kcnt2</i>	potassium channel, subfamily T, member 2	7.4	2.03E-03
<i>Kctd9</i>	potassium channel tetramerization domain containing 9	-2.3	9.29E-03

**Calcium channels**

<i>Cacna1a</i>	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	-3.0	1.40E-02
<i>Cacna1b</i>	calcium channel, voltage-dependent, N type, alpha 1B subunit	-5.5	4.54E-02
<i>Cacna1c</i>	calcium channel, voltage-dependent, L type, alpha 1C subunit	2.2	3.95E-03
<i>Cacna1h</i>	calcium channel, voltage-dependent, T type, alpha 1H subunit	2.7	7.70E-04
<i>Cacna1i</i>	calcium channel, voltage-dependent, alpha 1I subunit	2.6	9.87E-03
<i>Cacna2d3</i>	calcium channel, voltage-dependent, alpha2/delta subunit 3	-4.1	1.38E-02
<i>Cacnb1</i>	calcium channel, voltage-dependent, beta 1 subunit	-2.2	2.01E-02
<i>Cacnb3</i>	calcium channel, voltage-dependent, beta 3 subunit	-6.4	1.07E-02
<i>Cacnb4</i>	calcium channel, voltage-dependent, beta 4 subunit	-3.6	1.63E-03
<i>Cacng2</i>	calcium channel, voltage-dependent, gamma subunit 2	-6.1	1.29E-02
<i>Cacng3</i>	calcium channel, voltage-dependent, gamma subunit 3	-12.2	1.05E-02

**Sodium channels**

<i>Scn1a</i>	sodium channel, voltage-gated, type I, alpha	-8.8	6.04E-03
<i>Scn2a1</i>	sodium channel, voltage-gated, type II, alpha 1	-4.0	4.71E-03
<i>Scn2b</i>	sodium channel, voltage-gated, type II, beta	-11.6	5.63E-03
<i>Scn3a</i>	sodium channel, voltage-gated, type III, alpha	-4.9	1.98E-02

<i>Scn3b</i>	sodium channel, voltage-gated, type III, beta	-7.7	1.23E-02
<i>Scn7a</i>	sodium channel, voltage-gated, type VII, alpha	7.5	4.71E-03
<i>Scn9a</i>	sodium channel, voltage-gated, type IX, alpha	-14.9	1.38E-02
<b>Trp channels</b>			
<i>Trpc5</i>	transient receptor potential cation channel, subfamily C, member 5	30.1	2.74E-03
<i>Trpc6</i>	transient receptor potential cation channel, subfamily C, member 6	-3.0	2.08E-02
<i>Trpc7</i>	transient receptor potential cation channel, subfamily C, member 7	-4.1	1.01E-02
<i>Trpv2</i>	transient receptor potential cation channel, subfamily V, member 2	-2.3	9.96E-03
<i>Trpv4</i>	transient receptor potential cation channel, subfamily V, member 4	2.2	4.47E-03

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\*pFDR < 0.05 and fold change >2 or < -2.

**Table 4.** Top 20 up- and down-regulated genes in the AM versus the SCG of adult mice by microarray analysis.

Gene Symbol	Description	Fold change (linear)	pFDR
<b>Up-regulated</b>			
<i>Cyp11b1</i>	cytochrome P450, family 11, subfamily b, polypeptide 1	22442	3.01E-07
<i>Srd5a2</i>	steroid 5 alpha-reductase 2	21977	2.07E-07
<i>Cyp11a1</i>	cytochrome P450, family 11, subfamily a, polypeptide 1	19438	4.11E-07
<i>Hsd3b1</i>	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	10373	2.00E-06
<i>Star</i>	steroidogenic acute regulatory protein	9955	2.07E-07
<i>Cyp21a1</i>	cytochrome P450, family 21, subfamily a, polypeptide 1	8609	1.00E-06
<i>Adh1</i>	alcohol dehydrogenase 1 (class I)	1340	2.00E-06
<i>Kcnk9(Task 3)</i>	potassium channel, subfamily K, member 9	1244	1.60E-05
<i>Abcb1b</i>	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	966	2.00E-06
<i>Akr1cl</i>	aldo-keto reductase family 1, member C-like	913	2.00E-06
<i>Akr1b7</i>	aldo-keto reductase family 1, member B7	850	5.10E-05
<i>Pnmt</i>	phenylethanolamine-N-methyltransferase	500	3.70E-05
<i>Dlk1</i>	delta-like 1 homolog (Drosophila)	438	5.90E-07
<i>Ndufa4l2</i>	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	233	4.00E-06
<i>Slc18a1</i>	solute carrier family 18 (vesicular monoamine), member 1	156	2.40E-

			05
<i>Scarb1</i>	scavenger receptor class B, member 1	148	4.00E-06
<i>Chga</i>	chromogranin A	116	3.90E-05
<i>Gdf10</i>	growth differentiation factor 10	114	8.00E-05
<i>Tacr2</i>	tachykinin receptor 2	106	7.30E-05
<i>Kcnk3(Task 1)</i>	potassium channel, subfamily K, member 3	105	3.20E-05
<b>Down-regulated</b>			
<i>Htr3a</i>	5-hydroxytryptamine (serotonin) receptor 3A	-1920	3.46E-07
<i>Tubb3</i>	tubulin, beta 3 class III	-831	8.00E-06
<i>Htr3b</i>	5-hydroxytryptamine (serotonin) receptor 3B	-828	2.00E-06
<i>Sv2c</i>	synaptic vesicle glycoprotein 2c	-627	1.00E-05
<i>Tspan8</i>	tetraspanin 8	-624	2.00E-06
<i>Prph</i>	peripherin	-622	2.00E-05
<i>Ddah1</i>	dimethylarginine dimethylaminohydrolase 1	-598	2.00E-06
<i>Tubb2b</i>	tubulin, beta 2B class IIB	-378	2.00E-05
<i>Ret</i>	ret proto-oncogene	-313	1.90E-05
<i>Sncg</i>	synuclein, gamma	-289	2.40E-05

<i>Avil</i>	advillin	-276	3.20E-05
<i>Napb</i>	N-ethylmaleimide sensitive fusion protein attachment protein beta	-257	2.00E-06
<i>Ngfr</i>	nerve growth factor receptor (TNFR superfamily, member 16)	-244	3.00E-06
<i>Ppp1r1c</i>	protein phosphatase 1, regulatory (inhibitor) subunit 1C	-212	1.00E-06
<i>Nefl</i>	neurofilament, light polypeptide	-195	1.60E-05
<i>Fxyd7</i>	FXVD domain-containing ion transport regulator 7	-188	2.00E-06
<i>Tubb2a</i>	tubulin, beta 2A class IIA	-173	2.60E-05
<i>Nrip3</i>	nuclear receptor interacting protein 3	-156	2.00E-06
<i>Areg</i>	amphiregulin	-146	1.60E-05
<i>Rab6b</i>	RAB6B, member RAS oncogene family	-134	1.00E-05

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**Table 5.** Hypoxia-related differential gene expression in the AM compared to the SCG of adult mice by microarray analysis\*.

Gene Symbol	Description	Fold change (linear)	pFDR
<b>Phd/Hif pathway and targets</b>			
<i>Hif1a</i>	hypoxia inducible factor 1, alpha subunit	2.1	7.46E-03
<i>Epas1(Hif2a)</i>	endothelial PAS domain protein 1	6.6	1.31E-03
<i>Arnt</i>	aryl hydrocarbon receptor nuclear translocator	2.8	7.19E-04
<i>Arnt2</i>	aryl hydrocarbon receptor nuclear translocator 2	15.5	5.10E-05
<i>Egl3(Phd3)</i>	egl-9 family hypoxia inducible factor 3	-4.6	3.44E-03
<i>Vegfa</i>	vascular endothelial growth factor A	5.1	4.64E-03
<b>Mitochondria</b>			
<i>Ndufa4</i>	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4	-4.6	3.67E-03
<i>Ndufa4l2</i>	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	232.6	4.00E-06
<i>Ndufaf5</i>	NADH dehydrogenase (ubiquinone) complex I, assembly factor 5	-2.0	1.29E-03
<i>Ndufb6</i>	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6	-2.5	6.00E-03
<i>Ndufs4</i>	NADH dehydrogenase (ubiquinone) Fe-S protein 4	-2.1	1.05E-02
<i>Sdhb</i>	succinate dehydrogenase complex, subunit D, integral membrane protein	-2.6	3.54E-03
<i>Cybb</i>	cytochrome b-245, beta polypeptide	3.8	4.11E-04
<i>Cox4i2</i>	cytochrome c oxidase subunit IV isoform 2	2.1	1.72E-03



<i>Cox5a</i>	cytochrome c oxidase subunit Va	-4.4	2.58E-04
<i>Cox5b</i>	cytochrome c oxidase subunit Vb	-2.2	1.35E-02
<i>Cox6b1</i>	cytochrome c oxidase, subunit VIb polypeptide 1	-3.1	4.10E-03
<i>Cox7a2</i>	cytochrome c oxidase subunit VIIa 2	-2.5	1.14E-02
<i>Cox8b</i>	cytochrome c oxidase subunit VIIIb	6.4	5.48E-04
<i>Coa3</i>	cytochrome C oxidase assembly factor 3	-2.8	4.69E-03
<i>Ucp1</i>	uncoupling protein 1 (mitochondrial, proton carrier)	-3.7	3.11E-02
<i>Ucp2</i>	uncoupling protein 2 (mitochondrial, proton carrier)	10.1	5.54E-04
<i>Ucp3</i>	uncoupling protein 3 (mitochondrial, proton carrier)	2.1	1.14E-03
<i>Slc25a27</i>	solute carrier family 25, member 27	-5.0	7.00E-05
<b>TCA cycle/anaplerosis/biotin-related</b>			
<i>Pcx</i>	pyruvate carboxylase	6.1	6.21E-04
<i>Pdha1</i>	pyruvate dehydrogenase E1 alpha 1	-3.0	4.32E-04
<i>Pdk4</i>	pyruvate dehydrogenase kinase, isoenzyme 4	-2.6	1.12E-02
<i>Slc1a5</i>	solute carrier family 1 (neutral amino acid transporter), member 5	-3.4	1.60E-02
<i>Acaca</i>	acetyl-coenzyme A carboxylase alpha	-3.7	8.01E-04
<i>Ldha</i>	lactate dehydrogenase A	-2.8	2.40E-03

<i>Ldhb</i>	lactate dehydrogenase B	-9.3	7.70E-05
<i>Gls</i>	glutaminase	-4.4	1.24E-03
<i>Tgm2</i>	transglutaminase 2, C polypeptide	2.1	4.65E-03
<i>Aco1</i>	aconitase 1	2.2	4.74E-03
<i>Idh3a</i>	isocitrate dehydrogenase 3 (NAD+) alpha	-2.1	1.09E-03
<i>Suclg2</i>	succinate-coenzyme A ligase, GDP-forming, beta subunit	3.9	1.52E-04
<b>Others</b>			
<i>Ppara</i>	peroxisome proliferator activated receptor alpha	-2.4	2.61E-04
<i>Ppargc1a</i>	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	-6.0	3.96E-04
<i>Olfr78/Olfr560</i>	olfactory receptor 78; olfactory receptor 560	-2.3	9.02E-03

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\*pFDR < 0.05 and fold change >2 or < -2.

**Table 6.** Differential expression of ion channel genes in the AM compared to the SCG of adult mice by microarray analysis\*.

Gene Symbol	Description	Fold change (linear)	pFDR
<b>Potassium channels</b>			
<i>Kcna1</i>	potassium voltage-gated channel, shaker-related subfamily, member 1	-28.4	4.27E-04
<i>Kcna2</i>	potassium voltage-gated channel, shaker-related subfamily, member 2	-12.3	1.60E-03
<i>Kcna6</i>	potassium voltage-gated channel, shaker-related, subfamily, member 6	-13.9	1.94E-04
<i>Kcnab1</i>	potassium voltage-gated channel, shaker-related subfamily, beta member 1	-10.4	1.57E-04
<i>Kcnab2</i>	potassium voltage-gated channel, shaker-related subfamily, beta member 2	-8.2	5.00E-05
<i>Kcnb2</i>	potassium voltage gated channel, Shab-related subfamily, member 2	-5.0	2.81E-04
<i>Kcnc4</i>	potassium voltage gated channel, Shaw-related subfamily, member 4	-4.5	3.40E-04
<i>Kcnd1</i>	potassium voltage-gated channel, Shal-related family, member 1	-3.5	8.80E-04
<i>Kcnd2</i>	potassium voltage-gated channel, Shal-related family, member 2	-21.8	1.95E-04
<i>Kcng4</i>	potassium voltage-gated channel, subfamily G, member 4	-2.6	1.16E-02
<i>Kcnh1</i>	potassium voltage-gated channel, subfamily H (eag-related), member 1	-11.8	4.60E-05
<i>Kcnh5</i>	potassium voltage-gated channel, subfamily H (eag-related), member 5	-24.4	1.60E-05
<i>Kcnh7</i>	potassium voltage-gated channel, subfamily H (eag-related), member 7	-22.7	2.40E-05
<i>Kcnh8</i>	potassium voltage-gated channel, subfamily H (eag-related), member 8	-6.8	7.70E-05
<i>Kcnip3</i>	Kv channel interacting protein 3, calsenilin	16.2	5.00E-05

<i>Kcnip4</i>	Kv channel interacting protein 4	-9.2	6.94E-04
<i>Kcnj3</i>	potassium inwardly rectifying channel, subfamily J, member 3	-56.3	4.50E-05
<i>Kcnj6</i>	potassium inwardly rectifying channel, subfamily J, member 6	2.2	8.71E-04
<i>Kcnj8</i>	potassium inwardly rectifying channel, subfamily J, member 8	2.3	8.72E-03
<i>Kcnj10</i>	potassium inwardly rectifying channel, subfamily J, member 10	-5.9	7.14E-04
<i>Kcnj13</i>	potassium inwardly rectifying channel, subfamily J, member 13	-9.4	2.20E-05
<i>Kcnj16</i>	potassium inwardly rectifying channel, subfamily J, member 16	-2.9	7.69E-04
<i>Kcnk2</i>	potassium channel, subfamily K, member 2	12.1	1.41E-04
<i>Kcnk3(Tas k1)</i>	potassium channel, subfamily K, member 3	105.4	3.20E-05
<i>Kcnk9(Tas k3)</i>	potassium channel, subfamily K, member 9	1244.1	1.60E-05
<i>Kcnk10</i>	potassium channel, subfamily K, member 10	-6.1	9.80E-05
<i>Kcnk18</i>	potassium channel, subfamily K, member 18	-12.2	5.30E-04
<i>Kcnma1</i>	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	3.5	8.40E-05
<i>Kcnmb2</i>	potassium large conductance calcium-activated channel, subfamily M, beta member 2	6.0	5.09E-04
<i>Kcnmb4</i>	potassium large conductance calcium-activated channel, subfamily M, beta member 4	-3.6	9.57E-04
<i>Kcnn2</i>	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	2.0	2.29E-03
<i>Kcnn3</i>	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3	-6.1	1.19E-04

<i>Kcnn4</i>	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	-3.6	1.74E-04
<i>Kcnq2</i>	potassium voltage-gated channel, subfamily Q, member 2	-3.2	4.39E-04
<i>Kcnq3</i>	potassium voltage-gated channel, subfamily Q, member 3	-16.4	3.12E-04
<i>Kcnq5</i>	potassium voltage-gated channel, subfamily Q, member 5	-19.8	8.40E-05
<i>Kcnt1</i>	potassium channel, subfamily T, member 1	-7.7	5.00E-05
<i>Kctd9</i>	potassium channel tetramerization domain containing 9	-2.6	5.76E-04
<i>Kctd16</i>	potassium channel tetramerization domain containing 16	-2.7	6.83E-04
<b>Calcium channels</b>			
<i>Cacna1a</i>	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	-2.6	5.76E-04
<i>Cacna1b</i>	calcium channel, voltage-dependent, N type, alpha 1B subunit	-6.2	3.67E-04
<i>Cacna1c</i>	calcium channel, voltage-dependent, L type, alpha 1C subunit	5.5	3.63E-04
<i>Cacna1d</i>	calcium channel, voltage-dependent, L type, alpha 1D subunit	14.3	7.00E-05
<i>Cacna1h</i>	calcium channel, voltage-dependent, T type, alpha 1H subunit	6.4	8.67E-04
<i>Cacna2d1</i>	calcium channel, voltage-dependent, alpha2/delta subunit 1	3.2	3.68E-03
<i>Cacna2d3</i>	calcium channel, voltage-dependent, alpha2/delta subunit 3	-18.5	8.50E-05
<i>Cacnb1</i>	calcium channel, voltage-dependent, beta 1 subunit	-2.7	2.09E-03
<i>Cacnb2</i>	calcium channel, voltage-dependent, beta 2 subunit	4.6	1.08E-04

<i>Cacnb3</i>	calcium channel, voltage-dependent, beta 3 subunit	-5.8	2.90E-04
<i>Cacnb4</i>	calcium channel, voltage-dependent, beta 4 subunit	-6.2	1.90E-05
<i>Cacng2</i>	calcium channel, voltage-dependent, gamma subunit 2	-10.0	6.20E-05
<i>Cacng3</i>	calcium channel, voltage-dependent, gamma subunit 3	-4.9	7.60E-04
<i>Cacng4</i>	calcium channel, voltage-dependent, gamma subunit 4	-2.4	4.31E-04
<b>Sodium channels</b>			
<i>Scn1a</i>	sodium channel, voltage-gated, type I, alpha	-13.7	4.80E-05
<i>Scn2a1</i>	sodium channel, voltage-gated, type II, alpha 1	-6.0	1.96E-04
<i>Scn2b</i>	sodium channel, voltage-gated, type II, beta	-13.6	9.90E-05
<i>Scn3a</i>	sodium channel, voltage-gated, type III, alpha	-2.3	4.34E-03
<i>Scn3b</i>	sodium channel, voltage-gated, type III, beta	-6.8	7.21E-04
<i>Scn7a</i>	sodium channel, voltage-gated, type VII, alpha	-6.2	4.05E-04
<i>Scn9a</i>	sodium channel, voltage-gated, type IX, alpha	-42.2	1.30E-05
<i>Scn11a</i>	sodium channel, voltage-gated, type XI, alpha	-2.1	5.84E-03
<b>Trp channels</b>			
<i>Trpc1</i>	transient receptor potential cation channel, subfamily C, member 1	-2.4	4.09E-03
<i>Trpc5</i>	transient receptor potential cation channel, subfamily C, member 5	32.5	4.80E-

			05
<i>Trpc6</i>	transient receptor potential cation channel, subfamily C, member 6	-3.0	2.59E-03
<i>Trpc7</i>	transient receptor potential cation channel, subfamily C, member 7	-3.2	1.82E-03
<i>Trpm3</i>	transient receptor potential cation channel, subfamily M, member 3	-2.9	4.37E-03
<i>Trpv2</i>	transient receptor potential cation channel, subfamily V, member 2	-3.3	3.72E-04

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\*pFDR < 0.05 and fold change >2 or < -2.

**Table 7.** Differential gene expression in the CB, AM, and SCG analyzed by real-time quantitative PCR in adult mice.

Gene	CB vs. SCG	AM vs. SCG
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Symbol	A. microarray		B. whole tissue		C. TH <sup>+</sup> cells		A. microarray		B. whole tissue		C. TH <sup>+</sup> cells	
	Fold change	pFDR	Fold change <sup>#</sup>	p *	Fold change <sup>#</sup>	p *	Fold change	pFDR	Fold change <sup>#</sup>	p *	Fold change <sup>#</sup>	p *
<b>Phd/Hif pathway</b>												
<i>Epas1(Hif 2a)</i>	102.0	0.00249	190.2 ± 38.5	*	914.2 ± 270.3	*	6.6	0.00131	6.9 ± 0.7	*	16.4 ± 5.2	*
<i>Hif1a</i>	1.8	0.05001	6.4 ± 0.6	*	1.9 ± 0.5		2.1	0.00746	2.9 ± 0.4	*	2.6 ± 0.7	*
<i>Egln3(Phd 3)</i>	-4.1	0.01886	-2.2 ± 0.5	*	CB NE		-4.6	0.00344	-2.9 ± 0.2	*	-21.1 ± 16.7	*
<b>Mitochondrial ETC subunits</b>												
<i>Ndufa4l2</i>	720.9	0.00039	1003.8 ± 264.0	*	4015.5 ± 849.8	*	232.6	0.00000	257.6 ± 41.1	*	512.8 ± 184.5	*
<i>Ndufa4</i>	-1.9	0.21813	1.0 ± 0.1		1.5 ± 0.2		-4.6	0.00367	1.0 ± 0.1		1.2 ± 0.1	
<i>Cox4i2</i>	10.1	0.00603	360.0 ± 69.1	*	1334.4 ± 226.9	*	2.1	0.00172	25.4 ± 5.1	*	15.2 ± 12.4	
<i>Cox4i1</i>	-1.5	0.09280	1.5 ± 0.2	*	1.1 ± 0.1		-1.7	0.01749	1.3 ± 0.1		-1.5 ± 0.2	
<i>Cox8b</i>	11.8	0.01295	512.9 ± 143.9	*	4699.9 ± 1024.0	*	6.4	0.00055	255.3 ± 29.4	*	8672.1 ± 2019.0	*
<b>Metabolic enzymes and transporters</b>												
<i>Pcx</i>	4.9	0.01312	20.7 ± 3.8	*	23.5 ± 2.8	*	6.1	0.00062	8.7 ± 0.8	*	-1.3 ± 0.5	
<i>Pdha1</i>	-2.5	0.01900	-1.4 ± 0.2		-2.1 ± 1.2		-3.0	0.00043	-1.2 ± 0.2		-4.7 ± 1.4	*
<i>Slc7a5</i>	-3.6	0.02241	-2.4 ± 0.4	*	-3.5 ± 0.6	*	1.7	0.08917	1.9 ± 0.3	*	-3.9 ± 1.9	*
<i>ldh1</i>	-3.5	0.01335	-1.8 ± 0.2	*	-6.7 ± 2.1	*	1.6	0.07287	2.6 ± 0.4	*	-3.3 ± 0.7	*
<i>ldh3a</i>	-3.1	0.00550	-1.2 ± 0.1	*	1.9 ± 0.4	*	-2.1	0.00109	-1.3 ± 0.1	*	-1.3 ± 0.4	



<i>Idh3b</i>	-4.6	0.038 26	-1.6 ± 0.2	*	1.2 ± 0.2		-1.5	0.002 57	-1.2 ± 0.2		-1.4 ± 0.4
<i>Acly</i>	-4.4	0.077 42	-1.5 ± 0.3		-4.6 ± 0.7	*	1.5	0.191 80	1.3 ± 0.1		-4.8 ± 1.1 *
<i>Acacb</i>	2.7	0.009 57	18.9 ± 3.7	*	##		1.2	0.370 25	3.8 ± 0.9	*	6.3 ± 3.1 *
<b>Ion channels</b>											
<i>Kcnk3(Tas k1)</i>	2.5	0.021 74	13.8 ± 2.6	*	74.8 ± 12.0	*	105.4	0.000 03	23.1 ± 2.4	*	19.7 ± 5.4 *
<i>Kcnk9(Tas k3)</i>	83.5	0.002 19	2371.4 ± 522.9	*	18431.8 ± 5917.5	*	1244.1	0.000 02	6527.6 ± 1268.1	*	6647.1 ± 1736.0 *
<i>Kcnip3</i>	5.7	0.001 54	32.3 ± 3.7	*	75.7 ± 10.8	*	16.2	0.000 05	18.6 ± 1.3	*	30.1 ± 4.5 *
<i>Trpc5</i>	30.1	0.002 74	4258.8 ± 628.5	*	132974.6 ± 48426.0	*	32.5	0.000 05	1014.1 ± 77.6	*	1100.1 ± 799.9 *
<i>Cacna1h</i>	2.7	0.000 77	41.6 ± 5.5	*	60.9 ± 11.3	*	6.4	0.000 87	21.8 ± 2.8	*	8.7 ± 3.5 *
<b>Others</b>											
<i>Ucp2</i>	2.4	0.063 61	7.9 ± 1.3	*	10.4 ± 1.5	*	10.1	0.000 55	13.1 ± 2.5	*	22.1 ± 2.9 *
<i>Olfr78<sup>###</sup></i>	1.3	0.550 79	8.5 ± 1.6	*	5.3 ± 1.0	*	-2.3	0.009 02	-22.6 ± 4.0	*	-141.4 ± 11.6 *

**A.** For comparison, the results from microarray analysis are also listed. **B.** Real-time quantitative PCR analysis using RNA isolated from whole tissue. **C.** Real-time PCR analysis using RNA isolated from TH<sup>+</sup> cells from each tissue. \* p<0.05 compared to the SCG; # mean ± sem, n = 3-5/group; ### microarray detects Olfr78 and Olfr560, whereas real-time PCR detects Olfr78 only; CB NE, no expression was detected from CB TH+ cells; ##: Not available (highly variable, no conclusion).

**Abbreviations:** *Epas1(Hif2α)*, endothelial PAS domain protein 1; *Hif1α*, hypoxia inducible factor 1, alpha subunit; *Egln3(Phd3)*, egl-9 family hypoxia inducible factor 3; *Ndufa4l2*, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2; *Ndufa4*, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4; *Cox4i2*, cytochrome c oxidase subunit IV isoform 2; *Cox4i1*, cytochrome c oxidase subunit IV isoform 1; *Cox8b*, cytochrome c oxidase subunit VIIIb; *Pcx*, pyruvate carboxylase; *Pdha1*, pyruvate dehydrogenase E1 alpha 1; *Slc7a5*, solute carrier family 7 (cationic amino acid transporter, y+ system), member 5; *Idh1*, isocitrate dehydrogenase 1 (NADP+), soluble; *Idh3a*, isocitrate dehydrogenase 3 (NAD+) alpha; *Idh3b*, isocitrate

dehydrogenase 3 (NAD<sup>+</sup>) beta; *Acly*, ATP citrate lyase; *Acacb*, acetyl-coenzyme A carboxylase beta; *Kcnk3(Task1)*, potassium channel, subfamily K, member 3; *Kcnk9(Task3)*, potassium channel, subfamily K, member 9; *Kcnip3*, Kv channel interacting protein 3, calsenilin; *Trpc5*, transient receptor potential cation channel, subfamily C, member 5; *Cacna1h*, calcium channel, voltage-dependent, T type, alpha 1H subunit; *Ucp2*, uncoupling protein 2 (mitochondrial, proton carrier); *Olfir78*, olfactory receptor 78.