Anesthesiology 2010; 113:1270–9

The Human Carotid Body

Expression of Oxygen Sensing and Signaling Genes of Relevance for Anesthesia

Malin Jonsson Fagerlund, M.D., Ph.D.,* Jessica Kåhlin, M.D.,† Anette Ebberyd, B.M.A.,‡ Gunnar Schulte, Ph.D.,§ Souren Mkrtchian, M.D., Ph.D.,‖ Lars I. Eriksson, M.D., Ph.D., F.R.C.A.#

ABSTRACT

Background: Hypoxia is a common cause of adverse events in the postoperative period, where respiratory depression due to residual effects of drugs used in anesthesia is an important underlying factor. General anesthetics and neuromuscular blocking agents reduce the human ventilatory response to hypoxia. Although the carotid body (CB) is the major oxygen sensor in humans, critical oxygen sensing and signaling pathways have been investigated only in animals so far. Thus, the aim of this study was to characterize the expression of key genes and localization of their products involved in the human oxygen sensing and signaling pathways with a focus on receptor systems and ion channels of relevance in anesthesia.

Methods: Six CBs were removed unilaterally from patients undergoing radical neck dissection. The gene expression and cell-specific protein localization in the CBs were investigated with DNA microarrays, real-time polymerase chain reaction, and immunohistochemistry.

Results: We found gene expression of the oxygen-sensing pathway, heme oxygenase 2, and the K^+ channels TASK (TWIK-related acid sensitive K^+ channel)-1 and BK (large-conductance potassium channel). In addition, we show the expression of critical receptor subunits such as γ-aminobutyric acid A ($\alpha 2$, $\beta 3$, and $\gamma 2$), nicotinic acetylcholine receptors ($\alpha 3$, $\alpha 7$, and $\beta 2$), purinoceptors (A_{2A} and $P2X_2$), and the dopamine D_2 receptor.

Conclusions: In unique samples of the human CB, we here demonstrate presence of critical proteins in the oxygen-sensing and signaling cascade. Our findings demonstrate similarities to, but also important differences from, established animal models. In addition, our work establishes an essential platform for studying the interaction between anesthetic drugs and human CB chemoreception.

What We Already Know about This Topic

- Although molecular mechanisms of the oxygen-sensing apparatus in the carotid body have been partly probed in animals, the mechanisms in humans are unknown.

What This Article Tells Us That Is New

- Six carotid bodies were removed during radical neck dissection and subjected to high-resolution gene analysis and immunohistochemistry.
- There are similarities but also key differences in genetic expression of putative components of oxygen sensing in human carotid body compared with that in other species.

OXYGEN is an essential substrate for all mammalian cells, and early detection of hypoxia is critical to protect organs and the whole body against cell damage and death. The carotid body (CB) is the global oxygen sensor that promptly regulates ventilation and cardiorespiratory reflexes

◇ This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 9A.

* Assistant Professor, † Resident, # Professor and Academic Chair, Section for Anesthesiology and Intensive Care Medicine, Department of Physiology and Pharmacology, Karolinska Institutet, and Department of Anesthesiology and Intensive Care Medicine, Karolinska University Hospital, Stockholm, Sweden. ‡ Clinical Laboratory Scientist, ‖ Associate Professor, Section for Anesthesiology and Intensive Care Medicine, § Associate Professor, Section for Receptor Biology and Signaling, Department of Physiology and Pharmacology, Karolinska Institutet.

Received from the Department of Physiology and Pharmacology, Section for Anesthesiology and Intensive Care Medicine, Karolinska Institutet, and the Department of Anesthesiology and Intensive Care Medicine, Karolinska University Hospital, Stockholm, Sweden. Submitted for publication June 29, 2010. Accepted for publication August 23, 2010. Supported by the Stockholm County Council (Sweden), the Swedish Research Council (Stockholm) Project nos. K2008-53X-13405-09-3 and K2008-68P-20810-01-4, and the Swedish Society of Medicine (Stockholm), Karolinska Institutet and Knut & Alice Wallenberg Foundation Grant no. KAW2008.0149. Drs. Fagerlund and Kåhlin contributed equally to this work. Presented in part at the Annual Meeting of the European Society of Anaesthesiology, Euroanaesthesia, Helsinki, Finland, June 12–15, 2010.

Address correspondence to Dr. Fagerlund: Department of Physiology and Pharmacology, Section for Anesthesiology and Intensive Care Medicine, Karolinska Institutet, SE-171 76 Stockholm, Sweden. malin.jonsson.fagerlund@ki.se. This article may be accessed for personal use at no charge through the journal website, www.Anesthesiology.org.

during acute hypoxia.[1] Many drugs used in anesthesia depress regulation of breathing during acute hypoxia (*e.g.*, propofol, halogenated inhaled general anesthetics, and neuromuscular blocking agents [NMBAs]).[2–5] Although such agents clearly blunt the acute hypoxic ventilatory response (HVR) in humans, the mechanism(s) by which they interfere with oxygen sensing and signaling remains an enigma.

In brief, the acute HVR is triggered by stimulation of peripheral chemoreceptor cells (type 1 cells) in the CB.[1] The oxygen-sensing mechanism(s) in the CB is not fully understood but has been proposed to involve metabolic mitochondrial inhibition, production of oxygen radicals, inhibition of the hypoxia-induced factor pathway, or decreased carbon monoxide production by heme oxygenase 2 (HO-2) associated with the large-conductance potassium (BK) K^+ channel.[6] Early events in oxygen sensing involve closure of oxygen-sensitive K^+ channels in the CB type 1 cell, a subsequent membrane depolarization, and opening of voltage-dependent Ca^{2+} channels. The resultant Ca^{2+} influx triggers release of neurotransmitters, with a subsequent increase of activity in the afferent carotid sinus nerve. This activates areas in the brainstem responsible for regulation of breathing.[1] Among the multitude of transmitters released, acetylcholine and adenosine triphosphate (ATP) seem to be important excitatory transmitters, whereas dopamine and γ-aminobutyric acid (GABA) have modulatory and inhibitory functions, respectively.[6,7] General anesthetics and nondepolarizing NMBAs target ion channels such as $GABA_A$ receptors, nicotinic acetylcholine receptors (nAChRs), and leak K^+ channels.[8–10] In addition, propofol and nondepolarizing NMBAs blunt oxygen signaling in CB of many animal species, and the halogenated inhaled anesthetics halothane and sevoflurane have a distinct action on K^+ channels in the CB type 1 cells.[11–14] Thus, drugs used in anesthesia have the potential to target critical steps in global oxygen-sensing and signaling pathways of the CB. Although components of the oxygen sensing and signaling pathways in experimental animals have been partially characterized, the pathways of the human CB are at present unknown.[1,6]

Consequently, as a first step toward a better understanding of these fundamental mechanisms, we here characterize components of the human oxygen-sensing and signaling pathways with a focus on GABAergic, cholinergic, purinergic, and dopaminergic receptor systems and K^+ channels of relevance in anesthesia.

Materials and Methods

Patients and Materials

The study was approved by the ethics committee on human research at Karolinska Institutet (Stockholm, Sweden). Six patients (male, American Society of Anesthesiologists classification I-II, aged 36–68 yr) scheduled for unilateral neck dissection to treat head and neck cancer were included in the study after informed written consent. None of the patients had received radiation or chemotherapy before surgery, none had a tumor involving the CB, and none was using nicotine. Exclusion criteria were severe cardiopulmonary (New York Heart Association Functional Classification more than 2), neurologic, or metabolic disease and smoking. Demographic data are presented in table 1.

Table 1. Patient Characteristics

Patient No.	Age, yr	ASA	BMI
1	67	1	25.6
2	36	1	21.1
3	68	1	19.6
4	64	2	31.1
5	38	1	25.7
6	56	1	23.5

ASA = American Society of Anesthesiologists Physical Status Classification; BMI = body mass index.

During sevoflurane-narcotic based anesthesia with normoxic and normocapnic mechanical ventilation (Aisys; GE Healthcare, Madison, WI), the CB was removed by the head-and-neck surgeon after unilateral neck dissection. The CBs (weight range, 50–80 mg) were divided into pieces of equal size and immediately either frozen in liquid nitrogen for RNA isolation or fixed in 4% paraformaldehyde at 4°C for immunohistochemistry.

Total RNA Extraction and Purification

Snap frozen pieces of CBs weighing ~20–35 mg were stored at −80°C until RNA was prepared. For isolation of total RNA, samples were homogenized using a Potter-Elvehjem Teflon pestle homogenizer in 1 ml TRIzol® (Invitrogen, Carlsbad, CA) and allowed to stand for 5 min at room temperature. The homogenate was then mixed with 0.2 ml chloroform, vigorously shaken for 15 s, and incubated at room temperature for 2–3 min. The mixture was centrifuged for 5 min at 12,000*g* at 4°C, the aqueous phase was transferred to a fresh microcentrifuge tube, and 1 volume of 70% ethanol was added. Thereafter, RNA was isolated using an RNeasy kit (Invitrogen) according to the manufacturer's recommendations. The yields of RNA were from 1 to 2 μg. All RNA samples showed *A*260/280 ratios between 1.7 and 2.0. RNA integrity was assessed either by running samples on a denaturing agarose gel or by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Microarray Analysis

Labeling and hybridization were performed according to standard Affymetrix (Santa Clara, CA) procedure at the Karolinska Institute Bioinformatics and Expression core facility** using Affymetrix Human Genome U133 2.0 Plus oligonucleotide microarrays. Affymetrix microarrays were analyzed with the use of Affymetrix Microarray Suite Software (MAS) to estimate expression values for each probe. Using the statistical detection algorithms in MAS version

** Available at: http://www.bea.ki.se/. Accessed June 5, 2010.

5.0, we have separated transcripts of particular probe sets that are reliably detected (present or marginally present calls) from transcripts below the threshold of detection and therefore considered absent. Transcripts for which an absent call was determined were eliminated from further analysis.

Real-time Polymerase Chain Reaction

Isolated total RNA (0.5 μg) was used in a complementary DNA synthesis reaction using SuperScript II First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR; Invitrogen) and oligo(dT) primers. The resulting complementary DNA was amplified using a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA). Twenty-five microliters of amplification reaction included 200 ng complementary DNA template, TaqMan® Universal Master Mix, and a TaqMan® Gene Expression Assay for relevant genes (Applied Biosystems, Foster City, CA) (appendix 1). All samples were amplified in triplicate and normalized to three housekeeping genes (β-actin, TATA box binding protein, and 18S ribosomal RNA).

Immunohistochemistry

After fixation in 4% paraformaldehyde for 2–4 h, CB pieces were cryoprotected in 30% sucrose in phosphate-buffered saline at 4°C for 3–4 days. Subsequently, the CB pieces were rapidly frozen in TissueTek O.C.T. compound (Sakura, Zueterwoude, Netherlands) and stored at −80°C. Fourteen-micrometer sections were cut on a cryostat and mounted onto microscope slides.[15] For determination of morphology, some slides were stained with hematoxylin and eosin (Histolab, Gothenburg, Sweden), and the cellular structures of the CB were identified with light microscopy using a Zeiss Axioskop 2 plus equipped with an AxioCam digital camera (Zeiss, Munich, Germany).[15] Corresponding slides were chosen for immunohistochemistry. After incubation in phosphate-buffered saline and blocking in phosphate-buffered saline with bovine serum albumin and Triton X-100, primary and secondary antibodies were applied as described previously.[15] Primary antibodies against tyrosine hydroxylase (TH) and β-III-tubulin were used to identify type 1 cells and antibodies against glial fibrillary acidic protein were used to identify type 2 cells (appendix 2). Primary antibodies against the potential oxygen sensor HO-2, the TWIK-related acid sensitive K^+ channel-1 (TASK-1) and BK potassium channels, the α2, β3, γ2, and δ $GABA_A$ receptor subunits, the α3, α7, and β2 nAChR subunits, the ATP-receptor $P2X_2$, the adenosine A_{2A} receptor, and the dopamine D_2-receptor are described in appendix 2.

Double immunofluorescent staining with anti-TH or anti-β-III-tubulin and antibodies against relevant receptors was performed to demonstrate colocalization in type 1 cells.[15] The secondary antibodies used were Alexa Fluor 488, 555, and 647 antibodies (Invitrogen), selected to match the animal specificity of the primary antibodies and used at a concentration of 1:500. Slides without primary antibodies were used as negative controls. The slides were mounted using Vectashield, and 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) was used to counterstain the nuclei. Colocalization was confirmed with double immunofluorescent staining. The CB was then visualized and photographed using a laser-scanning microscope (LSM 710 and ZEN 2009 software; Zeiss Inc., Jena, Germany).[15]

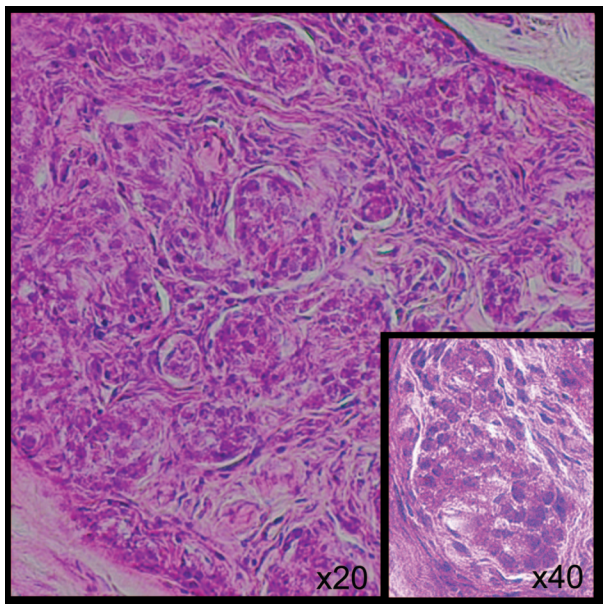

Fig. 1. Hematoxylin-eosin staining demonstrating the characteristic morphology of the carotid body. Higher magnification of one cell cluster demonstrating the typical appearance of type 1 cells with dark nuclei (*bottom right*).

Statistical Analysis

PCR data are normalized to the reference genes according to the common ΔC_t method using the formula $2^{\Delta C_t \text{ target gene } (40 - C_t \text{ target})/2\Delta C_t \text{ reference gene } (40 - C_t \text{ reference gene})}$, related to the expression of TH, and expressed as mean ± SEM (Prism 4.0; GraphPad Software, San Diego, CA).

Results

CBs from the six patients included in the study were analyzed to describe gene expression and protein localization. Because of technical limitations, we were not able to apply all methods used in the study of all CBs: the CB from patient 1 was used only for hematoxylin-eosin and immunohistochemical staining, and the quality of the RNA obtained from patients 2 and 5 did not match the specific requirements of the microarray analysis. Moreover, the fixed CB piece from patient 6 was not suitable for immunohistochemistry. We used CBs from patients 3, 4, and 6 for microarray, those from patients 2–6 for real-time PCR, and those from patients 1–5 for immunohistochemical staining.

Morphology

Hematoxylin-eosin staining revealed a typical CB appearance,[16] with dark type 1 cells arranged in an irregular cluster-like pattern (fig. 1). The size and arrangement of the type 1

Table 2. Expression of Selected Oxygen-Sensing and -Signaling Genes in the Human Carotid Body

Gene Symbol	mRNA		Proteins	
	Microarray (n = 3)	Real-time PCR (n = 5)	Immunohistochemistry (n = 4)*	Present
HO-2	3	5	5*	Y
TASK-1	3	5	4	Y
BK	3	5	3	Y
$GABA_A$ $\alpha1$	0	0	—	N
$GABA_A$ $\alpha2$	3	5	4	Y
$GABA_A$ $\alpha3$	0	0	—	N
$GABA_A$ $\beta2$	2	0	4	N
$GABA_A$ $\beta3$	3	5	4	Y
$GABA_A$ δ	0	2	0	N
$GABA_A$ $\gamma2$	0	2	4	Y
$GABA_A$ $\rho1$	1	0	—	N
$GABA_A$ $\rho2$	1	0	—	N
nAChR $\alpha3$	2	5	5*	Y
nAChR $\alpha4$	0	0	—	N
nAChR $\alpha7$	1	5	2	Y
nAChR $\beta2$	0	3	4	Y
A_{2A}	3	5	4	Y
$P2X_2$	0	5	4	Y
$P2X_3$	0	0	—	N
D_2	3	5	4	Y
TH	0	5	5*	Y

* CB data from Patient 1 was used for selected hematoxylin-eosin and immunohistochemical staining only. Thus, n = 5 for a total of three proteins, as indicated.

BK = big potassium; $GABA_A$ = γ-aminobutyric acid; HO-2 = heme oxygenase 2; N = not detected; nAChR = nicotinic acetylcholine receptor; PCR = polymerase chain reaction; TASK-1 = TWIK-related acid sensitive K^+; TH = tyrosine hydroxylase.

cell clusters was heterogeneous, both between individuals and in different parts of a single CB. There was no infiltration of tumor tissue in the hematoxylin-eosin preparations.

Gene Expression

The transcriptome of human CB was studied using Affymetrix Human Genome U133 2.0 Plus oligonucleotide microarray. MAS 5.0 analysis revealed approximately 25,000 probe sets with the assigned present or marginally present calls. After removal of redundant probe sets, the total number of genes expressed was approximately 13–14,000 (14,064, 13,253, and 13,658 genes expressed in patients 3, 4, and 6, respectively). This is consistent with the latest estimations of the average number of expressed genes in different human tissues, which varies from approximately 10,000 to 15 000.[17] Notably, the mouse CB transcriptome identified earlier is slightly smaller (approximately 10,000 genes),[18,19] which most likely reflects the higher complexity of the human CB.

Next, we analyzed the expressed gene lists obtained from the microarray assay for the presence of key genes that define the CB's chemosensory function and that are potential targets for drugs used in anesthesia, such as $GABA_A$, nicotinic, purinergic, dopaminergic receptors, K^+ channels, and potential oxygen sensors (table 2). To validate the microarray data, we performed real-time PCR analysis for all relevant genes using highly specific TaqMan® Gene Expression Assays (see *Materials and Methods*). The two methods yielded similar patterns of expression for all selected genes with the exception of $\alpha3$, $\alpha7$, and $\beta2$ nAChR subunits, for which microarray analysis did not assign present calls in all patients. The real-time PCR of these genes showed, however, that at least $\alpha3$ and $\alpha7$ had detectable levels of expression in all five patients tested. $\beta2$ expression was found in three of five cases. Similar trends were observed for $P2X_2$ and TH; the microarray data showed expression to be silent, whereas the real-time PCR identified both transcripts (albeit in rather low amounts) in all five patients (table 2). The expression of $GABA_A$ $\gamma2$ and δ receptor subunits was detected by PCR in only two patients, whereas microarray data were negative. Because neither analytical method used would allow estimation of absolute expression values, we have related the PCR gene expression data to the expression of TH, which is considered to be very low at normoxia (fig. 2).[19] Such analysis demonstrates low abundance of $GABA_A$ receptor subunits as well as $P2X_2$ transcripts, which were detected only by real-time PCR. In general, such discrepancies between microarray and PCR data might be explained by the known tendency of microarray analysis to underestimate the extent of gene expression compared with real-time PCR, which is undoubtedly a more sensitive and specific analytical tool.[20]

Immunohistochemistry

Immunohistochemistry was used to confirm results of real-time PCR and microarray and to provide information on specific cell localization of proteins. As described previously, TH and β-III-tubulin were used as type 1 cell markers and glial fibrillary acidic protein as type 2 cell marker[15,21] (fig.

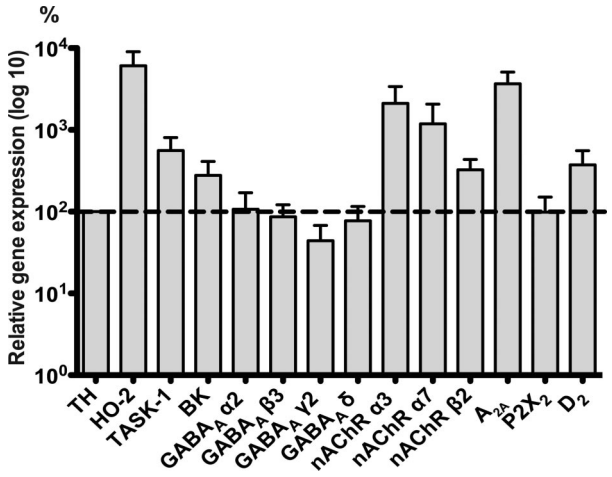

Fig. 2. Relative expression of the selected oxygen-sensing and signaling genes in the human carotid body. Gene expression was determined by real-time polymerase chain reaction (PCR). Data are normalized to the reference genes (see Materials and Methods) according to the common ΔC_t method using the formula $2^{\Delta C_t \text{ target gene } (40 - C_t \text{ target})/2\Delta C_t \text{ reference gene } (40 - C_t \text{ reference gene})}$ and related to the expression of tyrosine hydroxylase (TH) ± SEM. C_t is mean critical threshold, the PCR cycle number at which the increase in fluorescence is exponential. The PCR efficiency (amplification rate at each cycle) was assumed to be close to 2. BK = large-conductance potassium channel; GABA = γ-aminobutyric acid; HO-2 = heme oxygenase-2; nAChR = nicotinic acetylcholine receptor; TASK-1, TWIK-related acid sensitive K^+ channel-1.

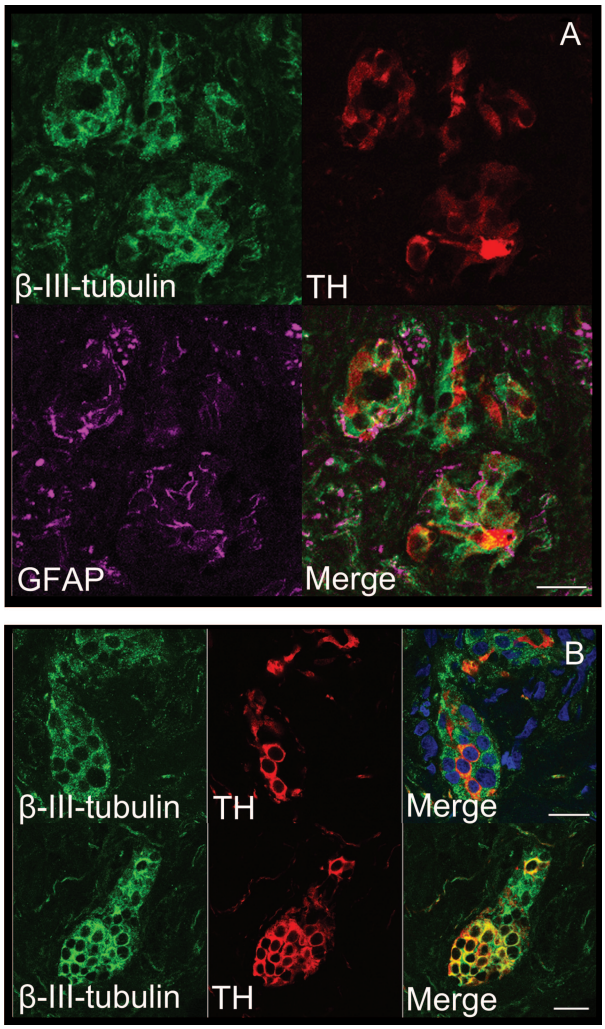

Fig. 3. Morphological overview of the human carotid body. (*A*) Immunohistochemical staining of one carotid body cell cluster demonstrating localization of different cell types: β-III-tubulin marks both type 1 cells and nerve endings, tyrosine hydroxylase (TH) marks certain type 1 cells, and glial fibrillary acidic protein (GFAP) marks type 2 cells. (*B*) The expression of TH in type 1 cells displays a large variability within each individual: TH is very sparse in some clusters (*top panel*) and more generally distributed in others (*bottom panel*). Scale *bar*, 20 μm.

3A). We detected sparsely distributed TH-positive cells in type 1 cell clusters, in line with previous findings in the human CB (fig. 3).[22,23] In some clusters, however, all cells were positive for TH (fig. 3B). β-III-tubulin, on the other hand, was positive in the majority of cells in all type 1 cell clusters, and we consequently used this marker to assess colocalization of relevant proteins. Positive immunostaining in the human CB was seen for $GABA_A$ receptor subunits α2, β3, and γ2, nAChR subunits α3, α7 and β2, ATP-receptor $P2X_2$, adenosine receptor A_{2A}, dopamine D_2 receptor, K^+ channels TASK-1 and BK, and HO-2 (figs. 4 and 5, table 2). The immunostaining for all proteins was positive in all patients tested with the exception of BK and the α7 subunit of the nAChR, where most of the CBs were positive (table 2). The immunostaining for the $GABA_A$ receptor δ subunit was negative (table 2). Based on morphology and colocalization with β-III-tubulin, we conclude that these proteins are localized in type 1 cells or in adjacent nerve endings but not in type 2 cells. The pattern for the α3 subunit of nAChR and D_2 is inconsistent with the rest; cells appear to be densely filled with the protein, suggesting a more intracellular localization than for the other proteins (fig. 5). A summary of all results is presented in table 2.

Discussion

Hypoxia is a common cause of serious anesthesia-related adverse events in the early postoperative period, the most frequent underlying mechanisms being respiratory depression and airway obstruction.[24–27] The residual effects of general anesthetics and NMBAs on regulation of breathing and airway protection seem to have a key role in postanesthetic mortality and morbidity.[28–30] Several studies on regulation of breathing in humans have indirectly pointed toward a specific interaction of anesthetics with peripheral chemoreceptors, yet there are no studies to show mechanisms through which anesthetics might perturb chemoreception in human CB.[2–5] This study is the first to demonstrate human oxygen-sensing and signaling proteins that are also potential targets for drugs used in anesthesia. The major findings are that key sensing and signaling proteins previously found in animal studies and known to be of importance in the

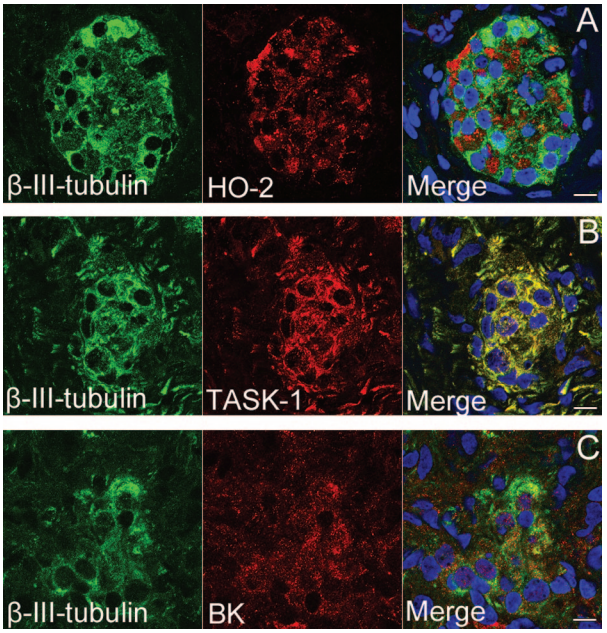

Fig. 4. Cell localization of oxygen sensing proteins in the human carotid body. Colocalization of β-III-tubulin (*green*) with (*A*) heme oxygenase 2 (HO-2), (*B*) TWIK-related acid sensitive K^+ channel-1 (TASK-1), and (*C*) large-conductance potassium (BK) K^+ channels (*red*) are homogenously distributed in carotid body type 1 cells or at surrounding nerve endings. In all figures "Merge" is a combination of the protein of interest and β-III-tubulin. 4′,6-Diamidino-2-phenylindole staining (*blue*) marks the cell nucleus. *Scale bar*, 10 μm.

context of anesthesia are present in the human CB. On both the messenger RNA (mRNA) and protein levels, we demonstrate presence of relevant GABAergic, nicotinergic, purinergic, and dopaminergic receptors, as well as the potential oxygen sensor HO-2 and the TASK-1 and BK K^+ channels.

It is essential to assess multiple signaling proteins because studies on animal CBs during the last few decades have demonstrated a signaling protein cascade between the oxygen-sensitive CB type 1 cell and the postsynaptic carotid sinus nerve in response to hypoxia.[1,6] This cascade involves closure of oxygen-sensitive K^+ channels in response to hypoxia followed by depolarization of the membrane of the CB type 1 cell and a Ca^{2+}-dependent neurotransmitter release. There are clear expressional and functional species differences regarding the identity of the oxygen-sensitive K^+ channels; various voltage-dependent (Kv) channels are important in mouse and rabbit CB, whereas the leak TASK-1 and BK K^+ channels are important in rat.[6,31] The neurotransmitters released during hypoxia, expression of the corresponding receptors, and especially their functions are also species dependent.[15,32] The most important excitatory transmitters at present seem to be acetylcholine acting on postsynaptic nAChRs, adenosine activating presynaptic A_{2A} receptors, and ATP acting on both presynaptic and postsynaptic $P2X_2$ and $P2\times_3$ ATP-receptors. Dopamine, on the other hand, has an inhibitory or modulatory role *via* postsynaptic dopamine D_2 receptors.[1,6] Activation of postsynaptic $GABA_A$ receptors has recently been demonstrated to have an important inhibitory role in CB chemotransmission.[7] Thus, both oxygen-sensitive K^+ channels and important receptors display large species variability and, with the exception of the dopamine D_2 receptor, they have not been characterized in human CB. So far, the only receptors reported to be present in the human CB are histamine H1 and H3, dopamine D_2, NTR1, TH, and serotonin.[22,23,33,34] Those immunohistochemical studies were also done on tissue taken up to 48 h postmortem from a mixed population of humans in terms of age (*e.g.*, age from 4 months to 91 yr), which raises questions concerning how representative they are.

Several physiologic studies have demonstrated the importance of CB function for the HVR in humans. Studies of patients undergoing carotid endarterectomy or bilateral neck dissection show that the hypoxic response is blunted after a unilateral CB denervation and, furthermore, markedly reduced or even abolished after a bilateral denervation. It is noteworthy that the human HVR does not recover over time after CB denervation or resection, which contrasts to animals, in which the aortic bodies gradually take over and compensate for the loss of CB function.[35] Moreover, there is a large interindividual variability in the normal ventilatory response to acute hypoxia; in some persons, the HVR is even absent.[36] The reason for this interindividual variance in humans is poorly understood, and molecular studies on human CB chemoreception are therefore needed. Improved knowledge in this field might also have implications for postoperative care of patients in whom HVR is reduced or absent.

In this unique material, we chose to investigate each CB individually rather than pooling them together, as is commonly done in animal research because of the small size of the organ.[18,19] Because characterization of gene expression on the transcriptional level is often insufficient for prediction of protein translation, we characterized each CB on both mRNA and protein levels. We chose an immunohistochemical method to detect target proteins, which gave the added benefit of showing the cell type in which the expression occurs. This is of paramount importance because the CB tissue contains various cell types, including glomus type 1 cells and sustentacular type 2 cells; thus, we could make some differentiation between cell types. In addition, relying only on immunohistochemistry would have increased the risk of false positive results.[37]

In all patients, the α2 and β3 $GABA_A$ receptor subunits, the α3 nAChR subunit, the adenosine A_{2A} and dopamine D_2 receptors, as well as the TASK-1 and BK K^+ channels and HO-2 were expressed as both mRNAs and proteins. The α7 and β2 nAChR subunits were present in most patients, whereas the α1 and α3 $GABA_A$ receptor subunits, the α4 nAChR subunit, and the $P2X_3$ receptor were not detected. It is noteworthy that microarray data suggested that TH and $P2X_2$ were absent, but both PCR and protein analyses showed them to be present in all patients. This might reflect low levels of expression of these genes during normoxia,

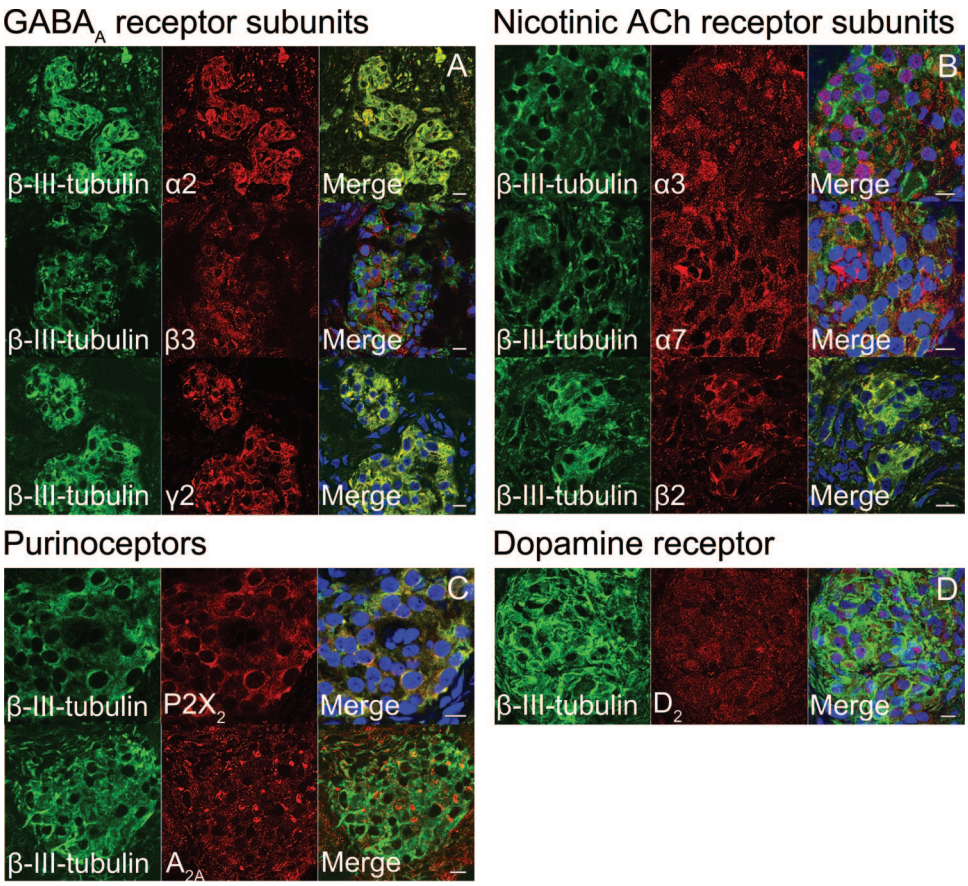

Fig. 5. Localization of signaling proteins in the human carotid body. Colocalization of β-III-tubulin (*green*) with γ-amino butyric acid A ($GABA_A$) receptor subunits (*A*), nicotinic acetylcholine (ACh) receptor subunits (*B*), purinoceptors (*C*), and the dopamine receptor D_2 (*red*) (*D*). Colocalization with β-III-tubulin indicates that the localization of these receptors and receptor subunits is in carotid body type 1 cells or at surrounding nerve endings. In all figures "Merge" is a combination of the receptor or receptor subunit of interest and β-III-tubulin. In some figures, 4′,6-diamidino-2-phenylindole staining (*blue*) marks the cell nucleus. *Scale bar*, 10 μm.

which, in combination with the above-mentioned issue of the sensitivity of microarray technique, might lead to such apparent contradictions in the experimental data. In addition, very few CB type 1 cells were immunopositive for TH, and the mRNA levels were also barely detectable, which is in line with previous findings that TH is much more sparse in the human CB than in those of experimental animals.[22,23] Interestingly, the gene expression of TH almost doubled during hypoxia in mouse,[19] but whether this occurs also in humans remains to be investigated. The $GABA_A$ receptor subunits δ, ρ1, and ρ2 were present only in single patients, and at very low levels in one modality, and were therefore considered not to be present in the human CB. Comparison of our data from humans with data from previous animal studies reveals large similarities between species but also interesting and potentially crucial differences. Here we demonstrate expression of the α2 subunit of the $GABA_A$ receptor, which is also expressed in rat CB but not in cat CB.[38,39] The α4 nAChR subunit was not found in the human material but has been detected at both the mRNA and protein levels in mouse and cat CB, and a pharmacological study indicates the presence of the α4β2 nAChR subtype.[15,40,41] Furthermore, in contrast to previous animal studies, we found no expression of the ATP-receptor $P2X_3$ in the human CB.[42,43] Thus, there are distinct differences in the expression of various receptors and receptor subunits in animal and human CB.

Whether this is of functional importance remains to be evaluated, but this study suggests that general conclusions about human CB oxygen-sensing and signaling function cannot be drawn solely on the basis of data from animals. Based on our findings in human CB, we speculate about potential target receptors/ion channels underlying the effects of drugs used in anesthesia on acute HVR and CB chemoreception. Although potentiation and activation of the $GABA_A$ receptor channel are the main mechanisms of anesthetic action of propofol, it also potently depresses CB chemoreception and HVR.[3,4,9,12,14] The most common $GABA_A$ receptors in the central nervous system consist of two α, two β, and one γ subunit, and in human CB, the α2, β3, and γ2 receptor subunits are expressed. Thus, it might be argued that propofol reduces CB chemoreception as a result of an interaction with an α2β3γ2 $GABA_A$ receptor, which is also one of the most common $GABA_A$ receptor subtypes expressed in the central nervous system.[9] Inhaled halogenated

anesthetics target both K^+ channels and nAChRs, which also are present in the human CB.[9] Finally, nondepolarizing NMBAs have the potential to inhibit neuronal nAChRs present in human CB.[13,44] It must be emphasized that these are hypotheses based on a combination of our data from the study of human CB and previous results from studies on human and animal CB and can as such be seen only as speculations. To achieve a more comprehensive understanding of human CB oxygen sensing, signaling, and interactions with drugs used in anesthesia, functional studies using human material will be needed. A better understanding of the human CB is important not only in the context of anesthesia but also for a larger patient population, such as those with chronic obstructive pulmonary disease or obstructive sleep apnea syndrome and in patients in critical condition after trauma.

In conclusion, in unique samples of human CB, we here demonstrate presence of critical proteins in the oxygen-sensing and signaling cascade. Our findings demonstrate similarities to but also important differences from established animal models. In addition, our work establishes an essential platform for studying the interaction between anesthetic drugs and human CB chemoreception.

The authors thank Jan Lundgren, M.D., Ph.D. (Associate Professor, Department of Oto-rhino-laryngology, Karolinska University Hospital, Solna, Sweden), for help in conducting this project. We also thank the head-and-neck surgeons at the Department of Oto-rhino-laryngology, Karolinska University Hospital, who were involved in the surgical care of the patients.

References

1. Weir EK, López-Barneo J, Buckler KJ, Archer SL: Acute oxygen-sensing mechanisms. N Engl J Med 2005; 353: 2042–55
2. Eriksson LI: The effects of residual neuromuscular blockade and volatile anesthetics on the control of ventilation. Anesth Analg 1999; 89:243–51
3. Nieuwenhuijs D, Sarton E, Teppema L, Dahan A: Propofol for monitored anesthesia care: Implications on hypoxic control of cardiorespiratory responses. ANESTHESIOLOGY 2000; 92:46–54
4. Blouin RT, Seifert HA, Babenco HD, Conard PF, Gross JB: Propofol depresses the hypoxic ventilatory response during conscious sedation and isohypercapnia. ANESTHESIOLOGY 1993; 79:1177–82
5. Eriksson LI, Sato M, Severinghaus JW: Effect of a vecuronium-induced partial neuromuscular block on hypoxic ventilatory response. ANESTHESIOLOGY 1993; 78:693–9
6. López-Barneo J, Ortega-Sáenz P, Pardal R, Pascual A, Piruat JI, Durán R, Gómez-Díaz R: Oxygen sensing in the carotid body. Ann N Y Acad Sci 2009; 1177:119–31
7. Nurse CA: Neurotransmitter and neuromodulatory mechanisms at peripheral arterial chemoreceptors. Exp Physiol 2010; 95:657–67
8. Fagerlund MJ, Eriksson LI: Current concepts in neuromuscular transmission. Br J Anaesth 2009; 103:108–14
9. Rudolph U, Antkowiak B: Molecular and neuronal substrates for general anaesthetics. Nat Rev Neurosci 2004; 5:709–20
10. Campagna JA, Miller KW, Forman SA: Mechanisms of actions of inhaled anesthetics. N Engl J Med 2003; 348: 2110–24
11. Pandit JJ, Buckler KJ: Differential effects of halothane and sevoflurane on hypoxia-induced intracellular calcium transients of neonatal rat carotid body type I cells. Br J Anaesth 2009; 103:701–10
12. Ponte J, Sadler CL: Effect of thiopentone, etomidate and propofol on carotid body chemoreceptor activity in the rabbit and the cat. Br J Anaesth 1989; 62:41–5
13. Jonsson M, Wyon N, Lindahl SG, Fredholm BB, Eriksson LI: Neuromuscular blocking agents block carotid body neuronal nicotinic acetylcholine receptors. Eur J Pharmacol 2004; 497:173–80
14. Jonsson MM, Lindahl SG, Eriksson LI: Effect of propofol on carotid body chemosensitivity and cholinergic chemotransduction. ANESTHESIOLOGY 2005; 102:110–6
15. Kåhlin J, Eriksson LI, Ebberyd A, Fagerlund MJ: Presence of nicotinic, purinergic and dopaminergic receptors and the TASK-1 K+-channel in the mouse carotid body. Respir Physiol Neurobiol 2010; 172:122–8
16. Mills SE: Histology for Pathologists, 3rd Edition. Philadelphia, Lippincott Williams & Wilkins, 2007
17. Ramskold D, Wang ET, Burge CB, Sandberg R: An abundance of ubiquitously expressed genes revealed by tissue transcriptome sequence data. PLoS Comput Biol 2009; 5:e1000598
18. Balbir A, Lee H, Okumura M, Biswal S, Fitzgerald RS, Shirahata M: A search for genes that may confer divergent morphology and function in the carotid body between two strains of mice. Am J Physiol Lung Cell Mol Physiol 2007; 292:L704–15
19. Ganfornina MD, Pérez-García MT, Gutiérrez G, Miguel-Velado E, López-López JR, Marín A, Sánchez D, González C: Comparative gene expression profile of mouse carotid body and adrenal medulla under physiological hypoxia. J Physiol 2005; 566:491–503
20. Wang Y, Barbacioru C, Hyland F, Xiao W, Hunkapiller KL, Blake J, Chan F, Gonzalez C, Zhang L, Samaha RR: Large scale real-time PCR validation on gene expression measurements from two commercial long-oligonucleotide microarrays. BMC Genomics 2006; 7:59
21. Izal-Azcárate A, Belzunegui S, San Sebastián W, Garrido-Gil P, Vázquez-Claverie M, López B, Marcilla I, Luquin MA: Immunohistochemical characterization of the rat carotid body. Respir Physiol Neurobiol 2008; 161:95–9
22. Lazarov NE, Reindl S, Fischer F, Gratzl M: Histaminergic and dopaminergic traits in the human carotid body. Respir Physiol Neurobiol 2009; 165:131–6
23. Kummer W, Habeck JO: Chemoreceptor A-fibres in the human carotid body contain tyrosine hydroxylase and neurofilament immunoreactivity. Neuroscience 1992; 47: 713–25
24. Cooper AL, Leigh JM, Tring IC: Admissions to the intensive care unit after complications of anaesthetic techniques over 10 years. 1. The first 5 years. Anaesthesia 1989; 44:953–8
25. Leigh JM, Tytler JA: Admissions to the intensive care unit after complications of anaesthetic techniques over 10 years. 2. The second 5 years. Anaesthesia 1990; 45:814–20
26. Lunn JN, Hunter AR, Scott DB: Anaesthesia-related surgical mortality. Anaesthesia 1983; 38:1090–6
27. Rose DK, Cohen MM, Wigglesworth DF, DeBoer DP: Critical respiratory events in the postanesthesia care unit. Patient, surgical, and anesthetic factors. ANESTHESIOLOGY 1994; 81:410–8
28. Arbous MS, Meursing AE, van Kleef JW, de Lange JJ, Spoormans HH, Touw P, Werner FM, Grobbee DE: Impact of anesthesia management characteristics on severe morbidity and mortality. ANESTHESIOLOGY 2005; 102:257–68
29. Adabag AS, Wassif HS, Rice K, Mithani S, Johnson D, Bonawitz-Conlin J, Ward HB, McFalls EO, Kuskowski MA,

Kelly RF: Preoperative pulmonary function and mortality after cardiac surgery. Am Heart J 2010; 159:691–7

30. Murphy GS, Brull SJ: Residual neuromuscular block: Lessons unlearned. Part I: definitions, incidence, and adverse physiologic effects of residual neuromuscular block. Anesth Analg 2010; 111:120–8
31. Gonzalez C, Vaquero LM, López-López JR, Pérez-García MT: Oxygen-sensitive potassium channels in chemoreceptor cell physiology: Making a virtue of necessity. Ann NY Acad Sci 2009; 1177:82–8
32. Monti-Bloch L, Eyzaguirre C: A comparative physiological and pharmacological study of cat and rabbit carotid body chemoreceptors. Brain Res 1980; 193:449–70
33. Porzionato A, Macchi V, Amagliani A, Castagliuolo I, Parenti A, De Caro R: Neurotensin receptor 1 immunoreactivity in the peripheral ganglia and carotid body. Eur J Histochem 2009; 53:135–42
34. Habeck JO, Pallot DJ, Kummer W: Serotonin immunoreactivity in the carotid body of adult humans. Histol Histopathol 1994; 9:227–32
35. Honda Y: Respiratory and circulatory activities in carotid body-resected humans. J Appl Physiol 1992; 73:1–8
36. Weil JV: Variation in human ventilatory control-genetic influence on the hypoxic ventilatory response. Respir Physiol Neurobiol 2003; 135:239–46
37. Moser N, Mechawar N, Jones I, Gochberg-Sarver A, Orr-Urtreger A, Plomann M, Salas R, Molles B, Marubio L, Roth U, Maskos U, Winzer-Serhan U, Bourgeois JP, Le Sourd AM, De Biasi M, Schröder H, Lindstrom J, Maelicke A, Changeux JP, Wevers A: Evaluating the suitability of nicotinic acetylcholine receptor antibodies for standard immunodetection procedures. J Neurochem 2007; 102:479–92
38. Zhang M, Clarke K, Zhong H, Vollmer C, Nurse CA: Postsynaptic action of GABA in modulating sensory transmission in co-cultures of rat carotid body *via* GABA(A) receptors. J Physiol 2009; 587:329–44
39. Igarashi A, Zadzilka N, Shirahata M: Benzodiazepines and GABA-GABAA receptor system in the cat carotid body. Adv Exp Med Biol 2009; 648:169–75
40. Conde SV, Monteiro EC: Activation of nicotinic ACh receptors with alpha4 subunits induces adenosine release at the rat carotid body. Br J Pharmacol 2006; 147:783–9
41. Higashi T, McIntosh JM, Shirahata M: Characterization of nicotinic acetylcholine receptors in cultured arterial chemoreceptor cells of the cat. Brain Res 2003; 974:167–75
42. Prasad M, Fearon IM, Zhang M, Laing M, Vollmer C, Nurse CA: Expression of P2X2 and P2X3 receptor subunits in rat carotid body afferent neurones: Role in chemosensory signalling. J Physiol 2001; 537:667–77
43. Rong W, Gourine AV, Cockayne DA, Xiang Z, Ford AP, Spyer KM, Burnstock G: Pivotal role of nucleotide P2X2 receptor subunit of the ATP-gated ion channel mediating ventilatory responses to hypoxia. J Neurosci 2003; 23:11315–21
44. Jonsson M, Gurley D, Dabrowski M, Larsson O, Johnson EC, Eriksson LI: Distinct pharmacologic properties of neuromuscular blocking agents on human neuronal nicotinic acetylcholine receptors: A possible explanation for the train-of-four fade. ANESTHESIOLOGY 2006; 105:521–33

Appendix 1. Gene Names and Symbols*

Gene Symbol (Current Study)	Gene Symbol (Official)	Gene Name (Official)
Oxygen sensing		
HO-2	*HMOX2*	Heme oxygenase 2
TASK-1	*KCNK3*	Potassium channel, subfamily K, member 3
BK	*KCNMA1*	Potassium large conductance calcium-activated channel, subfamily M, α member 1
Oxygen signaling		
$GABA_A$ α1	*GABRA1*	γ-aminobutyric acid A receptor, α1
$GABA_A$ α2	*GABRA2*	γ-aminobutyric acid A receptor, α2
$GABA_A$ α3	*GABRA3*	γ-aminobutyric acid A receptor, α3
$GABA_A$ β2	*GABRB2*	γ-aminobutyric acid A receptor, β2
$GABA_A$ β3	*GABRB3*	γ-aminobutyric acid A receptor, β3
$GABA_A$ δ	*GABRD*	γ-aminobutyric acid A receptor, δ
$GABA_A$ γ2	*GABRG2*	γ-aminobutyric acid A receptor, γ2
$GABA_A$ ρ1	*GABRR1*	γ-aminobutyric acid A receptor, ρ1
$GABA_A$ ρ2	*GABRR2*	γ-aminobutyric acid A receptor, ρ2
nAChRα3	*CHRNA3*	Cholinergic receptor, nicotinic, α3
nAChRα4	*CHRNA4*	Cholinergic receptor, nicotinic, α4
nAChRα7	*CHRNA7*	Cholinergic receptor, nicotinic, α7
nAChRβ2	*CHRNB2*	Cholinergic receptor, nicotinic, β2
A_{2A}	*ADORA2A*	Adenosine A2A receptor
$P2X_2$	*P2RX2*	Purinergic receptor P2X, ligand-gated ion channel, 2
$P2X_3$	*P2RX3*	Purinergic receptor P2X, ligand-gated ion channel, 3
D_2	*DRD2*	Dopamine receptor D2
TH	*TH*	Tyrosine hydroxylase

* International Human Gene Nomenclature Committee. Physician Quality Reporting Initiative (PQRI). Available at: http://www.gene.ucl.ac.uk/nomenclature. Accessed August 12, 2010.

BK = big potassium; $GABA_A$ = γ-aminobutyric acid; HO-2 = heme oxygenase 2; N = not detected; nAChR = nicotinic acetylcholine receptor; PCR = polymerase chain reaction; TASK-1 = TWIK-related acid sensitive K^+; TH = tyrosine hydroxylase.

Appendix 2. TaqMan® Gene Expression Assays and Primary Antibodies

Gene Symbol	TaqMan® Assay ID	Antibodies			
		Host	Company	Number	Dilution
HO-2	Hs00157969_m1	Rabbit	Santa Cruz	Sc 11361	1:100
TASK-1	Hs00605529_m1	Goat	Santa Cruz	Sc32065	1:50
BK	Hs00266938_m1	Rabbit	Alomone	APC-021	1:100
$GABA_A$ α1	Hs00168058_m1	—	—	—	—
$GABA_A$ α2	Hs00169069_m1	Goat	Santa Cruz	Sc 7350	1:100
$GABA_A$ α3	Hs00168073_m1	—	—	—	—
$GABA_A$ β2	Hs00241451_m1	—	—	—	—
$GABA_A$ β3	Hs00241459_m1	Mouse	NeuroMab	N87/25	1:500
$GABA_A$ δ	Hs00181309_m1	Rabbit	Millipore	AB9752	1:50–1:100
$GABA_A$ γ2	Hs00168093_m1	Goat	Santa Cruz	Sc 131935	1:200
$GABA_A$ ρ1	Hs00266687_m1	—	—	—	—
$GABA_A$ ρ2	Hs00266703_m1	—	—	—	—
nAChR α3	Hs00609519_m1	Rabbit	Santa Cruz	Sc 5590	1:100
nAChR α4	Hs00181247_m1	—	—	—	—
nAChR α7	Hs01063372_m1	Rabbit	Santa Cruz	Sc 5544	1:50
nAChR β2	Hs00181267_m1	Goat	Santa Cruz	Sc 1449	1:100
$A2_A$	Hs00169123_m1	Rabbit	Santa Cruz	Sc13937	1:100
$P2X_2$	Hs00247255_m1	Goat	Santa Cruz	Sc12212	1:50
$P2X_3$	Hs00175689_m1	—	—	—	—
D_2	Hs01024460_m1	Rabbit	Millipore	AB 5084P	1:200
TH	Hs01002182_m1	Rabbit	Millipore	AB 152	1:500
		Mouse	Millipore	MAB318	1:200
GFAP		Rabbit	Dako	Z0334	1:500
β-III-tubulin		Chicken	Millipore	AB9354	1:250

Alomone = Alomone Labs (Jerusalam, Israel); BK = big potassium; Dako = Dako Denmark A/S (Glostrup, Denmark); $GABA_A$ = γ-aminobutyric acid; GFAP = glial fibrillary acidic protein; HO-2 = heme oxygenase 2; Millipore = Millipore Bioscience Research Reagents (Temecula, CA); NeuroMab = University of California, Davis/National Institutes of Health NeuroMab Facility; nAChR = nicotinic acetylcholine receptor; TASK-1 = TWIK-related acid sensitive K^+; Santa Cruz = Santa Cruz Biotechnology (Santa Cruz, CA); TH = tyrosine hydroxylase.