MOLECULAR IDENTIFICATION AND FUNCTIONAL ROLE OF VOLTAGE-GATED SODIUM CHANNELS IN RAT CAROTID BODY CHEMORECEPTOR CELLS. REGULATION OF EXPRESSION BY CHRONIC HYPOXIA IN VIVO

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ABSTRACT

We have assessed the expression, molecular identification and functional role of Na⁺ channels (Na_v) in carotid bodies (CB) obtained from normoxic and chronically hypoxic adult rats. Veratridine evoked release of catecholamines (CA) from an *in vitro* preparation of intact CBs obtained from normoxic animals, the response being Ca²⁺ and Na⁺-dependent and sensitive to tetrodotoxin (TTX). TTX inhibited by 25-50% the CA release response evoked by graded hypoxia. Immunoblot assays demonstrated the presence of Na_v α subunit (~220 kDa) in crude homogenates from rat CBs, being evident an upregulation (60%) of this protein in the CBs obtained from chronically hypoxic rats (10% O₂; 7 days). This upregulation was accompanied by an enhanced TTX-sensitive release response to veratridine, and by an enhanced ventilatory response to acute hypoxic stimuli. RT-PCR studies demonstrated the expression of mRNA for Na_v1.1, Na_v1.2, Na_v1.3, Na_v1.6 and Na_v1.7 isoforms. At least three isoforms, Na_v1.1, Na_v1.3 and Na_v1.6 co-localized with tyrosine hydroxylase in all chemoreceptor cells. RT-PCR and immunocytochemistry indicated that Na_v1.1 isoform was upregulated by chronic hypoxia in chemoreceptor cells. We conclude that Na_v upregulation represents an adaptive mechanism to increase chemoreceptor sensitivity during acclimatization to sustained hypoxia as evidenced by enhanced ventilatory responses to acute hypoxic tests.

Running Title: Na⁺ channels in rat carotid body chemoreceptor cells

Key Words: chemoreceptor cell; voltage-gated sodium channels; gene expression; RT-PCR; catecholamine release; immunocytochemistry.

INTRODUCTION

Carotid bodies (CB) are the main oxygen chemoreceptors involved in respiratory and cardiovascular control. When CBs are stimulated by arterial hypoxic hypoxia they initiate the ventilatory chemoreflex which increases breathing in order to prevent further reductions in arterial PO_2 . Cellular mechanisms underlying O_2 sensing by CB chemoreceptor cells are incompletely understood, but the inhibition of O_2 -sensitive K^+ channels present in the plasma membrane of the chemoreceptor cells by hypoxia (Lopez-Barneo et al., 1988; Gonzalez et al., 1992) is the trigger for cell depolarization, leading to activation of voltage-gated Ca^{2+} -channels. The resultant Ca^{2+} entry elevates intracellular Ca^{2+} levels which stimulates neurotransmitter release onto carotid sinus nerve sensory nerve endings projecting to the brainstem (Gonzalez et al., 1994). Among neurotransmitters present in chemoreceptor cells, catecholamines (CA), particularly dopamine, are very abundant in all studied species, and its rate of release has been shown by many laboratories to be proportional to the intensity of CB stimulation and paralleled by the action potential frequency in the carotid sinus nerve (CSN; Fidone et al., 1982; Obeso et al., 1989; Rigual et al., 1991; see Gonzalez et al., 1994). Tyrosine hydroxylase (TH), the rate-limiting enzyme in CA synthesis, is the standard specific marker for chemoreceptor cells.

Studies regarding the nature of the O_2 -sensitive K^+ channels in preparations of isolated chemoreceptor cells have shown variabilities among species on the molecular identity of the channels (Peers 1990 vs. Sanchez et al., 2002), and in some instances there are clear discrepancies on the proposed functional role for a given K^+ channel subtype within a particular species (Wyatt et al., 1995 vs. Buckler, 1997). Controversial data also exist on the presence

and density of voltage-dependent Na⁺ channels in chemoreceptor cells. Na⁺ channels are present in high density in adult rabbit chemoreceptor cells where they can generate repetitive action potentials of large amplitude (Lopez-Lopez et al., 1989) and play a role in the genesis of chemoreceptor cell Ca²⁺ signal and neurotransmitter release responses during hypoxic stimulation, as their inhibition by TTX caused a very significant reduction in the release of CA during hypoxic stimulation (Rocher et al., 1994). In rat chemoreceptor cells the situation regarding Na⁺ channels is controversial. According to Fieber and McCleskey, (1993) and Peers (1994) freshly dissociated rat chemoreceptor cells lack Na⁺ channels, yet Stea et al. (1992) in long term cultured cells found that all of them express Na⁺ currents albeit at low density; finally Lopez-Lopez et al. (1997) and Hempleman (1995) found that Na⁺ channels (currents) are present in only a small percentage of short-term cultured cells (see Lopez-Lopez and Peers, 1997). In addition, in the studies of Hempleman (1995) and Stea et al. (1992) it was shown that sustained hypoxia produced upregulation of Na⁺ channels. However, there are major experimental differences that render difficult to envision the functional significance of their findings. In the study of Hempleman (1995) hypoxia meant rats pups hypoxic from second day of conception until use (5-8 days of age) and in the study of Stea et al. (1992) hypoxia meant chemoreceptor cells from normoxic pups (5-12 days of age) cultured in a 6% O₂ atmosphere for 13 days, aiming to mimic postnatal exposure to hypoxia. To our knowledge there are not data available on the functionality of the CB in neonatal rats treated with Hempleman's (1995) protocol, but it is well known that if neonatal animals are kept in a hypoxic atmosphere, the CB does not mature, being its response to hypoxia drastically

blunted or absent (Eden and Hanson, 1987; Sterni et al., 1999). The paradox resides in the fact that there is an apparent upregulation of Na⁺ channels in a situation where the function of the CB is down regulated (Hempleman's data) and in a different situation that intends to mimic upregulation of the CB function (Stea et al.'s data). Thus, although there is an apparent agreement (the induction of Na⁺ channels with sustained hypoxia), the observations are difficult to integrate with the known functional behavior of the entire CB.

Voltage-gated sodium channels (Na_v) are large glycoproteins consisting of three subunits, one α subunit and two β subunits (β 1 and β 2). The α subunit ($\approx$ 220 kDa) forms the voltage-gated sodium selective aqueous pore and the β subunits modify channel properties and interact with cytoskeletal and extracellular matrix proteins (Catterall, 2000). To date, at least nine different α subunits genes have been identified in mammals, SCN1-11 (Goldin et al., 2000; Ogata and Ohishi, 2002), with different channel isoforms exhibiting specific developmental, tissue and cellular distributions, and associated pathologies (Mandel, 1992; Barchi, 1995). Na⁺ channel isoforms have characteristic kinetics, voltage-dependency and pharmacological properties (Smith and Goldin, 1998; Cummins et al., 2001) and can be pharmacologically distinguished by their sensitivity to TTX: most sodium channels are blocked by low concentrations of TTX and are defined as TTX-sensitive (TTX-s) while others are resistant to TTX and are defined as TTX-resistant channels (TTX-r) (Hille, 2001).

Controversies regarding the expression and functional significance of Na⁺ channels in rat chemoreceptor cells using electrophysiological approaches and absence of data in *in vivo* or intact CB preparations prompted the present

study using intact CB preparations and a wide fan of neurochemical and molecular approaches. We have used RT-PCR, immunoblotting, and immunocytochemical techniques to assess the presence of Na⁺ channels in the CB, to define their molecular identity and to locate them in chemoreceptor cells from normoxic and chronically hypoxic adult rats. Additionally, we have used neurochemical and pharmacological techniques to define the functional significance of Na⁺ channels in the neurotransmitter release response to hypoxia from chemoreceptor cells in a preparation of intact CB. We have found the expression of five α subunits isoforms (Na_v1.1, Na_v1.2, Na_v1.3, Na_v1.6 and Na_v1.7) in the rat CB, two of them, Na_v1.1 and Na_v1.3 being inducible by chronic hypoxia. Na⁺ channel proteins are expressed in all chemoreceptor cells because they co-localize with TH. In intact CBs from normoxic and chronically hypoxic rats, veratridine (a Na⁺ channel activator) elicited a Na⁺ and Ca²⁺-dependent and TTX-sensitive release of CA from chemoreceptor cells, and further TTX significantly reduced the release of CA elicited by hypoxia in both normoxic and chronically hypoxic CBs.

METHODS

Exposure to Chronic Hypoxia and Surgical Procedures. Adult female Wistar rats (body wt 250-300 g) were used in all experiments. A group of 24 rats was sequentially introduced and kept 7 days in a glass chamber continuously fluxed with a gas mixture (10 -11% O₂ in N₂; PO₂ ≈80 mmHg). Accumulation of CO₂ was prevented by the continuous flow of the gas mixtures and the presence in the chamber floor of a layer of soda lime. The rats had free access to food and water, and remained in this atmosphere except for 30 min/3 days

for routine cleaning and maintenance. At day 7 a group of six animals was removed to isolate their CBs simultaneously to a control group (six normoxic rats at 20% O₂ in N₂ in the same environment). Control and chronically hypoxic rats were anesthetized with sodium pentobarbitone (60 mg/Kg body wt; i.p.), the carotid bifurcations excised and the carotid bodies cleaned of surrounding tissue under dissecting microscope in a Lucite chamber filled with ice-cold Tyrode solution (in mM: 140 NaCl, 5 KCl, 2 CaCl₂, 1.1 MgCl₂, 5 glucose and 10 HEPES) adjusted to pH 7.4. All of our efforts were made to minimize animal stress and to reduce to a minimum the number of animals used. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Valladolid.

[³H]-CA synthesis and measurement of labeled CA release. The endogenous deposits of CA of CBs were isotopically labeled by incubating the organs in a medium containing 30 μM 3,5-[³H]tyrosine (48 Ci/mmol s.a.), 1mM ascorbic acid, and 100μM DL-6-methyl-5,6,7,8-tetrahydropterine as cofactors for dopamine-β-hydroxylase and TH, respectively. After labeling, individual CBs were placed in vials containing 4 ml of solution (in mM: NaCl, 116; NaHCO₃, 24; KCl, 5; CaCl₂, 2; MgCl₂, 1.1; Hepes, 10; glucose, 5; pH 7.4) equilibrated with 20%O₂ / 5%CO₂, rest N₂. CBs were kept in a shaker bath at 37 °C for the rest of the experiment. Solutions were continuously bubbled with the above gas mixture saturated with water vapor, except when hypoxia was applied as stimulus. The CB superfusing solutions were renewed every 10 min and analyzed for their content in [³H]-CA by adsorption to alumina and posterior counting of the eluates in a scintillation spectrometer. Specific protocols for hypoxic stimulations as well as for drug applications are provided in the Results

sections. In most of the experiments control and experimental CBs were stimulated twice (stimulus 1 and 2; S1 and S2) with any given stimulus, but in the experimental CBs the second application of the stimulus included an additional variable (usually the presence of a drug). The evaluation of the effect of the drug was assessed by comparing the ratios of the amplitude of the release responses in experimental CBs (S1/S2) with that obtained in control organs. The amplitude of the release response for any given stimulus was quantified as the [^3H]-CA released (dpm) above basal conditions; dividing the stimulus induced dpm released by the total dpm present in the tissue allowed normalization of the release data as fractional release to minimize variability due mostly to organ size. Data are presented as means $\pm$ SEM and statistical significance of the observed differences was assessed by the Student's *t* test for unpaired data.

In selected experiments we performed HPLC-ED analysis of the alumina eluates to identify chemically the ^3H -labelled released material. The alumina eluates were concentrated to dryness in a vacuum concentrator and resuspended in 100 μl of mobile phase (in mM: 10 NaH_2PO_4 , 0.6 sodium octane sulfonate, and 0.1 EDTA, with 16% methanol, pH 3.2-3.6) containing 0.2 nmol of unlabeled CA as an internal standard. Aliquots of 10-45 μl were injected into the HPLC system and fractions of the column effluent were collected every minute and counted in the liquid scintillation counter.

Western Blot analysis. For analysis of $\text{Na}_v \alpha$ subunit expression, frozen CBs from normoxic and hypoxic rats were homogenized in lysis buffer containing detergents and protease inhibitors (Triton X-100, 1%; sodium cholate, 1%; SDS, 0.1%; trypsin inhibitor, leupeptin, aprotinin, and pepstatin 1

$\mu\text{g/ml}$; and Na_3VO_4 1mM). All procedures were performed on ice, and centrifugation was performed at 4°C. The solubilized proteins were mixed and boiled in 2 x sample buffer (0.125 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 10% 2-mercaptoethanol and 0.02% bromophenol blue). Protein suspensions were electrophoretically fractionated on 8% SDS-polyacrylamide gels and protein bands transferred to PVDF membranes by electroblotting in a Mini Trans-blot cell transfer apparatus (Bio-Rad, CA) under standard procedures. The membranes were blocked in 5% non-fat dry milk in TBS (10mM Tris-HCl, 150mM NaCl, pH 7.5) and then incubated for 2 h at room temperature in primary antibody solutions: polyclonal anti-pan $\alpha\text{-Na}_v$ (dilution 1:250; Chemicon) and monoclonal anti β -actin (dilution 1:5000; Abcam). Membranes were reincubated with secondary HRP-conjugated anti-rabbit IgG (1:10000; Amersham) and anti-mouse IgG (1:10000; Amersham), respectively. Signals were visualized by enhanced chemiluminescence (ECL, Amersham). The density of bands on Western blots was quantified by a PDI scanner and Kodak 1D Image Analysis software. Optical densities of Na_v signals were compared and normalized with β -actin signals (bands in the $\approx$ 45 kDa region) to assess equal protein loading. Normalized values were then averaged for all the replicated gels and used to calculate the relative change of hypoxic to normoxic values of the same gel.

Immunohistochemistry and Immunocytochemistry. To identify the cells expressing Na_v channels, double immunolabeling using TH as chemoreceptor cell marker was performed in both, CB sections and freshly dissociated chemoreceptor cells. For immunohistochemistry studies, anesthetized rats were perfused by gravity (100 cm) through a thin nylon tube

inserted into the left ventricle with 0.01M heparinized phosphate-buffered saline (PBS), followed by freshly prepared fixative solution (4% paraformaldehyde in 0.1M PB). The pair of carotid bodies was then removed under a dissecting microscope and immersed in the same fixative for an additional 6-8h at 4°C. The CBs were transferred to 30% sucrose in PBS at 4°C for 24 h. Each CB was serially cut at 10 μ m sections on a cryostat, mounted in slides and blocked for 30 min in blocking solution (0.01M PBS containing 2% normal goat serum and 0.1% Triton X-100). Sections were simultaneously incubated overnight (4 °C) with rabbit polyclonal antibodies to pan α subunit of Na_v channel, directed against a conserved epitope that recognizes all vertebrate Na_v1 isoforms, (1:50; Chemicon) and mouse monoclonal anti-TH (TH 1:1000; Abcam). Then, sections were washed with PBS and reincubated with Alexa 594-conjugated goat anti-rabbit (1:2000) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (1:1000) for 2h. After washed in PBS and distilled water, coverslips were mounted in a photobleaching protective medium (Vectashield H-1000, Vector Laboratories) and the sections were examined with the appropriated filters for immunofluorescence. Incubation without primary or secondary antibodies yielded only background levels of signal (data not shown). Counterstaining with cresyl violet was used to reveal the general histology.

A similar protocol was used in freshly dissociated CB cells plated onto poli-L-lysine-treated glass coverslips, obtained by previously described methods (Lopez-Lopez et al., 1997). Cell cultures were washed in PBS and fixed by 4% paraformaldehyde in 0.1M PB solution for 10 min at room temperature. After additional washes (3x10 min) with PBS, cells were incubated in blocking solution: PBS containing 2% normal goat serum and 0.1% Triton X-100 at room

temperature for 30 min and then overnight (4°C) incubated simultaneously with primary rabbit polyclonal antibodies to pan Na_v channel α subunit or against either the specific α subunit type 1.1, 1.3 or 1.6 (dilutions 1:50) and monoclonal antibody against TH (1:1000). Finally, the Na channels α subunits and TH were visualized by incubation with Alexa 594-conjugated goat anti-rabbit IgG antibody and FITC-conjugated goat anti-mouse IgG antibody, respectively. Incubation without primary antibodies yielded only background levels of signal (data not shown). To confirm specificity of the antibodies, control experiments were performed where the primary antibodies were preincubated for 120 min with antigenic peptides. After this step cells were washed and incubated with secondary antibodies conjugated with fluorescent probes. Coverslips were mounted on glass slides as above described. Sections and cells were photographed by fluorescence microscopy with appropriated filters, using a Zeiss Axioscop 2 (*not plus*) microscope equipped with a digital camera (CoolSnap cf) and analyzed with Metamorph 6.3 software. To measure the fluorescence intensity for each pair of preparations (e.g., normoxic TH vs. hypoxic TH) the exposure time and the gain of the camera for the acquisition of images was identical. Only images with similar backgrounds were used. Labelled chemoreceptor cells were selected and average pixel intensity for each cell was computed. Data from all the cells in the acquired images were averaged to obtain the Average Pixel Intensity. The analysis of fluorescence intensity for the Na_v was identically performed for each pair of preparations (e.g., Na_v1.1 normoxic vs. Na_v1.1 hypoxic), but to restrict our analysis to TH positive cells we had open the correspondent image stained for the enzyme and only the TH positive cells were computed.

RNA extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from frozen CBs using the one-step Trizol® method (Gibco, BRL) following manufacturer's protocol. During the retrieval and RNA extraction procedures, precautions were taken to prevent RNase activity. Total RNA samples (from individual CB) were reverse transcribed into cDNA using MultiScribe reverse transcriptase (MuLV; Applied Biosystems) and random hexamers, following standard protocols. Aliquots of first-strand cDNA (4µl) were amplified by PCR in a 20 µl reaction mix containing buffer, dNTPs, MgCl₂, Taq-polymerase and primers at appropriated concentrations. Amplification was carried out using Gold Taq polymerase and the protocol involved an initial denaturation at 95°C for 2 min to activate Taq, followed by cycles of denaturing at 95°C for 30s, annealing at 60°C for 30s and extension at 72°C for 1 min. Gene specific sense and antisense primers for six Na_v genes (SCN1A, 2A, 3A, 4A, 8A and 9A) and β-actin were designed using Primer Express software (Applied Biosystem; see Table I). PCR reactions were carried out in a Perkin Elmer 9700 thermocycler (Applied Biosystem). PCR products were collected at the number of cycles before reaching saturation (30 cycles) and analyzed on 1% agarose gels containing ethidium bromide under UV light. Predicted products sizes were assessed by comparison with a DNA molecular weight marker (100 bp ladder; BioRad, Spain) that was routinely run on the gels. Controls without reverse transcriptase were used to demonstrate the absence of contaminating DNA. Parallel experiments were performed for equal amounts of total RNA collected from CBs of normoxic or hypoxic rats. The quantification of the change in Na_v channel expression after prolonged exposure to hypoxia was performed by image analysis. The intensity of the

ethidium bromide fluorescence was measured, corrected by housekeeping gene signal (β -actin) and reported in arbitrary units with respect to that of the controls (set as 100). Prior to the experiments with CB mRNA, the designed primers were tested in brain tissue mRNA to check their adequacy.

Measurement of ventilation by whole-body plethysmography.

Ventilation was measured in conscious freely-moving rats by whole body plethysmography. The system (Emka Technologies, Paris, France) consists of 5 liters metacrylate chambers continuously fluxed (2 l/min) with temperature being maintained in the chamber within the thermo-neutral range (22-24°C). Tidal volume (V_T ; ml/Kg) respiratory frequency (f ; breaths/min) and minute ventilation (V_E ; ml/min/Kg) were measured. Briefly, the rats were placed in the plethysmographic chamber and breathed room air for at least 30 min until adapted to the chamber ambient and they acquired a standard resting behavior. Thereafter we started recording of the normoxic ventilatory parameters during 20 min, followed by fluxing of the chamber with a gas mixture containing 7% O_2 (rest N_2 ; 2 l/min) for an additional period of 20 min and recording of ventilation. The pressure change within the chamber reflecting tidal volume (V_T) was measured with a high-gain differential pressure transducer. Ideally the frequency of pressure fluctuations is identical to breathing movements; spurious fluctuations of the pressure due to animal movements were electronically rejected. The amplitude of the pressure oscillations is proportionally related to V_T ; a calibration of the system by injections of 0.2 to 0.5 ml air into the chamber allowed a direct estimation of V_T . Pressure signals were fed to a computer for visualization and storage for later analysis with EMKA software.

Drugs and chemicals. All chemicals were of analytical grade and were obtained from Sigma (Sigma-Aldrich, Spain) except for TTX, purchased from Tocris (Tocris Bioscience, UK). The primary antibodies were obtained from Chemicon (for Na_v channels; the antigenic epitopes are available in the web of the company) or AbCam (for TH the monoclonal antibody was grown against full length native protein purified from rat pheochromocytoma) and the secondary HPR-conjugated and fluorescent antibodies were from Amersham and Molecular Probes, respectively. Antibodies were dissolved in deionized water, aliquoted and kept at –20°C until use.

Statistics and data analysis. All data are expressed as mean values ± standard error of the mean (SEM) for the number of cells analyzed (n). Statistical significance was calculated using unpaired Student's *t* test. Differences were considered to be significant at *p* < 0.05

RESULTS

Evidence for the presence of functional Na⁺ channels in rat CB chemoreceptor cells.

a) Effects of veratridine on the release of ³H-CA from intact rat CB.

Veratridine is an activator of voltage-sensitive sodium channels that holds the channels open even at resting membrane potential (Hille, 2001), and therefore causes cell depolarization, voltage-dependent Ca²⁺ channel activation and triggering of Ca²⁺-dependent responses such as the release of neurotransmitters (Rubin, 1982). Incubation of intact CBs in normoxic medium with 50 μM veratridine during 20 min produced an increase of the release of ³H-CA with the time course shown in left histogram block of **Figure 1A**. Evoked

release (above dotted line) amounted to 6350 dpm which represents $\approx 7\%$ of ^3H -CA content in the CB. On removal of veratridine, the release of ^3H -CA declined slowly toward control values within the next 40 min. In the right histogram block of **Figure 1A** it is evidenced that veratridine effect was nearly fully reversed by the sodium channel blocker TTX ($1\ \mu\text{M}$); the small increase in the release of ^3H -CA seen on simultaneous removal of veratridine and TTX from the incubating medium, probably reflects different washout time courses of the drug from the tissue. Identification of the ^3H -catechols released by HPLC showed that DA, together with its catabolite DOPAC, amounted to nearly 90% of all released catechols (Table II), indicating that the ^3H -catechols released came mainly from chemoreceptor cells, since intraglomerular sympathetic endings synthesize only norepinephrine. Following the protocol shown in Figure 1A we tested the effect of veratridine in $0\ \text{Na}^+$ and in $0\ \text{Ca}^{2+}$ media, and the results are summarized in **Figure 1B**. Findings evidence that the ability of veratridine to elicit release of neurotransmitters was inhibited by more than 85% in Na^+ or Ca^{2+} -free solutions or in the presence of TTX, indicating that Na^+ entry through TTX-sensitive voltage-gated Na^+ channels, does indeed cause cell depolarization, calcium influx through voltage-dependent calcium channels and exocytotic release.

b) TTX inhibits the release of ^3H -CA elicited by hypoxia. The potential participation and quantitative contribution of Na^+ channels in the release of ^3H -CA induced by hypoxia was studied using the protocol shown in **Figure 2A**. Control CBs were low PO_2 stimulated twice by incubating the organs during 10 min in solutions equilibrated with 7% O_2 ($\text{PO}_2 \approx 46\ \text{mmHg}$) and experimental CBs were similarly stimulated but the 10 min prior to the second hypoxic

stimulation and the 10 min period of this second stimulus the incubating solution contained in addition 1 μ M TTX. Data indicate that TTX inhibited the release of 3 H-CA elicited by hypoxia (defined by the areas delimited by the curves and corresponding dotted lines); this inhibition was also observed with other intensities of hypoxic stimulation (10% O₂ equilibrated solution, PO₂ $\approx$ 66 mmHg; 5% O₂ equilibrated solution, PO₂ $\approx$ 33 mmHg). **Figure 2B** shows mean S2/S1 ratios obtained for the three hypoxic intensities in control and experimental CBs; in experimental CBs the second hypoxic stimulus was applied in the presence of TTX. The Na⁺ channel blocker inhibited by nearly 50% the release response elicited by the less intense hypoxia and around 30% the response elicited by the other two hypoxic stimuli. Increasing TTX concentration to 5 μ M did not augment the degree of inhibition (data not shown). The magnitude of TTX inhibition of the release response elicited by hypoxia is nearly identical to that previously observed for the same stimulus in the rabbit CB (Rocher et al., 1994) and to that found by Kidokoro and Richie (1980) in the embryologically-related rat adrenomedullary chromaffin cells in response to high external K⁺ and ACh. In agreement with the interpretation given by those authors, present findings would indicate that voltage dependent Na⁺ channels provide an amplification mechanism to the response elicited by hypoxia, with an apparently greater significance at low levels of hypoxic stimulation.

Demonstrated the presence of functional Na⁺ channels in chemoreceptor cells and their participation in the release of neurotransmitters elicited by hypoxia we decided to identify the isoform of channel(s) expressed in the cells, the distribution of the channels among chemoreceptor cells and the effect of in vivo chronic hypoxia on the Na⁺ channel expression and the participation of the

channels in the release response to hypoxia in CBs obtained from chronically hypoxic rats. In the paragraphs that follow are presented the findings.

Protein expression of voltage-gated Na⁺ channel and hypoxia-induced rise, evaluated by Western Blot in rat CBs. Western blots were carried out using protein extracted from CBs removed from control and chronically hypoxic (10-11% O₂, 7 days) rats. **Figure 3A and B** show representative bands from Western blot analyses of Na⁺ channel protein expression in CBs (usually 1 CB per lane) and superior cervical ganglia (usually 1/10 of SCG per lane). Tissue homogenates were fractionated on 8% SDS-polyacrylamide gel and immunoblot assayed with a Na⁺ channel α subunit antibody (anti-pan Na_v) demonstrated a single protein band of the expected molecular weight ($\approx$ 220 kDa) in CBs and SCG from control and chronically hypoxic rats. The band was less intense in the control normoxic (Figure 3B, lanes 1-3) than in the chronically hypoxic CBs (Figure 3B, lanes 4-6), suggesting that chronic hypoxia in vivo causes an up regulation of the expression of the Na⁺ channels. Such up regulation was not noticeable in the SCG. The density of β -actin band also appear to increase, an expected finding due to the hypertrophy of the CB during hypoxia. A semiquantitative analysis was performed in 12 control and 12 chronically hypoxic CBs defining the ratio of densities of Na_v band to the correspondent β -actin band. **Figure 3C** shows that taking the mean ratio of control CBs as unit, the ratio for chronically hypoxic CBs rose to 1.57 ± 0.08 ($p < 0.001$). Immunoblots of rat CB proteins were also probed with anti-Na_v1.1, Na_v1.3 and Na_v1.6 specific antibodies but only Na_v1.6 showed up as a faint signal due to lower abundance of individual channels than that of the general population of CB sodium channels labelled by a pan-specific antibody.

RT-PCR identification of α subunit Na_v gene expression in normoxic and chronically hypoxic rat CBs. To determine the molecular identity of the Na^+ channel isoforms present in rat CB, expression of member genes of the SCN channel family was investigated by RT-PCR using unique primers designed from rat sequences (see Table I). The specificity of the custom designed primers for SCN1A ($\text{Na}_v1.1$), SCN2A ($\text{Na}_v1.2$), SCN3A ($\text{Na}_v1.3$), SCN4A ($\text{Na}_v1.4$), SCN8A ($\text{Na}_v1.6$), SCN9A ($\text{Na}_v1.7$) were tested on tissues previously shown to express these isoforms (brain and superior cervical ganglia). As I_{Na} in rat chemoreceptor cells both from neonatal and adult rats is fully blocked by TTX (Stea et al., 1992; Lopez-Lopez et al., 1997) and because veratridine opens TTX-sensitive and TTX-resistant Na_v and TTX blocked nearly completely veratridine effect (see Figure 1) we excluded SCN5A ($\text{Na}_v1.5$), SCN10A ($\text{Na}_v1.8$) and SCN11A ($\text{Na}_v1.9$) in our study in the CB because they are TTX resistant, and SCN7A (Na_x) since it has been shown that does not form a functional Na channel (Ogata and Ohishi, 2002). Total RNA extracted from the rat CB and SCG, was reverse transcribed into cDNA, PCR amplified, and amplicons were visualized by ethidium bromide in agarose gels. **Figure 4A** shows a representative agarose gel electrophoresis of the six TTX-sensitive α subunits Na_v for a control CB. **Figure 4B** shows representative gels obtained from 2 control and 2 experimental CBs; the two control CBs were obtained from two different rats and the 2 experimental from 2 chronically hypoxic rats. The figure also includes a gel for β -actin, as a standard housekeeping gene, obtained from the same CBs and a gel for TH obtained from different couples of control and experimental CBs. In PCR amplified products corresponding to 1/10 of CB cDNA we observed fragments of predicted sizes (~400-500 bp) for all α -

Na_v transcripts (except Na_v1.4, a skeletal muscle specific channel), β -actin (327 bp) and TH (234 bp) in all lanes; a high level of up regulation of the rate limiting enzyme in CA synthesis was clearly noticeable in all samples from chronically hypoxic rats. From Figures **4A and 4B** it is evident that Na_v1.1, Na_v1.3 and Na_v1.6 are the best amplified; it is also apparent that Na_v1.1 and Na_v1.3 are up regulated in chronic hypoxia. Indeed, when the ratio of the densities of the Na_v to β -actin bands was obtained we found a nearly 80% increase for Na_v1.1 and a 40% increase for Na_v1.3 with no change in the expression of Na_v1.2, Na_v1.6 and Na_v1.7 (**Figure 4C**)

Immunostaining of CB sections for TH and Na_v channels and cresyl violet counterstaining. To determine the localization of protein expression of Na_v channels in individual cell types present in the rat CB, immunohistochemical studies were performed using specific antibodies to sodium channels (PanNa_v that recognizes all α subunit isoforms of neuronal Na channels), and TH, the chemoreceptor cell specific marker. **Figure 5** shows sections obtained from a control (upper row) and a chronically hypoxic CB (lower row). In both instances and in all sections it can be appreciated the typical histological traits of the CB: a very important area of the section occupied by empty spaces that represent blood vessels, mostly capillaries, and the parenchymatous tissue forming clusters around the vessels. By comparing the cresyl violet counterstained sections it is evident the higher density of capillaries in the section of the chronically hypoxic CB, which in turn produces a better defined image of the cell clusters, evidenced in the middle sections stained for TH. Induction of TH in chronically hypoxic CBs contributes to make clusters more conspicuous. Some of the filamentous TH-positive images seen in the periphery of the sections

might represent sympathetic endings which run in the capsule of the CB. In the sections stained for Na_v the same clustering is clearly noticeable, indicating that the vast majority (if not all) of TH-positive chemoreceptor cells express Na⁺ channels, but in addition, other cell types also are positive for Na⁺ channels and therefore the sharp images of the clusters is less clear than in the sections stained for TH; nonetheless, as it was the case for TH, the clustering is most evident in the section obtained from the chronically hypoxic CB due to the up regulation of Na⁺ channels.

Localization of Na⁺ channel isoforms in rat chemoreceptor cells in culture. To identify if chemoreceptor cells express different Na_v isoforms in rat CB, we performed immunocytochemical experiments with antibodies pan-Na_v and specific for Na_v1.1, Na_v1.3 and Na_v1.6 in dissociated cells in conjunction with antibodies to TH as a specific marker of chemoreceptor cells. Findings are presented in **Figure 6**. We have chosen images from cultures in which cells were completely dissociated and cultures in which cell cluster were more evident. In the left half of Figure 6 are shown images obtained from different cultures of dissociated CBs isolated from control normoxic animals, evidencing that the staining with antibodies to the Na⁺ channels is more extensive than the staining with the TH antibody, i.e. there are non-chemoreceptor cells (probably smooth muscle cells) that express Na⁺ channels, existing also cells negative to Na⁺ channels. On the contrary, we did not find any TH-positive cells that were negative to any Na⁺ channel antibody. The right half of the Figure shows images obtained from four different cultures prepared with CB from chronically hypoxic rats. As it was the case with normoxic cultures, all TH-positive cells were also positive to the Na⁺-channel antibodies, and there were many cells

that being positive to Na⁺-channel antibodies were TH-negative. Blanks obtained by carrying the reaction in the absence of primary antibody or with preabsorbed antibody yield black images (not shown). In a new group of experiments we have quantified the intensity of the fluorescence emitted by the normoxic and hypoxic preparations by selecting exposure times that provided an adequate level of signal in the normoxic preparations stained for each antibody. The intensity of the fluorescence signal on preparations stained with Na_v antibodies was quantified exclusively in TH⁺ cells. The data are presented in Table III evidencing that the fluorescence for TH in chemoreceptor cells from hypoxic rats increased a factor of 2.5 (p<0.001). In comparison to chemoreceptor cells obtained from normoxic animals, the intensities of fluorescence in cells obtained from hypoxic CBs were 1.38 (p<0.001) for the pan-Na_v antibody and 1.18 (p<0.001) for Na_v1.1 antibody. For Na_v1.3 antibody the fluorescence signal was low both, in normoxic and in hypoxic cells, being 1.06 times greater in hypoxic than in the control cells (p>0.05). For Na_v1.6 the fluorescence signal was even lower and no attempts were made to quantify its intensity because there were not differences at the mRNA level.

Functional role of sodium channels in CB chemoreceptor cells from chronically hypoxic rats. To ascertain that modulation of Na⁺ channel expression is at the basis of the increased excitability during acute O₂ deprivation, we used further the in vitro whole CB preparation from chronically hypoxic animals and applied veratridine or acute hypoxia in absence or presence of TTX to block Na⁺ channels. **Figure 7A** shows the mean evoked release of ³H-CA elicited by 50μM veratridine in the absence and in the presence of 1μM TTX in groups of CBs obtained from control and chronically

hypoxic rats. As in figures 1 and 2 the evoked ^3H -CA release was defined as release above basal and expressed as total dpm. Hypoxic CBs show an increased response to veratridine indicating that induced sodium channels in chronically hypoxic CBs are functional channels. However, taking into account that ^3H -CA content in hypoxic CBs was enhanced by $\approx 400\%$ (159785 ± 9105 vs. 42250 ± 5800 dpm; $n = 10$ in both cases), there is not a significant difference in the response to veratridine when normalized by ^3H -CA tissue content. In both control and chronically hypoxic CBs, the effect of veratridine was almost totally abolished by TTX ($p < 0.001$). **Figure 7B** shows that $1\mu\text{M}$ TTX inhibited the release induced by mild and moderate hypoxia (incubation in 7% O_2 and 10% O_2 -equilibrated media) in chronically hypoxic CBs in percentages comparable to those seen in control organs (see Figure 2B), but absolute amount released was much greater in the CBs of chronically hypoxic rats (9137 ± 1156 vs. 2262 ± 685 dpm in controls) and therefore the TTX-sensitive release amounted to 678 dpm in control animals and to 2714 dpm in chronically hypoxic CBs.

Ventilatory responses to acute hypoxic tests in control and chronically hypoxic rats. In a final group of experiments we verified that our protocol of chronic hypoxic exposure does indeed produce sensitization of the overall chemoreception process. Using whole body plethysmography we measured basal ventilation (20% O_2 atmosphere) as well as ventilatory responses to acute hypoxic tests (7% O_2 , 20 min). **Figure 8** shows that ventilatory frequency both in normoxia (73.03 ± 2.47 vs. 74.39 ± 3.14 breaths/min; $n = 12$) and hypoxia (112.30 ± 3.86 vs. 114.04 ± 3.35 breaths/min; $n = 12$) was indistinguishable in control and chronic hypoxic rats. However, the tidal volume was significantly

higher in chronic hypoxic than in control rats both, when breathing room air (9.89 ± 0.51 vs. 7.77 ± 0.29 ml/kg; $p < 0.001$; $n = 12$) and during the hypoxic test (14.15 ± 0.57 vs. 10.66 ± 0.48 ml/kg; $p < 0.001$; $n = 12$). The net result was a very significant increase in minute ventilation in the chronic hypoxic animals, that represented a gain of the chemoreflex in chronic hypoxic animals that was a 35% greater than in control animals.

DISCUSSION

In the present study we demonstrate that adult rat chemoreceptor cells express functional Na^+ channels, because their activation with veratridine promotes a neurotransmitter release response that is Na^+ and Ca^{2+} dependent and TTX sensitive (Figure 1); Na^+ channels participate in the activation of chemoreceptor cells produced by hypoxia, as TTX inhibits partially the release of ^3H -CA induced by hypoxia (Figure 2). Western blot and RT-PCR findings show that the CB expresses five isoforms of TTX-sensitive Na^+ channels, and out of them $\text{Na}_v1.1$ and $\text{Na}_v1.3$ where up regulated in the CBs of rats exposed during 7 days to a hypoxic atmosphere (Figures 3 and 4). Immunocytochemically, we have obtained evidence showing that voltage-gated Na^+ channels are expressed in the entire population of adult rat chemoreceptor cells and confirmed the up regulation by chronic hypoxia of the Na_v as a whole, and specifically of the $\text{Na}_v 1.1$ isoform, observing in addition other non-identified cell types in the CB that are positive to Na^+ channel antibodies (Figures 5 and 6). We demonstrate that TTX-sensitive Na^+ channels are functional in chronically hypoxic CBs contributing to the genesis of the release response elicited by hypoxia in a percentage comparable to that seen in control normoxic

CBs but much greater in absolute terms (Figure 7). Finally, we demonstrate that indeed our protocol of chronic hypoxic exposure produces sensitization of the CB to acute tests of hypoxia (Figure 8)

At the outset of the Discussion we want to state unambiguously that we have used the release of CA as a “marker” of the activity of chemoreceptor cells, without any further implication on the possible functional significance of these biogenic amines. As shown in Table II, DA, which is specifically located in chemoreceptor cells (NE is also located in sympathetic endings; Gonzalez et al., 1994), represents nearly 90% of the total CA released, and therefore the release of CA directly reflects the degree of activation of these cells as indeed it has been shown in intact preparations of the CB (see Gonzalez et al., 1992), in isolated chemoreceptor cells (Montoro et al., 1996; Jackson and Nurse, 1997) and in CB slices (Ortega Saenz et al., 2003). In fact, data presented in this article indicate once again that sensitization of the CB chemoreceptor reflex (Figure 8) is associated with a higher dopaminergic activity of chemoreceptor cells. We also want to state that we have undertaken the molecular approach to study Na⁺ channels in rat chemoreceptor cells because there is no other study of this nature in the CB, and because we considered that techniques alternative to electrophysiology should be used to solve the controversies that have emerged in electrophysiological studies (see Introduction).

In the context of current literature there are several aspects of our findings that deserve further commentary, namely, the diversity of Na⁺ channels isoforms present in rat chemoreceptor cells, the correspondence of our findings with priorly published electrophysiological data on Na⁺ channels and finally the

functional significance of our data, both in normoxia and chronically hypoxic animals.

The rat CB expresses 5 TTX-sensitive Na_v isoforms (SCN1A, SCN2A, SCN3A, SCN8A and SCN9A) as they can be detected by RT-PCR in total RNA extracted from the intact organ. The presence of TTX resistant channels was not explored because recorded Na⁺ currents in rat chemoreceptor cells are sensitive to TTX (e.g., Stea et al., 1992; Lopez-Lopez et al., 1997) and because, as shown in this study, the release ³H-CA elicited by veratridine was fully inhibited by TTX. In immunocytochemical (frozen sections) and Western blot (whole CB homogenate) experiments carried out with a polyclonal antibody capable of reacting with a sequence common to all Na_v α subunit, we provide unequivocal evidence for the presence of the conducting subunit of Na⁺ channels in the TH-positive chemoreceptor cell clusters of the CB, showing further that chronic hypoxia augments their expression. Western blots with antibodies to specific Na⁺ channel isoforms did not provide satisfactory bands to each isoform due to the low abundance of the singled channel protein, but immunocytochemistry carried out in fresh cultured dissociated CB cells indicates that the α subunits Na_v1.1, Na_v1.3 and Na_v1.6 are indeed expressed in all TH-positive cells, existing in addition other cells in the organ that express one or more of these isoforms. Quantitatively, and consistent with the PCR studies, the intensity of the immunoreactivity for Na_v1.1 was significantly augmented in chemoreceptor cells obtained from CBs of chronically hypoxic rats with minor increases in the Na_v1.3 isoform. In the central nervous system Na_v1.1 and Na_v1.3 channels are expressed widely being preferentially located in the soma and dendrites of the neurons, while Nav1.6 located in axons and terminals

seem to play a major role in the conduction of neural impulses and in the regulation of neurotransmitter release (Caldwell et al., 2000). In general, differential location of isoforms of Na^+_v suggests that each isoform permits local regulation of electrical excitability (Mandel, 1992; Caldwell et al., 2000). In the case of chemoreceptor cells that are round or ovoid, although occasionally they form finger-like processes (Nishi and Stensaas, 1974; McDonald, 1981), is more difficult to postulate differential regulation of excitability. Why, then, so many different types of α subunits present in rat CB chemoreceptor cells? We can speculate that the diversity might be related to the specific regulation of the expression of different isoforms. We can hypothesize that $\text{Na}_v1.6$ would be constitutively expressed, with the other isoforms, particularly $\text{Na}_v1.1$, being expressed on physiological demand such as in chronic hypoxia and therefore be contributing to the sensitization and acclimatization-adaptation to chronic hypoxia (Stea et al., 1992; Bisgard and Neubauer, 1995). It has been shown that some K^+ channel subtypes are also regulated by chronic hypoxia, this regulation been related to the sensitization-adaptation to sustained hypoxia (Wyatt et al., 1995; Kaab et al., 2005).

In comparing our findings with prior literature in the rat CB we are forced to limit our considerations to electrophysiological data, as the present work is the first study aimed to identify functionally and molecularly Na^+ channels in this species. However, Na^+ currents have not been invariably found in rat chemoreceptor cells (see Introduction). The reported absence, limited or universal expression of Na^+ channels (currents) in chemoreceptor cells of this species needs an explanation at the light of our present findings showing that all chemoreceptor cells express the channels. We envision two major potential

causes for the discrepancies between previously published data; first, the age of the rats used and second, the difference of preparation. In spite of recognized developmental differences in the expression of β subunits of Na^+ channel (Sutkowski and Catterall, 1990) age of the animals does not appear to explain the inconsistencies among the data of the literature and here presented findings: in neonatal (Hempleman, 1995) and adult rats (Lopez-Lopez et al., 1997) only some cells expressed Na^+ currents or none of the cells expressed them (Fieber and McCleskey, 1993), and in young rats all cells (Stea et al., 1992) or no cells (Peers, 1994) expressed Na^+ currents. Second, differences in preparations. In all electrophysiological experiments the CBs were enzymatically dissociated and cells were cultured for different periods of time; yet, no differences in the amplitude of the currents was observed between 3h and 4 days (Hempleman, 1995) or 2 to 15 days (Stea et al., 1992) old cultures. We would suggest that the differences might arise from the enzymatic dispersion protocol, as it has been shown that smooth muscle cells dissociated with two alternative enzymatic protocols exhibit both I_{Na} and I_{Ca} current with one of the protocols and only Ca^{2+} currents with the other (Berra-Romani et al., 2005). Thus, although most studies use a dissociation protocol similar to that described in our laboratory (Perez-Garcia et al., 1992), slight modifications or spurious proteolytic contaminants of different batches of enzymes might be responsible for the different percentages of cells expressing Na^+ currents. It should be noted, however that our laboratory with the same dissociation protocol has recorded Na^+ currents in all chemoreceptor cells of the rabbit (Ureña et al., 1989) and in only $\approx 10\%$ of the cells of the rat; yet, different organ size (rabbit CB is ≈ 7 times larger than the rat organ) and different primary and

spatial structures of the Na^+ channels between the two species might render rabbit Na^+ channel more resistant to proteolytic action. However, these considerations do not account for our observations that all freshly (24-48 hours) dissociated rat chemoreceptor cells express the conducting α subunit of the Na^+ channels. Out of the three membrane spanning subunits of Na^+ channels, the $\beta 2$ subunit can be removed from purified rat brain Na^+ channels without alterations in the properties of the channels, whereas removal of $\beta 1$ subunits causes loss of all functional activities (Messner and Catterall, 1986); similarly in skeletal muscle Na^+ channels removal of $\beta 1$ subunits caused a marked reduction (>60%) of the current amplitude (Nuss et al., 1995). Therefore a proteolytic damage of the $\beta 1$ subunits would satisfactory explain our present results, i.e., immunocytochemical demonstration of the α subunits in all chemoreceptor cells, and might contribute to explain discrepancies among electrophysiological studies.

Last point in our Discussion deals with the physiological significance of our findings. In the experiments presented here in the intact rat CB it is evidenced that Na_v channels in chemoreceptor cells in normoxic CBs are functional, contributing to the neurotransmitter release elicited by natural hypoxic stimulation. Our data imply that Na^+ currents contribute to increase the amplitude of the action potentials, the amount of Ca^{2+} entering the cells and the Ca^{2+} dependent exocytosis of ^3H -CA (Kidokoro and Ritchie, 1980; Gonzalez et al., 1992). It is worth mentioning that some cells obtained from normoxic CBs (e.g. cell in Figure 3 in Buckler and Vaughan-Jones 1994) generate repetitive action potentials with clear overshoots suggestive of a contribution of Na^+ currents in their genesis; higher overshoots are more evident in the action

potentials recorded in chemoreceptor cells cultured in hypoxia (Stea et al., 1995). The increase in the expression of Na_v with chronic hypoxia seen in our *in vivo* experiments conform with the findings *in vitro* of Stea et al. (1992, 1995) and go further in showing, first, that the *in vitro* observations have a correlate in *in vivo* conditions and second, that the amount of neurotransmitter release elicited by Na^+ channel activation with veratridine and hypoxia and sensitive to TTX increases by a factor of nearly 4 times. Thus, although the CB enlarges with chronic hypoxia, most of the organ growth is due to increase in blood vessels volume (Laidler and Kay, 1975) and, therefore, the increased release of neurotransmitters would produce a higher neurotransmitter concentration in the synaptic cleft, which would generate the sensitization of the CB chemoreflex in chronic hypoxia (Figure 8; Bisgard and Neubauer, 1995, Bisgard, 2000; Gonzalez et al., 2003). Thus, the here observed induction of Na^+ channels in chemoreceptor cells would constitute a unitary piece of cellular machinery contributing to match higher neurotransmitter synthesis with higher neurotransmitter utilization rates, and therefore to support the sensitization seen in the CB in sustained hypoxia and evidenced by a higher frequency of action potentials in the CSN, the sensory nerve of the CB, and a higher ventilatory response to acute hypoxic tests (Figure 8; Bisgard 1995; 2000).

In conclusion, our observations indicate that rat chemoreceptor cells express at least three isoforms of TTX-sensitive voltage dependent Na^+ channels constitutively, $\text{Na}_v1.1$, $\text{Na}_v1.3$ and $\text{Na}_v1.6$, with $\text{Na}_v1.1$ channel being induced by sustained hypoxia. Our observations also indicate that sodium channels are functional, contributing to the genesis of the neurotransmitter release response elicited by hypoxia in normal animals. And further, induction of

Na⁺ channels contributes to augment the neurotransmitter release response during acute hypoxic stimulation in chronic hypoxic animals and thereby to the genesis of sensitization seen in this condition.

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FIGURE LEGENDS

Figure 1. Effect of TTX and removal of Ca^{2+} or Na^{+} on the veratridine-evoked release of ^3H -CA from intact rat CB. **A.** Time course of veratridine effect on ^3H -CA release from 6 CBs. Veratridine was applied for 20 min as indicated in the drawing. TTX superfusion started 10 min prior to and was maintained during veratridine application. **B.** Total evoked release (equivalent to dpm above dotted line in part A), was expressed as % total ^3H -CA content. Ca^{2+} was removed from the medium 30 min prior to veratridine application and remained Ca^{2+} -free until the end of the experiment. Na^{+} was removed 10 min prior to veratridine application. Values are means $\pm$ SEM for 6-10 CBs (** $p < 0.001$).

Figure 2. Effect of TTX on release of ^3H -CA elicited by graded hypoxia. **A.** Mean time courses of release obtained in 6 control CBs challenged twice with a hypoxic stimulus (7% O_2 -equilibrated solutions) as drawn, and in 6 experimental CBs similarly stimulated but prior to and during second hypoxic challenge $1\mu\text{M}$ TTX was present in the incubating solution. **B.** Results of 3 series of similar experiments using indicated levels of hypoxia. Bars represent ratios of total evoked release obtained in the second challenge to that obtained in the first one (S_2/S_1) for control (open bars) and experimental CBs (hatched bars). Values are means $\pm$ SEM for 6-10 CBs (** $p < 0.01$; *** $p < 0.001$).

Figure 3. Western blot for $\text{Na}_v \alpha$ subunits in normoxic and chronically hypoxic rat CBs. **A.** Shows a representative immunoblot obtained with a pan Na_v antibody (dilution 1:250) showing the region from 40 kDa to the top of the gel: a

single band of expected molecular weight (≈ 220 kDa) is detected by the antibody. **B.** Shows a comparison between normoxic (CB-N; lanes 1-3) and chronic hypoxic (CB-CH; lanes 4-6) CB Na_v band in the 220 kDa region. Normoxic (SCG-N) and hypoxic superior cervical ganglion (SCG-CH) were used as control tissue. Optical densities of Na_v were normalized relative to β -actin signal and averaged for all the replicated gels. **C.** Shows relative quantification of intensity bands obtained from 12 control CBs (open bar) and 12 hypoxic CBs (hatched bar). Values are means $\pm$ SEM (* $p < 0.01$).

Figure 4. RT-PCR assessment of $\alpha\text{-Na}_v$ gene expression in normoxic and chronically hypoxic rat CBs. **A.** Expression of TTX-sensitive Na_v α subunits mRNA (SCN) in normoxic rat CB. Figure shows a representative 1% agarose gel electrophoresis of PCR products obtained with primers listed in Table I. Each lane corresponds to 1/10 CB mRNA. A 100 bp DNA ladder is shown. **B.** Representative agarose gel electrophoresis of PCR products obtained from normoxic and hypoxic CBs for different Na_v α subunits (upper panels), β -actin (lower panel; housekeeping gene) and TH. **C.** Semiquantitative analysis of Na_v channel isoforms expression. Intensities of Na_v channel bands were normalized to those of the β -actin. Data are means $\pm$ SEM from six independent experiments (* $p < 0.05$; *** $p < 0.001$).

Figure 5. Rat CB sections immunostained for TH and Na_v channels and counterstained with cresyl violet. **Upper row**, sections from a control (normoxic) rat CB. **Lower row**, sections from a chronically hypoxic rat CB. Both sections demonstrate the intensity and localization of the Na_v channels visualized with

Alexa594-conjugated secondary antibody and TH visualized with a FITC-conjugated secondary antibody. Counterstaining with cresyl violet reveals the general histology of the organ. Incubation without primary antibodies yielded no signal (data not shown). Calibration bars, 50 μ m.

Figure 6. Localization of pan- Na^+ channels and isoforms $\text{Na}_v1.1$, $\text{Na}_v1.3$ and $\text{Na}_v1.6$ by immunofluorescence in cultured rat chemoreceptor cells. **Left three columns** correspond to cultures obtained from CB of normoxic rats and show bright field and double-labeling images revealing co-localization of Na_v channels and TH (chemoreceptor cell marker). **Right three columns** show images obtained from four different cultures from chronically hypoxic rat CBs. Calibration bars represent 30 μ m.

Figure 7. Effect of chronic hypoxia on the release of ^3H -CA elicited by veratridine and on the effect of TTX upon the release of ^3H -CA elicited by hypoxia in CBs obtained from chronically hypoxic animals. **A.** Response to 50 μM veratridine applied for 20 min and its blockade by TTX (1 μM) in CBs from control (normoxic CB) and chronically hypoxic rats (hypoxic CB). Bars represent the release evoked by veratridine calculated as in Figure 1. Values are means $\pm$ SEM for 6-10 CBs (** $p < 0.001$). **B.** Effect of 1 μM TTX on release of ^3H -CA elicited by hypoxia in CBs from chronically hypoxic rats. Protocols as in Figure 2. Values are means $\pm$ SEM for 6-10 CBs (* $p < 0.05$; ** $p < 0.01$).

Figure 8. Whole body plethysmography of unrestrained normoxic and chronically hypoxic rats. The three panels from left to right show mean

frequency, mean tidal volume and minute ventilation in a group of 12 normoxic or control rats and 12 chronically hypoxic rats. Notice first, that frequency of breathing both in normoxia and in the acute hypoxic test was identical in both groups, secondly that there were very significant differences in tidal volumes in both conditions (while breathing room air and 7% O₂) and thirdly, that it resulted in a significant increase in the minute ventilation in the chronically hypoxic rats. For each animal and for the entire population of animals tidal volume and minute ventilation data were normalized to unit body weight. Data are means $\pm$ SEM (n=12; ***p< 0.001).

TABLE I. mRNA primer sequences.

Gene Bank	Target mRNA	Primer sequences 5'-3'	Tm/°C	% CG	Product length
NM_030875	SCN1A Na _v 1.1	Fw: GCAAGCTGTCCGCTGGTAATATA	61	48	436 bp
		Rv: AGTGATCGTGATATCAACCTGAAG	59	42	
NM_012647	SCN2A Na _v 1.2	Fw: GCTGCAGCTCTCATTACACAC	62	55	494 bp
		Rv: GGCTAAACAATACTGCAGGGAAAA	59	42	
NM_013119	SCN3A Na _v 1.3	Fw: TATCCGTGTCAACTGGACTCTAAG	61	46	422 bp
		Rv: GATTACTGGAGAACTTGTGGACT	59	42	
NM_013178	SCN4A Na _v 1.4	Fw: GCCTGCGCTCTCTGACTTG	61	60	407 bp
		Rv: CCTGACATTGGTACCCGG	61	63	
L39018	SCN8A Na _v 1.6	Fw: TTCAATGCGGTTTCCATCCT	55	45	401 bp
		Rv: GACTGCAGGCCATGGTTCA	59	58	
U79568	SCN9A Na _v 1.7	Fw: TCAGCGTGCTTACAGACGGTA	60	52	402 bp
		Rv: CTAATGGCTGTGCTGCCTTTG	60	52	
NM_012740	TH	Fw: CCCCAGCGCCCCCTCGCCACAGC	74	72	234 bp
		Rv: GCATTCCCATCCCTCTCCTCAAA	62	52	
NM_031144	β-actin	Fw: AAGATCCTGACCGAGCGTGG	62	54	327 bp
		Rv: CAGCACTGTGTTGGCATAGAGG	61	60	

(TH, tyrosine hydroxylase)

TABLE II. ³H-catechols released in basal conditions and in veratridine stimulated rat CBs. Values are means ± SEM for 5 determinations. CBs were incubated in presence of 50 µM veratridine for 10 min and the eluates collected for analysis. Individual catechols were separated and identified by HPLC. Dopamine (DA) includes its main catabolite dihydroxyphenylacetic acid (DOPAC).

		NORMOXIA		CHRONIC HYPOXIA	
BASAL RELEASE (fmol/CB)	³ H-NE	2.0 ± 0.2	14.1 %	4.0 ± 0.4	5.5 %
	³ H-DA + ³ H-DOPAC	12.2 ± 1.2	85.9 %	69.0 ± 7.3	94.5 %
50 µM VERATRIDINE (fmol/CB)	³ H-NE	9.4 ± 1.8	9.7 %	12.6 ± 1.1	3.0 %
	³ H-DA + ³ H-DOPAC	87.6 ± 20.8	90.3 %	422.2 ± 42.0	97.0 %

Table III. Fluorescence intensity of TH, pan- Na_v and $\text{Na}_v1.1$ and $\text{Na}_v1.3$ isoforms in chemoreceptor cells from normoxic and chronically hypoxic animals immunostained with the correspondent antibodies. Chemoreceptor cells for TH staining were obtained from four different cultures. Cells analysed for fluorescence intensity for Na_v channels staining were chemoreceptor cells (TH^+ cells) obtained in all the cases from two different cultures.

PROTEIN	CONDITIONS	AVERAGE PIXEL INTENSITY	n
TH	Normoxia	459 ± 7	289
	Hypoxia	1124 ± 35	120
pan Na_v	Normoxia	1026 ± 34	59
	Hypoxia	1309 ± 57	57
$\text{Na}_v1.1$	Normoxia	611 ± 11	96
	Hypoxia	697 ± 27	72
$\text{Na}_v1.3$	Normoxia	215 ± 7	39
	Hypoxia	227 ± 12	28

A. **B.** **C.**

Normoxic Rat CB

Hypoxic Rat CB

A. **B.**

□ Normoxic rats ■ Hypoxic rats