Enhanced Firing in NTS Induced by Short-Term Sustained Hypoxia Is Modulated by Glia-Neuron Interaction

Daniela Accorsi-Mendonça, Carlos E.L. Almado, Leni G.H. Bonagamba, Jaci A. Castania, Davi J.A. Moraes, and Benedito H. Machado

Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, 14049-900 Ribeirão Preto, SP, Brazil

Humans ascending to high altitudes are submitted to sustained hypoxia (SH), activating peripheral chemoreflex with several autonomic and respiratory responses. Here we analyzed the effect of short-term SH (24 h, FIO₂10%) on the processing of cardiovascular and respiratory reflexes using an *in situ* preparation of rats. SH increased both the sympatho-inhibitory and bradycardiac components of baroreflex and the sympathetic and respiratory responses of peripheral chemoreflex. Electrophysiological properties and synaptic transmission in the nucleus tractus solitarius (NTS) neurons, the first synaptic station of afferents of baroreflexes and chemoreflexes, were evaluated using brainstem slices and whole-cell patch-clamp. The second-order NTS neurons were identified by previous application of fluorescent tracer onto carotid body for chemoreceptor afferents or onto aortic depressor nerve for baroreceptor afferents. SH increased the intrinsic excitability of NTS neurons. Delayed excitation, caused by A-type potassium current (IK_A), was observed in most of NTS neurons from control rats. The IK_A amplitude was higher in identified second-order NTS neurons from control than in SH rats. SH also blunted the astrocytic inhibition of IK_A in NTS neurons and increased the synaptic transmission in response to afferent fibers stimulation. The frequency of spontaneous excitatory currents was also increased in neurons from SH rats, indicating that SH increased the neurotransmission by presynaptic mechanisms. Therefore, short-term SH changed the glia-neuron interaction, increasing the excitability and excitatory transmission of NTS neurons, which may contribute to the observed increase in the reflex sensitivity of baroreflex and chemoreflex in *in situ* preparation.

Key words: astrocytes; firing; intrinsic properties; NTS; sustained hypoxia; synaptic transmission

Introduction

Experimental hypoxic condition induces activation of peripheral chemoreflex, which increases sympathetic activity, blood pressure, and ventilation (Antunes et al., 2005; Moraes et al., 2011). Similar changes were also observed in unacclimatized humans after ascending to high altitude (Basu et al., 1996; Hackett and Roach, 2001; Roach and Hackett, 2001). These responses are due to the activation of glomus cells located in carotid body, which are pO₂-sensitive, in close contact with afferents of peripheral chemoreceptors (McDonald, 1983). The peripheral chemoreceptors afferents establish synaptic contact with second-order neurons in nucleus tractus solitarius (NTS), via tractus solitarius (TS; Donoghue et al., 1984; Claps and Torrealba, 1988; Mifflin, 1992; Chitravanshi and Sapru, 1995). After integrating signals from sensorial inputs, the NTS neurons of chemoreflex pathways

send projections to several other brain areas (brainstem, pons, hypothalamus, and cortex) to provide autonomic, respiratory, and behavioral adjustments to the hypoxic challenge (Accorsi-Mendonça and Machado, 2013).

Short-term sustained hypoxia (SH) for 24 h produces an increase in arterial pressure and expiration in rats (Moraes et al., 2014). SH also induces several adaptive changes in peripheral chemoreflex pathways, such as, enhancement of gene transcription and excitability of glomus cells in carotid body (Wang et al., 2000; Ortiz et al., 2009; Liu et al., 2013). Long-term SH for 10 d also affected NTS neurons, which presented an increased NTS neuronal discharge due to an evoked excitatory inputs to NTS neurons, as well as a decrease in K_{ATP} channel subunits expression (Kir 6.1 and Kir 6.2 subunits; Zhang et al., 2008, 2009). Furthermore, episodes of chronic intermittent hypoxia (CIH) also change the NTS synaptic plasticity, because there is a reduction in the afferent glutamatergic neurotransmission in NTS neurons in rats submitted to CIH (10 d), probably because of a decrease in the number of active synapses, as we previously documented (Almado et al., 2012). All these changes in the NTS neurons may contribute to the observed increase in sympathetic activity and high blood pressure observed in CIH rats (Zoccal et al., 2009).

Although the neural adaptations to sustained hypoxia was previously documented (Zhang et al., 2008 and 2009), the mechanisms underlying the synaptic response of NTS neurons to short-term SH and the functional implications of these changes

Received Nov. 6, 2014; revised March 6, 2015; accepted March 20, 2015.

Author contributions: D.A.-M., C.E.L.A., D.J.A.M., and B.H.M. designed research; D.A.-M., C.E.L.A., L.G.H.B., J.A.C., D.J.A.M., and B.H.M. performed research; D.A.-M., C.E.L.A., D.J.A.M., and B.H.M. analyzed data; D.A.-M., D.J.A.M., and B.H.M. wrote the paper.

This work was supported by FAPESP Grants 2009/50113-0 and 2013/06077-5, CNPQ Grant 472704/04-4, and FAPESP fellowship Grant 2011/24050-1 to D.J.A.M. We thank Ricardo M. Leão for his comments and suggestions.

The authors declare no competing financial interests.

Correspondence should be addressed to Dr Daniela Accorsi-Mendonça, Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900, 14049-900, Ribeirão Preto, SP, Brazil. E-mail: daniaccorsi@usp.br.

DOI:10.1523/JNEUROSCI.4598-14.2015

Copyright © 2015 the authors 0270-6474/15/356903-15\$15.00/0

are not yet fully understood. Here, we evaluated the changes in baroreflexes and chemoreflexes sensitivity using: (1) an *in situ* preparation of rats submitted to short-term SH (24 h, 10% O₂) and (2) whole-cell patch-clamp in brainstem slices. A fine electrophysiological approach was used to explore the effects of SH on intrinsic properties and synaptic transmission of second-order NTS neurons receiving afferent inputs from baroreceptors and chemoreceptors. The overall changes observed in NTS neurons of SH rats might explain at least in part, the multiple and complex neurovegetative dysfunctions associated to the mountain sickness.

Materials and Methods

The experimental protocols used in the present study were approved by the Institutional Ethical Committee on Animal Experimentation of the School of Medicine of Ribeirão Preto, University of São Paulo (Protocols CEUA: 019/2006, 070/2007, 064/2010).

SH model. Experiments were performed in 55 juvenile male Wistar rats (21- to 30-d-old), which were divided into two groups: (1) rats exposed to SH (FIO₂: 10%) for 24 h and (2) rats maintained under normoxic condition (FIO₂: 21%). Rats were maintained inside of acrylic chambers equipped with gas injectors and sensors for O₂, CO₂, humidity, and temperature inside the chamber (Oxycycler, Biospherix) and had access to food and water *ad libitum*. The room temperature was kept at 23 ± 2°C with a 12 h dark/light cycle.

Labeling of baroreceptor and chemoreceptor inputs to the NTS with fluorescent tracer (DiI). We used a fluorescent membrane tracer [1'-diiododecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen)] to identify the second-order NTS neurons that received baro- or chemoreceptor inputs. For this purpose, we applied the DiI onto aortic depressor nerve (ADN) fibers or carotid body (CB) as previously described by Accorsi-Mendonça et al. (2009). Briefly, animals were anesthetized with intraperitoneal injection of ketamine (0.6 mg kg⁻¹, União Química Farmacêutica Nacional S/A) + xylazine (0.6 mg kg⁻¹, União Química Farmacêutica Nacional S/A) and divided into two groups of rats: (1) tracer applied onto ADN and (2) tracer applied onto CB. During the surgery, the DiI crystals were gently applied bilaterally onto the CB or ADN fibers and they were coated with silicone elastomer (Kwik-Sil, WPI). One week after this surgery, rats were submitted to the SH or control condition during 24 h. At the end of this protocol, slices of brainstem were obtained to *in vitro* experiments.

In situ preparation. Immediately after of 24 h of SH experimental protocols, rats were prepared for the *in situ* Working Heart Brainstem Preparation (WHBP), as previously described (Paton, 1996; Moraes et al., 2013). Rats were deeply anesthetized with halothane (Astra Zeneca), transected caudal to the diaphragm, exsanguinated and submerged in a cooled Ringer solution (in mM: 125 NaCl, 24 NaHCO₃, 3 KCl, 2.5 CaCl₂, 1.25 MgSO₄, 1.25 KH₂PO₄, 10 dextrose). They were then decerebrated at the precollicular level, therefore rendered insentient, and skinned. The descending aorta was isolated and the lungs removed. Preparations were then transferred to a recording chamber and the descending aorta was cannulated with a double-lumen cannula and retrogradely perfused with Ringer's solution containing an oncotic agent (1.25% polyethylene glycol, Sigma-Aldrich) and a neuromuscular blocker (vecuronium bromide, 3–4 μg/ml, Cristália Produtos Químicos Farmacêuticos) using a peristaltic pump (Watson-Marlow 502 s, Falmouth). The mean perfusion pressure was maintained in the range 50–70 mmHg by adjusting the flow between 21 and 25 ml/min and by adding vasopressin (0.6–1.2 nM, Sigma-Aldrich) to the perfusate, which was continuously gassed with 5% CO₂ and 95% O₂ (White Martins), warmed to 31–32°C and filtered using a nylon mesh (pore size: 25 μm, Millipore).

Electrophysiological data acquisition. Sympathetic and respiratory motor nerves were isolated and recorded using bipolar glass suction electrodes with the aid of a micromanipulator (Narishige). Phrenic nerve (PN) activity was recorded from its central end. The activity of thoracic sympathetic nerve (tSN) was recorded from the sympathetic chain at the level of T8–T12. Abdominal nerve (AbN) was isolated from the abdominal muscles at thoracic–lumbar level, cut distally, and its activity re-

corded. Heart rate (HR) was derived from ECG. All signals were amplified, bandpass filtered (0.05–5 kHz), and digitized (5 kHz; CED Micro1401; Cambridge Electronic Design; CED) to a computer using Spike2 software v5 (CED).

Chemoreflex and baroreflex activation in *in situ* preparations. Peripheral chemoreceptors were stimulated by injections of potassium cyanide (KCN 0.05%, 50 μl) into the descending aorta of the *in situ* preparations via the perfusion cannula as previously described (Moraes et al., 2012). The sympatho-inhibitory and bradycardic responses to electrical stimulation of the ADN (0.2 ms, 3 pulses at 400 Hz) were used as an index of barosensitivity. The stimulus intensity was set at the intensity required to elicit a fall in the perfusion pressure >10 mmHg in response to ADN stimulation at 50 Hz (0.2 ms duration) for 5 s (threshold), as described by Moraes et al. (2012).

Data analysis of *in situ* preparations. The quantification of the sympathetic, bradycardic and respiratory responses to chemoreflex activation was performed in accordance with a previous description by Moraes et al. (2012). PN inspiratory response to chemoreflex activation was assessed by the difference between baseline PN burst frequency and the peak of response observed after the stimulus (ΔPN, expressed in Hz). The tSN (during inspiration and expiration) and AbN expiratory responses to peripheral chemoreflex activation was assessed by the measurement of the area under the curve, in a time window of ≤10 s after the stimulus, and expressed as percentage values (ΔtSN and ΔAbN in percentage) in relation to the activity before the stimulus. To evaluate the barosensitivity of tSN, we compared the reduction in the tSN activity during the ADN stimulus with an average of the tSN activity recorded during 10 respiratory cycles immediately preceding the stimulus. We calculated the area under the integrated activities of tSN and the ratio of the tSN activity between stimulation and the control condition was calculated as follows and used as an index of barosensitivity: percentage of inhibition = [(baseline tSN activity – tSN activity during stimulus)/baseline tSN activity] × 100. The HR responses (ΔHR) to chemoreflex and baroreflex activation were quantified by measuring the maximum bradycardia. We calculated the intensity required to produce 50% of inhibition of tSN and HR to ADN stimulation by fitting the responses to different intensities (threshold, two-, three-, and fourfold the threshold) using the following logistic equation (GraphPad Prism v4): $Y = a1(a2 - a1)/1 + 10(\log x0 - x)/p$, in which $a1$ is the minimal response; $a2$ is the maximum response; $\log(x0 - x)$: correspond to 50% of inhibition and p is the slope.

Data were compared using one-way ANOVA with Tukey *post hoc* testing or a Student's unpaired *t* test (GraphPad Prism) in accordance with the experimental protocol. Differences were considered significant at $p < 0.05$.

Brainstem slice preparation. At the end of the 24 h protocols of SH or control conditions the rats were decapitated and the brain was rapidly removed and submerged in modified ice-cold (4°C) artificial CSF (aCSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 1 MgCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, 25 glucose and 2 CaCl₂, with osmolality of ~300 mOsm/Kg.H₂O and pH 7.4 when bubbled with 95% O₂ and 5% CO₂. Brainstem transversal slices (250 μm thick) were cut using an oscillating slicer (Vibratome 1000 plus, Leica Biosystems) and kept in aCSF at 31°C for 30 min. Thereafter the slices were kept at room temperature (RT; 23–25°C) in normal aCSF containing the following (in mM): 125 NaCl, 2.5 KCl, 1 MgCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃. Before recordings, a single slice was submerged into the recording chamber, held in place with a nylon thread, and continuously perfused with aCSF at a flow of ~2–3 ml/min at RT.

Neurons in brainstem slice were visualized using infrared and differential interference contrast (IR-DIC) microscopy (Olympus BX51WI) through a 40× water-immersion objective (LUMPlan F1-IR, Olympus) and a CCD camera (C7500-50, Hamamatsu).

Whole-cell patch-clamp recordings. Whole-cell recordings were made with patch pipettes pulled from thick-walled borosilicate glass capillaries (Sutter Instruments), using a puller (P-97, Sutter Instruments). The patch pipettes were filled with an internal solution containing the following (in mM): 130 Kgluconate, 20 KCl, 2 Mg-ATP, 0.3 Na-ATP, 10 Na₂phosphocreatin, 5 EGTA, 10 HEPES, osmolality of ~310 mOsm/Kg. H₂O and pH 7.4 adjusted with KOH (Almado et al., 2012). The final

resistance of pipette ranged from 4 to 8 MΩ. The whole-cell configuration was obtained, signals were acquired using an amplifier (Axopatch 200B, Molecular Devices) connected to a data acquisition system (Digidata 1440A, Molecular Devices), and recorded in a microcomputer using software (Clampex, pClamp v10, Molecular Devices). The data obtained in voltage or current-clamp configuration were low-pass filtered at 2 kHz and sampled at 10 kHz, according with Nyquist theorem (sampling theorem). Series resistance (<30 MΩ) was checked regularly during the experiments and cells with large variations in series resistances were discarded. The membrane potential (V_m) of cells were held at -70 mV (voltage-clamp configuration) and recordings started 5 min after establishing the whole-cell configuration.

Electrophysiological measurements. Hyperpolarizing current (steps of -12.5 pA, from -12.5 to -50 pA with 2 s duration) was injected into NTS neuron to calculate the input resistance (R_{input}). To evoke action potential, two protocols in current-clamp configuration were used: (1) depolarizing injected current pulses of 12.5 mV with duration of 2 s from the resting membrane potential (holding current 0 pA) and (2) electrical stimulation of ipsilateral TS fibers (0.1 ms duration, 0.07 Hz) from the resting membrane potential (holding current 0 pA) using a concentric bipolar microelectrode (Frederick Haer) connected to an isolated stimulator (DS2A, Digitimer). The intensity of stimulation was selected using the voltage-clamp configuration. In this case, we increased the intensity of the stimulus to achieve the maximum current amplitude for each recorded neuron and then this intensity of stimulation was used to induce action potentials (Accorsi-Mendonça et al., 2011).

The transient outward potassium current (TOC) was induced using protocols described previously by Bailey et al. (2002). The TOC activation was assessed by conditioning step (-90 mV, 500 ms), followed by longer activation steps (from -100 to 10 mV, 1200 ms; see Fig. 4). The TOC inactivation was assessed by conditioning steps (from -100 to -30 mV, 500 ms), followed by a longer step (-10 mV, 1200 ms; Fig. 4).

In a voltage-clamp configuration we recorded the evoked EPSCs (eEPSCs) and the spontaneous postsynaptic currents (gapfree mode).

Data analysis of patch-clamp experiments. All experiments were recorded using the software Clampex (pClamp v10, Axon Instruments) and were analyzed off-line using the software Clampfit (pClamp v10, Axon Instruments) or Mini Analysis Program (Synaptosoft). The data were expressed as mean $\pm$ SEM using GraphPad Prism v4. Significance was established at $p < 0.05$.

The junction potential between the external and electrode solutions was ~ -14 mV and resting membrane potentials (RMP) were corrected with this value. The R_{input} was determined via linear regression applied to the linear portion of the voltage–current relationship after hyperpolarizing injected current. Membrane capacitance (C_m) was calculated by dividing the membrane time constant by R_{input} and the membrane time constant was measured by fitting a single exponential function to the data points in the hyperpolarizing phase of the same recording. The RMP, C_m and R_{input} , frequency, amplitude, half-width, rise time or decay time of the currents were compared using unpaired Student's t test (GraphPad Prism v4).

The number of action potential induced by positive injected current or TS stimulation from control or SH rats were compared using two-way ANOVA with Tukey *post hoc* testing (GraphPad Prism v4). The delay excitation was considered as a delay in the occurrence of action potentials after depolarized step preceded by a hyperpolarized step (Dekin and Getting, 1987; Dekin et al., 1987; Moak and Kunze, 1993).

The IK_A was isolated by digital subtraction of the steady-state current (SS) from TOC. TOC peak was measured 2–4 ms after capacitive transients in the long step and SS was measured near the end of the long step in each protocol (see Fig. 4).

Activation curve as well as inactivation curve for IK_A was fit with the Boltzmann function using data analysis Prism software (GraphPad Prism v4), according to previous studies from our laboratory (Moraes et al., 2013).

The conductance (G) of IK_A was calculated as follows:

$$G = I_{peak} / (V_m - E_K),$$

where I_{peak} is peak current, V_m is membrane voltage, and E_K is the potassium equilibrium potential calculated by Nernst equation, which in our experimental conditions was 98.6 mV.

The relationship between normalized conductance (G/G_{max}) and membrane potential were fitted by the Boltzmann function using data analysis Prism software v4 (GraphPad):

$$G/G_{max} = \frac{1}{1 + \exp \left[\frac{V_{1/2} - V_m}{V_c} \right]},$$

where $V_{1/2}$ is the voltage at half-maximal conductance and V_c is the slope factor.

Voltage-dependent inactivation of the TOC was fitted with Boltzmann function using data analysis Prism software v4 (GraphPad):

$$I/I_{max} = \frac{1}{1 + \exp \left[\frac{V_{1/2} - V_m}{V_c} \right]},$$

where I_{max} is the maximal current recorded.

The curves for IK_A analyzes (activation, inactivation, conductance or 4-AP-sensitive current curves) were compared using two-way ANOVA with Tukey *post hoc* testing (GraphPad Prism v4).

Drugs. The brainstem slice in the recording chamber was perfused with aCSF by a gravity-driven perfusion system (flow: 2–3 ml/min⁻¹). All drugs were diluted in the aCSF solution and the flow throughout the recording chamber containing the slices was regulated by a six-valve solenoid system (VC-6; Warner Instruments). Bicuculline free-base, a GABA_A receptor antagonist, was dissolved in dimethylsulphoxide (DMSO; Sigma-Aldrich); the final concentration of DMSO in the aCSF bath was in the range of 0.1%. 4-aminopyridine (4-AP; IK_A blocker), fluoracetate (FAC), bicuculline, and DMSO were obtained from Sigma-Aldrich. Tetrodotoxin (TTX; sodium channel blocker) was obtained from Tocris Bioscience.

Results

SH, baroreflex, and chemoreflex responses

In *in situ* preparations under the same conditions of flow rate, temperature, and perfusate content, we verified that preparations from SH rats developed higher levels of perfusion pressure [PP; control: 63 ± 1 mmHg ($n = 18$) vs SH: 78 ± 1.8 mmHg ($n = 17$); $p < 0.0001$] than preparations from control rats, indicating an increased vascular resistance in SH rats as described previously (Moraes et al., 2014). We next evaluated the autonomic and respiratory reflexes sensitivity in *in situ* preparations from control and SH groups.

The changes in tSN, HR, AbN, and PN activities were measured in response to stimulation of carotid chemoreceptors with intra-arterial injections of KCN, which increased tSN in both control and SH rats (Fig. 1A,B). The increase in the tSN was significantly enhanced in SH rats during expiratory phase [control: $101.3 \pm 1.7\%$ ($n = 9$) vs SH: $126.5 \pm 4.4\%$ ($n = 11$); $p < 0.001$], but not during inspiratory [control: $16.1 \pm 1.4\%$ ($n = 9$) vs SH: $16.7 \pm 2.1\%$ ($n = 11$); $p = 0.82$; Fig. 1A,B,C]. Accordingly, in SH rats the AbN expiratory response was also more pronounced than in control rats [control: $381.3 \pm 4.1\%$ ($n = 12$) vs SH: $463.7 \pm 13.6\%$ ($n = 10$); $p < 0.0001$; Fig. 1A,B,D], whereas the inspiratory [(frequency of PN) control: 0.36 ± 0.02 Hz ($n = 12$) vs SH: 0.38 ± 0.07 Hz ($n = 11$); $p = 0.77$] and bradycardic [control: -193 ± 6 bpm ($n = 12$) vs SH: -207 ± 9 bpm ($n = 11$); $p = 0.2$] responses were not different between the groups (Fig. 1A,B).

Stimulation of aortic depressor nerve (ADN), containing mainly baroreceptor afferent fibers (Brophy et al., 1999), in the *in situ* preparation resulted in a well described sympatho-inhibition

Figure 1. Peripheral chemoreflex responses in control and SH rats. Raw and integrated (J) recordings of AbN, tSN, and PN activities, and HR of a control (**A**) and a SH rat (**B**), representatives of their respective groups, illustrating the respiratory, sympathetic, and bradycardic responses elicited by the activation of peripheral chemoreceptors with intra-arterial injection of KCN (0.05%, 50 μ l). The percentage of average magnitude of the tSN (**C**) and AbN (**D**) reflex responses to peripheral chemoreflex activation in control and SH rats. I, Inspiration; E, expiration; PP, perfusion pressure. ** $p < 0.001$, ANOVA one-way; *** $p < 0.0001$, unpaired t test.

and bradycardia, as well as a prolongation of the expiratory duration (Baekey et al., 2008). The magnitudes of the responses are dependent on the respiratory phase in which the stimulus is applied, with the maximal greatest effect occurring immediately after inspiration, i.e., postinspiration. Considering that a comprehensive study of the respiratory modulation of baroreflex function has been described in *in situ* preparations (Baekey et al., 2008), we evaluated the effects of SH on the sympatho-inhibitory and bradycardic components of baroreflex within the postinspiratory phase of the respiratory cycle. SH rats presented higher sympatho-inhibitory [twofold the threshold: control: $64.2 \pm 2.2\%$ ($n = 6$) vs SH: $84.1 \pm 1.7\%$ ($n = 6$); $p < 0.0001$; Fig. 2A,B,C] and bradycardic [twofold the threshold: control: -30 ± 1.5 bpm ($n = 6$) vs SH: -46.6 ± 2.9 bpm ($n = 6$); $p = 0.0005$; Fig. 2A,B,D] responses to baroreflex activation than control rats, but no changes in the prolongation of expiratory period [control: 6.1 ± 1.2 s ($n = 6$) vs SH: 5.7 ± 0.4 s ($n = 6$), $p = 0.75$; Fig. 2A,B].

Consistent with the observation that the tSN and HR responses are more pronounced to baroreflex activation in SH rats, the intensity required to produce 50% of inhibition of tSN [control: 1.6 ± 0.1 the threshold ($n = 6$) vs SH: 1.0 ± 0.07 the threshold ($n = 6$); $p = 0.0006$] and HR [control: 1.9 ± 0.22 the threshold ($n = 6$) vs SH: 2.5 ± 0.14 the threshold ($n = 6$); $p = 0.04$] was lower in SH rats than in control rats (fit using a logistic equation; see methods). Therefore, our results demonstrate that SH affects the processing of both baroreflexes and chemoreflexes.

Electrophysiological properties of NTS neurons

Considering the effects of SH on baroreflexes and chemoreflexes, we then evaluated whether SH also affected the synaptic transmission at the NTS level, because NTS neurons receive the afferent fibers from arterial baroreflexes and chemoreflexes (Contreras et al., 1982; Seiders and Stuesse, 1984; Claps and Torrealba, 1988; Barraco et al., 1992; Finley and Katz, 1992; Mendelowitz et al., 1992; Machado,

Figure 2. Sympatho-inhibitory component of baroreflex in control and SH rats. Raw and integrated (*f*) recordings of tSN and PN activities and HR of a control (*A*) and a SH rat (*B*), representatives of their respective groups, illustrating the sympatho-inhibitory, bradycardic, and expiratory responses elicited by the activation of baroreflex before (basal) and during ADN stimulation (response). The percentage of average magnitude of the tSN inhibition (*C*) and HR (*D*) reflex responses to baroreflex activation in control and SH rats; ** $p < 0.001$, *** $p < 0.0001$, unpaired *t* test.

2001, 2004). Whole-cell patch-clamp recordings were obtained from 186 nonlabeled NTS neurons from 41 rats. The nonlabeled NTS neurons were located mainly in the commissural NTS level, as illustrated in Figure 3. In addition, we also performed whole-cell patch-clamp experiments in 19 second order NTS-labeled neurons from 10 rats. The second-order NTS neurons receiving afferents inputs from baroreceptors and chemoreceptors were morphologically identified with DiI tracer (see Fig. 5).

SH and passive properties of nonlabeled NTS neurons

SH produced no changes in the resting membrane potential [control: -68 ± 2.7 mV ($n = 31$) vs SH: -66 ± 2.4 mV ($n = 21$), $p = 0.6283$], input resistance [control: 1.16 ± 0.2 G Ω ($n = 21$) vs SH: 1.14 ± 0.11 Ω ($n = 25$), $p = 0.9366$] or capacitance of nonlabeled NTS neurons [control: 72.8 ± 8 pF ($n = 14$) vs SH: 52.4 ± 7 pF ($n = 20$), $p = 0.0736$].

SH and active properties of nonlabeled NTS neurons

In current-clamp configuration we measured the spike discharge frequency of NTS neurons after positive injected current (0, 12.5, 25, 37.5, and 50 pA) into neurons from control and SH rats (Fig. 3C). The NTS neurons from SH rats presented higher spike discharge frequency than control rats in response to all values of injected current (Fig. 3D). The reduced excitability of NTS neurons from control rats compared with NTS neurons from SH rats seems to be related to a larger afterhyperpolarization phase (AHP) of action potential (Storm, 1987), which may contribute to limit the cell firing in response to sustained depolarization (Madison and Nicoll, 1984; Lancaster and Nicoll, 1987). However, in the present study the amplitudes of AHP in NTS neurons in control and SH groups were similar [+37.5 pA injected current: control -56.7 ± 3 ($n = 11$) vs SH -54.6 ± 2.7 ($n = 19$), $p = 0.6204$],

Figure 3. Nonlabeled NTS neurons of SH animals present increased firing frequency in response to injected current. **A**, Photomicrograph showing brainstem slice at the NTS level viewed under IR-DIC. AP, Area postrema; TS, tractus solitarius; NTS, nucleus tractus solitarius. **B**, Photomicrograph showing one NTS neuron and the patch pipette (PP) on the surface of slice viewed on IR-DIC optic. **C*i***, Representative trace showing the number of action potential after inject current (50 pA during 2 s) into NTS neuron from control rat; note the delay excitation (black arrow). **C*ii***, Representative tracing showing the number of action potential after inject current (50 pA during 2 s) into NTS neuron from SH animals. **D**, Correlation between the injected current and the number of action potential in NTS neurons from control ($n = 14$) and SH animals ($n = 19$); $**p < 0.01$, ANOVA two-way.

indicating that the changes in NTS neurons from the SH rats are not related to the AHP amplitude. Moreover, in neurons from control rats we observed a delayed excitation (Fig. 3C, arrow), which was defined as a delay in the occurrence of action potentials after depolarized step preceded by a hyperpolarized step (Dekin and Getting, 1987; Dekin et al., 1987; Moak and Kunze, 1993). The delayed excitation was longer in control than SH rats [at +37.5 pA of injected current: control 89.7 ± 4.1 ms ($n = 11$) vs SH 14.55 ± 2.8 ms ($n = 17$); $p = 0.0310$].

SH and TOC in nonlabeled and labeled NTS neurons

Considering that the delayed excitation in NTS neurons is a consequence of TOC activation (Dekin and Getting, 1987; Dekin et

al., 1987; Moak and Kunze, 1993; Paton et al., 1993; Tell and Bradley, 1994; Sundaram et al., 1997; Uteshev and Smith, 2006; Suwabe and Bradley, 2009), herein we evaluated the possibility that SH reduces the TOC and affects the delayed excitation.

These experiments were performed in the presence of TTX in aCSF solution to block the voltage-dependent sodium currents. Depolarization voltage steps (from -100 to 10 mV) from a conditioning potential of -90 mV elicited large voltage-dependent TOC that decayed over time in nonlabeled NTS neurons from control and SH rats (Fig. 4A). However, the amplitude of TOC was significantly different between nonlabeled NTS neurons from control and SH rats [control: 1428 ± 600 pA, $V_m = 10$ mV ($n = 10$) vs SH: 334 ± 76 pA, $V_m = 10$ mV ($n = 10$); Fig. 4B].

Moreover, activated TOC presented similar voltage-dependent activation kinetics in nonlabeled NTS neurons from control [$V_{1/2} = -25.3 \pm 1.1$ mV; $V_c = 7.2 \pm 1.1$ ($n = 10$); Fig. 4C] or SH rats [$V_{1/2} = -31 \pm 2.2$ mV; $V_c = 7.4 \pm 2$ ($n = 9$); Fig. 4C]. The reduction in TOC amplitude after SH was also observed on morphologically identified NTS neurons receiving afferent inputs from the ADN or CB fibers (second-order NTS neurons; Fig. 5). There was no difference between SS currents of NTS neurons from control or SH rats (+10 mV: control 789 ± 190 pA vs SH 923 ± 180 pA, $p = 0.6346$).

The voltage-dependent inactivation was different between nonlabeled NTS neurons from control and SH rats (Fig. 4E,F). In nonlabeled NTS neurons from control group the peak inactivation current was higher (795 ± 183 pA, $V_m = -100$ mV; $n = 12$) compared with those from SH rats (342 ± 73 pA, $V_m = -100$ mV; $n = 11$). However, the kinetic of TOC inactivation of both groups was similar [control rats: $V_{1/2} = -74 \pm 2$ mV; $V_c = -8 \pm 2$ ($n = 12$); SH rats: $V_{1/2} = -74 \pm 4$ mV; $V_c = -11 \pm 4$ ($n = 11$); Fig. 4G].

Pharmacological characterization of TOC in nonlabeled NTS neurons

In our experiments TOC were consistent with the activation of an early, transient outward current, named IK_A , which turns on with depolarization but subsequently it is inactivated (Pongs, 1999). To identify the currents, we tested whether the TOC were sensitive to 4-AP (5 mM), a blocker of IK_A (Mathie et al., 1998). TOC was reduced by 4-AP (Fig. 4D,H), suggesting that the TOC recorded in nonlabeled NTS neurons resemble IK_A .

Glial inhibition and IK_A in nonlabeled NTS neurons

Recent study by Naskar and Stern (2014) demonstrated that the astrocytic inhibition induces a reduction of IK_A amplitude in hypothalamic magnocellular neurosecretory neurons, involving extrasynaptic NMDA receptor activation and Ca^{2+} -dependent, protein kinase C-dependent pathways. To determine whether or not astrocytic inhibition also decrease IK_A in nonlabeled NTS neurons, we performed experiments with FAC, a glial metabolism inhibitor (Fonnum et al., 1997). We incubated the slices with FAC (1 mM) during 1 h before the experiments and IK_A in nonlabeled NTS neurons was registered in the presence of TTX + FAC. The glial inhibition decreased the IK_A in control rats [aCSF: 1227 ± 129 pA, $V_m = 10$ mV ($n = 7$) vs aCSF + FAC: 605 ± 210 pA, $V_m = 10$ mV ($n = 7$), $p = 0.0246$; Fig. 6A], but produced no change in the current amplitude of SH rats [aCSF: 475 ± 110 pA, $V_m = 10$ mV ($n = 4$) vs aCSF + FAC: 427 ± 114 pA, $V_m = 10$ mV ($n = 6$); Fig. 6B].

SH and afferent fibers stimulation

Considering that SH changed the intrinsic electrophysiological properties of NTS neurons, afterward we evaluated the possibility that SH also affects the synaptic transmission at the NTS level. These experiments were performed in the presence of bicuculline (20 μ M) in aCSF solution, to isolate the excitatory transmission. At steady resting potentials, five ST stimuli evoked action potential in nonlabeled NTS neurons from control and SH rats (Fig. 7A). Nonlabeled NTS neurons from SH rats presented a higher frequency discharge after TS stimulation compared with control rats ($p < 0.0001$; Fig. 7B). Considering that increased numbers of action potential after TS stimulation could be related to: (1) a reduction of IK_A and (2) also to an increase in the excitatory transmission, we measured the EPSCs evoked by TS-stimulation to evaluate the possible contribution of synaptic transmission.

TS-eEPSCs of nonlabeled NTS neurons from SH rats presented higher amplitude compared with those from control rats [control: 204.3 ± 26 pA ($n = 28$) vs SH: 340 ± 58 pA ($n = 23$); Fig. 7C,D], but no changes were observed in the kinetic parameters, such as half-width (control: 7.11 ± 0.41 ms vs SH: 6.8 ± 0.40 ms; Fig. 7E), rise time (control: 3.04 ± 0.3 ms vs SH: 2.3 ± 0.3 ms; Fig. 7F), or decay time of events (control: 13.4 ± 0.85 ms vs SH: 13 ± 0.89 ms; Fig. 7G).

To evaluate whether or not the mechanism by which SH exerts its effect on evoked synaptic transmission involves an increase in presynaptic glutamate release and/or changes in the postsynaptic neuron, we used two approaches to study the synaptic plasticity: (1) comparison of the inverse of squared coefficient of variation ($1/CV^2$) of TS-eEPSCs amplitude (Bekkers and Stevens, 1990; Malinow and Tsien, 1990) and (2) frequency-dependent depression protocol (Miles, 1986; Glaum and Miller, 1995; Schild et al., 1995; Scheuer et al., 1996; Chen et al., 1999; Doyle and Andresen, 2001).

The analysis of $1/CV^2$ is a measurement of presynaptic changes in transmitter release (Malinow and Tsien, 1990). Our data show that $1/CV^2$ was similar in SH and control rats [control: 71 ± 19 ($n = 26$) vs SH: 87 ± 20 ($n = 23$), $p = 0.5749$], suggesting that the effect of SH on NTS neurons is not due to a presynaptic mechanisms. To test this possibility, we used a second approach to analyze the synaptic plasticity and to reach this goal we applied a train of five TS stimuli at 33 Hz, which produces a depression in the amplitude of evoked event. This depression in current amplitude is considered to be primarily mediated by presynaptic mechanisms and related to neurotransmitter release probability (Zucker and Regehr, 2002). We compared the amount of short-term synaptic depression of TS-eEPSCs in nonlabeled NTS neurons, from both control and SH rats. We observed that the magnitude of depression was similar in both groups [second, third, fourth, and fifth normalized TS-eEPSCs; control: 47 ± 3.8 , 34 ± 3.3 , 32 ± 4 , and $39 \pm 34.4\%$; ($n = 24$) vs SH: 51 ± 35.2 , 39 ± 5.1 , 31 ± 5.4 , and $33 \pm 7\%$, ($n = 17$); two-way ANOVA, $p = 0.3699$], indicating that SH produces no effect on release probability. Therefore, this analysis of synaptic plasticity confirmed that SH is not affecting presynaptic mechanism of nonlabeled NTS synaptic transmission.

SH and spontaneous synaptic transmission of NTS neurons

The effect of SH on spontaneous postsynaptic currents (sPSCs) was also evaluated. We measured the sPSCs in nonlabeled NTS neurons (Fig. 8A), which include excitatory and inhibitory currents (Accorsi-Mendonça et al., 2007). sPSCs of nonlabeled NTS neurons from SH rats presented a higher frequency compared with those from control rats [control: 3.4 ± 0.44 Hz ($n = 19$) vs SH: 7.1 ± 1.6 Hz ($n = 15$), $p = 0.0213$; Fig. 8B], with no change in amplitude (control: 20 ± 1.6 pA vs SH: 22 ± 2.8 pA; Fig. 8B) or half-width of events (control: 3.1 ± 0.13 ms vs SH: 3.2 ± 0.16 ms; Fig. 8B).

To verify whether the effect of SH on sPSCs was due to excitatory or inhibitory currents, we isolated pharmacologically the excitatory transmission with bicuculline, a GABA_A receptor antagonist, and the spontaneous EPSCs (sEPSCs; Fig. 8C) were recorded. sEPSCs of nonlabeled NTS neurons from SH rats presented a higher frequency compared with those from control rats [control: 2.7 ± 0.3 Hz ($n = 22$) vs SH: 4.54 ± 0.8 Hz ($n = 28$), $p = 0.0331$; Fig. 8D], with no change in amplitude (control: 19 ± 1.6 pA vs SH: 23 ± 2 pA; Fig. 8D) or half-width of events (control: 3.5 ± 0.2 ms vs SH: 2.9 ± 0.2 ms; Fig. 8D).

Figure 4. IK_A is decreased in nonlabeled NTS neurons from SH rats. *Ai*, Voltage-step protocols to activate outward currents in NTS neurons. The neurons were held at -90 mV for 500 ms followed by potentials step commands (-100 to 10 mV in 10 mV increments during 1 s). *Aii*, Representative traces showing the voltage-dependent activation of IK_A in NTS neuron from control animal. *Aiii*, Representative traces showing the voltage-dependent activation of IK_A in NTS neuron from SH animal. *B*, The relationship between the IK_A peak and voltage in NTS neurons from control ($n = 9$) and SH animals ($n = 7$); $**p < 0.01$ ANOVA two-way. *C*, Correlation between the normalized conductance and voltage in NTS neurons from control ($n = 9$) and SH animals ($n = 7$). *D*, Correlation between the 4-AP-sensitive current and the voltage in NTS neurons from control ($n = 6$) and SH animals ($n = 9$). *Ei*, Voltage-step protocols to inactivate TOC in NTS neurons. Prepulses varied in 10 mV increments from -100 to -30 mV during 500 ms, followed by a prolonged step (-10 mV during 1200 ms). *Eii*, Representative traces showing the voltage-dependent inactivation of IK_A in NTS neuron from control animal. *Eiii*, Representative traces showing the voltage-dependent inactivation of TOC in NTS neuron from SH animal. *F*, Correlation between the IK_A peak and the voltage of prepulse in NTS neurons from control ($n = 9$) and SH animals ($n = 6$). *G*, Correlation between the normalized conductance and the voltage of prepulse in NTS neurons from control ($n = 9$) and SH animals ($n = 7$). *H*, Correlation between the 4-AP-sensitive current and the voltage of prepulse in NTS neurons from control ($n = 5$) and SH animals ($n = 6$); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ANOVA two-way.

Figure 5. I_{K_A} is decreased in second-order NTS neurons from SH rats. **A**, Photomicrograph of NTS neuron that receive synaptic contacts from ADN showing the fluorescent afferent boutons viewed under IR-DIC optic. **B**, The same neuron under epifluorescence illumination. **C**, Overlay of fluorescent and IR-DIC image. Fluorescent ADN terminals (red color) identify baroreceptor second-order NTS neuron in slices. **D**, Correlation between the TOC peak and voltage in ADN-NTS neurons from control ($n = 7$) and SH animals ($n = 7$). **E**, Photomicrograph of the NTS neuron that receives synaptic contacts from CB showing the fluorescent afferent boutons viewed with IR-DIC optic. **F**, The same neuron under epifluorescence illumination. **G**, Overlay of fluorescent and IR-DIC image. Fluorescent CB terminals (red color) identify chemoreceptor second-order NTS neuron in a slice. **H**, Correlation between the TOC peak and voltage in CB-NTS neurons from control ($n = 4$) and SH animals ($n = 6$); *** $p < 0.001$, **** $p < 0.0001$, ANOVA two-way.

Figure 6. Glial inhibition and I_{K_A} in nonlabeled NTS neurons. **A**, Correlation between the TOC amplitude and voltage in NTS neurons from control rats during perfusion with aCSF ($n = 7$) or aCSF + FAC ($n = 7$). **B**, Correlation between the I_{K_A} amplitude and voltage in NTS neurons from SH rats during perfusion with aCSF ($n = 4$) or aCSF + FAC ($n = 6$); **** $p < 0.0001$, ANOVA two-way.

Considering that SH affected the frequency but not the amplitude of sEPSCs, we assumed that SH is essentially affecting presynaptic mechanisms. To test this hypothesis, we evaluated the effect of SH on the action potential-independent events, i.e., the miniature excitatory transmission. For this purpose, we isolated pharmacologically the miniature sEPSCs (msEPSCs; Fig. 8E) using bicuculline and TTX. msEPSCs of nonlabeled NTS neurons from SH rats presented a similar frequency compared with those from control rats [control: 1.2 ± 0.3 Hz ($n = 7$) vs SH: 1.74 ± 0.4 Hz ($n = 14$); Fig. 8F], with no change in amplitude (control: 15.2 ± 3 pA vs SH: 22 ± 3 pA; Fig. 8F) or half-width of events (control: 2.8 ± 0.4 ms vs SH: 2.6 ± 0.35 ms; Fig. 8F). These data show that SH produced no effect on a postsynaptic mechanism (mean amplitude of mEPSCs; Neher and Sakaba, 2001), confirming the hypothesis that the effects of SH are essentially due to changes in the presynaptic terminal.

Discussion

In the present study, we verified that short-term SH (24 h, FI O₂: 10%) enhanced the sympathetic, bradycardic, and respiratory responses to baroreflex and chemoreflex activation, and produced important changes at NTS synaptic transmission. There is evidence that SH contributes to development of high blood pressure (Calbet, 2003; Tamisier et al., 2005). In a recent study, we showed that SH produced sympathetic overactivity and hypertension in rats (Moraes et al., 2014), which can be induced by increased peripheral chemosensitivity or carotid body tonic activity as described in other hypertensive models, such as spontaneously hypertensive rats and heart failure (Abdala et al., 2012; McBryde et al., 2013; Ortega-Sáenz et al., 2013; Paton et al., 2013; Marcus et al., 2014). In the present study, we observed that SH produced an increase in expiratory response, but not inspiratory, and in expiratory-related sympatho-excitation to peripheral chemoreflex activation. We suggest that the enhanced expiratory response contributes to augmented sympathetic activity in SH rats as a consequence of a strengthened central coupling between the expiratory neurons of central pattern generator and medullary pre-sympathetic neurons (Moraes et al., 2014). These findings suggest a critical role for altered central respiratory-sympathetic coupling in the development of hypertension after SH. Considering the increased mean arterial pressure (Moraes et al., 2014) and chemosensitivity (present study) after SH, it was expected to find a reduction in the baroreflex sensitivity, as described in spontaneous hypertension (Head and Adams, 1988); however, our data show that SH produced an enhancement baroreflex sen-

sitivity because the sympatho-inhibition and bradycardia to ADN stimulation was greater in SH rats. Therefore, the increase in baroreflex sympatho-inhibition and bradycardia in SH rats may be an important protective mechanism to bring back to normal levels the acute increase in arterial pressure mediated by changes in the expiratory modulation of presympathetic neurons in the rostral ventrolateral medulla (Moraes et al., 2014), as previously documented in rats submitted to CIH during 10 d (Zoccal et al., 2009). This new concept is in agreement with our recent data showing that 24 h after the cessation of exposure to SH, the mean arterial pressure of SH rats returned to levels similar to those observed in control rats (Moraes et al., 2014), suggesting that the enhanced baroreflex function may play a key role in a fast normalization of the arterial pressure and also to prevent a greater increase in arterial pressure during SH.

Considering that SH affected the processing of cardiovascular and respiratory reflexes and NTS is the first synaptic and integration site for the afferents of baroreflexes and chemoreflexes in CNS, we hypothesized that after SH the changes in baroreceptor and chemoreceptor sensitivity are due to altered NTS neuronal activity. Therefore, we analyzed the SH effects on intrinsic properties and synaptic transmission of NTS neurons. We verified that SH has no effect on passive properties of NTS neurons, similarly to results using long-term SH protocol (Zhang et al., 2008, 2009).

The membrane excitability of NTS neurons was analyzed after injected positive current into these cells. SH increased firing after positive injected current and reduced the delay excitation, a well characterized delay between the onset and the first spike (Dekin et al., 1987). The presence of delayed excitation in NTS neurons from control rats is an indicative of I_{K_A} , a outward current with fast activation at subthreshold voltages followed by fast inactivation (Cai et al., 2007). Because the delayed excitation was reduced in neurons from SH rats, we suggested that SH decreases the I_{K_A} in NTS neurons and this hypothesis was tested using electrophysiological and pharmacological protocols. We evaluated the I_{K_A} in nonlabeled NTS neurons as well on morphologically identified NTS neurons that receive afferent inputs from the ADN or CB fibers (labeled second-order neurons). The voltage-dependent characteristics and 4-AP sensitivity of these currents confirmed the hypothesis that the delayed excitation was mediated by I_{K_A} in nonlabeled NTS neurons. This result was also observed in second-order NTS neurons related to baroreceptors and chemo-

Figure 7. Evoked synaptic activity is increased in nonlabeled NTS neurons from SH rats. **Ai**, Representative trace of action potentials after 5 TS stimuli (black circle) in NTS neuron from control animal. **Aii**, Representative trace of action potential after five TS stimuli (black circle) in NTS neuron from SH animal. **B**, Correlation between number of action potential and TS stimuli. **C**, Representative traces of eEPSCs after TS stimulus (black circles) in NTS neuron from control and SH rat. **D**, Average data of peak amplitude of TS-eEPSCs in NTS neurons from control and SH rat. **E**, Average data of half-width of TS-eEPSCs in NTS neurons from control and SH animal. **F**, Average data of rise time of TS-eEPSCs in NTS neurons from control and SH animal. **G**, Average data of decay time of TS-eEPSCs in NTS neurons from control and SH animal; * $p < 0.05$, unpaired t test; **** $p < 0.0001$, ANOVA two-way.

Figure 8. Spontaneous currents are increased in nonlabeled NTS neurons from SH rats. **A**, Representative traces of spontaneous currents in NTS neuron from control and SH rat. **B**, Average of frequency (**Bi**), amplitude (**Bii**), and half-width (**Biii**) of spontaneous currents in NTS neuron from control and SH rats. **C**, Representative traces of spontaneous excitatory currents in NTS neuron from control and SH rat. **D**, Average of frequency (**Di**), amplitude (**Dii**), and half-width (**Diii**) of spontaneous excitatory currents in NTS neuron from control and SH animals. **E**, Representative traces of miniature spontaneous excitatory currents in NTS neuron from control and SH rats. **F**, Average of frequency (**Fi**), amplitude (**Fii**), and half-width (**Fiii**) of miniature spontaneous excitatory currents in NTS neuron from control and SH rats; * $p < 0.05$, unpaired t test.

receptors, demonstrating that labeled NTS neurons also presented a IK_A reduction after SH.

The IK_A decreases the spontaneous firing, finely modulates the action potential threshold and interspike intervals (Li et al., 2006; Cai et al., 2007) and it was previously described in NTS neurons, mainly in inhibitory interneurons of NTS and baroreceptor second-order NTS neurons receiving C-fiber afferent inputs (Dekin and Getting, 1987; Dekin et al., 1987; Moak and Kunze, 1993; Paton et al., 1993; Tell and Bradley, 1994; Sundaram et al., 1997; Butcher and Paton, 1998; Bailey et al., 2002; Uteshev and Smith, 2006; Suwabe and Bradley, 2009). Our results demonstrated that SH decreases the IK_A in NTS neurons and consequently may facilitate the occurrence of the observed high-frequency of action potentials.

The mechanisms underlying the IK_A reduction after SH are not known, but recently Naskar and Stern (2014) demonstrated that IK_A is reduced in hypothalamic magnocellular neurosecretory neurons after astrocytic inhibition. The au-

thors showed that during dehydration, a condition known to produce a retraction of astrocytic process, the astrocytic glutamate GLT1 transporter was blunted, resulting in an abnormal increase in glutamate levels at the synaptic cleft. The endogenous extracellular glutamate activates extra-synaptic NMDA receptors, which leads to inhibition of IK_A in a Ca^{2+} and protein kinase C-dependent manner. To test the possibility that astrocyte can also modulate the IK_A in our experiments, we incubated the NTS slices with FAC, a glial metabolism inhibitor (Fonnum et al., 1997). FAC decreased IK_A in nonlabeled NTS neurons from control rats, but surprisingly produced no change in IK_A amplitude of SH rats, suggesting that SH exposure affects the functional coupling between astrocytes and intrinsic properties of NTS neurons, but the mechanisms underlying this effect of SH on astrocytes remain to be evaluated.

We must also consider the possibility that SH affects the subunits that constitute the potassium channels or even a decrease in

the number of channels mediating IKA in NTS neurons. Voltage-dependent potassium channels subunits are divided in eight subfamilies (Coetzee et al., 1999) and channels containing Kv3.4 subunits mediate IKA, which are downregulated by chronic hypoxia in glomus cells of the carotid body (Käbb et al., 2005). Considering that NTS presented high immunoreactivity for Kv3.4 subunit (Brooke et al., 2004), we suggest that SH decreases the Kv3.4 expression, reducing the channel conductance and increasing the excitability of NTS neurons. Such substantial difference in IKA between NTS neurons from control and SH rats may impact on the ability of afferent synaptic inputs to excite the postsynaptic neurons during SH. Moreover, the enhanced excitability of NTS neurons can be also related to an increase in the excitatory transmission and for this reason, we evaluated the synaptic transmission of NTS neurons. For this purpose, we stimulated TS afferent fibers and recorded the action potential in NTS neurons. In fact, the percentage of failure to induce action potential in SH rats (18%) was reduced compared with the control group (53%), indicating that after SH the inputs stimulation are much more efficient to produce action potential.

To clarify the mechanisms involved in TS-evoked discharge, we analyzed the synaptic currents as a parameter of evoked transmission in SH rats. The data show that TS-evoked currents in NTS neuron from SH rats presented higher amplitude compared with those from control rats, indicating that SH also affects the synaptic currents in nonlabeled NTS neurons. In accordance with the analysis of synaptic plasticity (comparison of $1/CV^2$ and synaptic depression protocol) we are assuming that its effect is not on afferent fibers. It is important to note that SH can affect astrocytes or even the recorded neuron. On the other hand, chronic SH (7 d) produces changes in both presynaptic and postsynaptic mechanisms, with consequent increase in the amplitude of TS-evoked current in NTS neurons (Zhang et al., 2009). In this context, previous study from our laboratory described that CIH during 10 d decreased the TS-evoked currents in NTS neurons (Almado et al., 2012) and suggested that the CIH-induced synaptic depression was due to the reduction in the number of active synapses. Therefore, the duration of hypoxia may activate different mechanisms affecting the synaptic transmission, contributing to changes in the excitability of neurons.

In the present study we also evaluated the spontaneous currents, an index of basal neurotransmission, and interestingly SH increased the frequency of total (inhibitory and excitatory currents) and excitatory spontaneous currents, with no change in amplitude or half-width of events, indicating that SH affected the spontaneous transmission by presynaptic mechanisms. These changes in spontaneous transmission are action-potential dependent, because there is no difference in mSEPSCs in NTS neurons from control or SH rats. In this regard, SH clearly induces a facilitatory effect on the spontaneous glutamatergic neurotransmission in the NTS.

We conclude that exposure to short-term SH increases the autonomic and respiratory responses of chemoreflexes and baroreflexes, probably due to the increased excitation at the second order NTS neurons associated to: (1) reduction in IKA consequent to a decrease in astrocytic modulation and (2) an increase in spontaneous excitatory synaptic transmission.

References

Abdala AP, McBryde FD, Marina N, Hendy EB, Engelman ZJ, Fudim M, Sobotka PA, Gourine AV, Paton JF (2012) Hypertension is critically dependent on the carotid body input in the spontaneously hypertensive rat. *J Physiol* 590:4269–4277. [CrossRef Medline](#)

Accorsi-Mendonça D, Bonagamba LG, Leão RM, Machado BH (2009) Are L-glutamate and ATP cotransmitters of the peripheral chemoreflex in the rat nucleus tractus solitarius? *Exp Physiol* 94:38–45. [CrossRef Medline](#)

Accorsi-Mendonça D, Castania JA, Bonagamba LG, Machado BH, Leão RM (2011) Synaptic profile of nucleus tractus solitarius neurons involved with the peripheral chemoreflex pathways. *Neuroscience* 197:107–120. [CrossRef Medline](#)

Accorsi-Mendonça D, Machado BH (2013) Synaptic transmission of baro- and chemoreceptors afferents in the NTS second order neurons. *Auton Neurosci* 175:3–8. [CrossRef Medline](#)

Accorsi-Mendonça D, Leão RM, Aguiar JF, Varanda WA, Machado BH (2007) Urethane inhibits the GABAergic neurotransmission in the nucleus of the solitary tract of rat brain stem slices. *Am J Physiol Regul Integr Comp Physiol* 292:R396–R402. [CrossRef Medline](#)

Almado CE, Machado BH, Leão RM (2012) Chronic intermittent hypoxia depresses afferent neurotransmission in NTS neurons by a reduction in the number of active synapses. *J Neurosci* 32:16736–16746. [CrossRef Medline](#)

Antunes VR, Braga VA, Machado BH (2005) Autonomic and respiratory responses to microinjection of ATP into the intermediate or caudal nucleus tractus solitarius in the working heart-brainstem preparation of the rat. *Clin Exp Pharmacol Physiol* 32:467–472. [CrossRef Medline](#)

Baekey DM, Dick TE, Paton JF (2008) Pontomedullary transection attenuates central respiratory modulation of sympathetic discharge, heart rate and the baroreceptor reflex in the in situ rat preparation. *Exp Physiol* 93:803–816. [CrossRef Medline](#)

Bailey TW, Jin YH, Doyle MW, Andresen MC (2002) Vanilloid-sensitive afferents activate neurons with prominent A-type potassium currents in nucleus tractus solitarius. *J Neurosci* 22:8230–8237. [Medline](#)

Barraco R, el-Ridi M, Ergene E, Parizon M, Bradley D (1992) An atlas of the rat subpostremal nucleus tractus solitarius. *Brain Res Bull* 29:703–765. [Medline](#)

Basu CK, Selvamurthy W, Bhaumick G, Gautam RK, Sawhney RC (1996) Respiratory changes during initial days of acclimatization to increasing altitudes. *Aviat Space Environ Med* 67:40–45. [Medline](#)

Bekkers JM, Stevens CF (1990) Presynaptic mechanism for long-term potentiation in the hippocampus. *Nature* 346:724–729. [CrossRef Medline](#)

Brooke RE, Atkinson L, Batten TF, Deuchars SA, Deuchars J (2004) Association of potassium channel Kv3.4 subunits with pre- and post-synaptic structures in brainstem and spinal cord. *Neuroscience* 126:1001–1010. [CrossRef Medline](#)

Brophy S, Ford TW, Carey M, Jones JF (1999) Activity of aortic chemoreceptors in the anesthetized rat. *J Physiol* 514:821–828. [CrossRef Medline](#)

Butcher JW, Paton JF (1998) K⁺ channel blockade in the NTS alters efficacy of two cardiorespiratory reflexes in vivo. *Am J Physiol* 274:R677–R685. [Medline](#)

Cai SQ, Li W, Sesti F (2007) Multiple modes of a-type potassium current regulation. *Curr Pharm Des* 13:3178–3184. [CrossRef Medline](#)

Calbet JA (2003) Chronic hypoxia increases blood pressure and noradrenaline spillover in healthy humans. *J Physiol* 551:379–386. [CrossRef Medline](#)

Chen CY, Horowitz JM, Bonham AC (1999) A presynaptic mechanism contributes to depression of autonomic signal transmission in NTS. *Am J Physiol* 277:H1350–H1360. [Medline](#)

Chitravanshi VC, Sapru HN (1995) Chemoreceptor-sensitive neurons in commissural subnucleus of nucleus tractus solitarius of the rat. *Am J Physiol* 268:R851–R858. [Medline](#)

Claps A, Torrealba F (1988) The carotid body connections: a WGA-HRP study in the cat. *Brain Res* 455:123–133. [CrossRef Medline](#)

Coetzee WA, Amarillo Y, Chiu J, Chow A, Lau D, McCormack T, Moreno H, Nadal MS, Ozaita A, Pountney D, Saganich M, Vega-Saenz de Miera E, Rudy B (1999) Molecular diversity of K⁺ channels. *Ann N Y Acad Sci* 868:233–285. [CrossRef Medline](#)

Contreras RJ, Beckstead RM, Norgren R (1982) The central projections of the trigeminal, facial, glossopharyngeal and vagus nerves: an autoradiographic study in the rat. *J Auton Nerv Syst* 6:303–322. [Medline](#)

Dekin MS, Getting PA (1987) *In vitro* characterization of neurons in the ventral part of the nucleus tractus solitarius: II. Ionic basis for repetitive firing patterns. *J Neurophysiol* 58:215–229. [Medline](#)

Dekin MS, Getting PA, Johnson SM (1987) *In vitro* characterization of neurons in the ventral part of the nucleus tractus solitarius: I. Identification of

- neuronal types and repetitive firing properties. *J Neurophysiol* 58:195–214. [Medline](#)
- Donoghue S, Felder RB, Jordan D, Spyer KM (1984) The central projections of carotid baroreceptors and chemoreceptors in the cat: a neurophysiological study. *J Physiol* 347:397–409. [CrossRef Medline](#)
- Doyle MW, Andresen MC (2001) Reliability of monosynaptic sensory transmission in brain stem neurons *in vitro*. *J Neurophysiol* 85:2213–2223. [Medline](#)
- Finley JC, Katz DM (1992) The central organization of carotid body afferent projections to the brainstem of the rat. *Brain Res* 572:108–116. [CrossRef Medline](#)
- Fonnum F, Johnsen A, Hassel B (1997) Use of fluorocitrate and fluoroacetate in the study of brain metabolism. *Glia* 21:106–113. [CrossRef Medline](#)
- Glaum SR, Miller RJ (1995) Presynaptic metabotropic glutamate receptors modulate omega-conotoxin-GVIA-insensitive calcium channels in the rat medulla. *Neuropharmacology* 34:953–964. [CrossRef Medline](#)
- Hackett PH, Roach RC (2001) High-altitude illness. *N Engl J Med* 345:107–114. [CrossRef Medline](#)
- Head GA, Adams MA (1988) Time course of changes in baroreceptor reflex control of heart rate in conscious SHR and WKY: contribution of the cardiac vagus and sympathetic nerves. *Clin Exp Pharmacol Physiol* 15:289–292. [CrossRef Medline](#)
- Käab S, Miguel-Velado E, López-López JR, Pérez-García MT (2005) Down regulation of Kv3.4 channels by chronic hypoxia increases acute oxygen sensitivity in rabbit carotid body. *J Physiol* 566:395–408. [CrossRef Medline](#)
- Lancaster B, Nicoll RA (1997) Properties of two calcium-activated hyperpolarizations in rat hippocampal neurones. *J Physiol* 389:187–203. [CrossRef Medline](#)
- Li Q, Bi HR, Zhang R, Zhu DL (2006) Kv3.4 channel is involved in rat pulmonary vasoconstriction induced by 15-hydroxyeicosatetraenoic acid. *Sheng Li Xue Bao* 58:77–82. [Medline](#)
- Liu X, He L, Dinger B, Stensaa L, Fidone S (2013) Sustained exposure to cytokines and hypoxia enhances excitability of oxygen-sensitive type I cells in rat carotid body: correlation with the expression of HIF-1 α protein and adrenomedullin. *High Alt Med Biol* 14:53–60. [CrossRef Medline](#)
- Machado BH (2001) Neurotransmission of the cardiovascular reflexes in the nucleus tractus solitarius of awake rats. *Ann N Y Acad Sci* 940:179–196. [CrossRef Medline](#)
- Machado BH (2004) Chemoreflex and sympathoexcitation. In: *Neural mechanisms of cardiovascular regulation* (Dun NJ, Machado BH, Pilowsky PM, eds), pp 59–80. Boston: Kluwer Academic.
- Madison DV, Nicoll RA (1984) Control of the repetitive discharge of rat CA1 pyramidal neurones *in vitro*. *J Physiol* 354:319–331. [CrossRef Medline](#)
- Malinow R, Tsien RW (1990) Presynaptic enhancement shown by whole-cell recordings of long-term potentiation in hippocampal slices. *Nature* 346:177–180. [CrossRef Medline](#)
- Marcus NJ, Del Rio R, Schultz EP, Xia XH, Schultz HD (2014) Carotid body denervation improves autonomic and cardiac function and attenuates disordered breathing in congestive heart failure. *J Physiol* 592:391–408. [CrossRef Medline](#)
- Mathie A, Woollorton JR, Watkins CS (1998) Voltage-activated potassium channels in mammalian neurons and their block by novel pharmacological agents. *Gen Pharmacol* 30:13–24. [CrossRef Medline](#)
- McBryde FD, Abdala AP, Hendy EB, Pijacka W, Marvar P, Moraes DJ, Sobotka PA, Paton JF (2013) The carotid body as a putative therapeutic target for the treatment of neurogenic hypertension. *Nat Commun* 4:2395. [CrossRef Medline](#)
- McDonald DM (1983) Morphology of the rat carotid sinus nerve: I. Course, connections, dimensions and ultrastructure. *J Neurocytol* 12:345–372. [CrossRef Medline](#)
- Mendelowitz D, Yang M, Andresen MC, Kunze DL (1992) Localization and retention *in vitro* of fluorescently labeled aortic baroreceptor terminals on neurons from the nucleus tractus solitarius. *Brain Res* 581:339–343. [CrossRef Medline](#)
- Mifflin SW (1992) Arterial chemoreceptor input to nucleus tractus solitarius. *Am J Physiol* 263:R368–R375. [Medline](#)
- Miles R (1986) Frequency dependence of synaptic transmission in nucleus of the solitary tract *in vitro*. *J Neurophysiol* 55:1076–1090. [Medline](#)
- Moak JP, Kunze DL (1993) Potassium currents of neurons isolated from medical nucleus tractus solitarius. *Am J Physiol* 265:H1596–H602. [Medline](#)
- Moraes DJ, Bonagamba LG, Costa KM, Costa-Silva JH, Zoccal DB, Machado BH (2014) Short-term sustained hypoxia induces changes in the coupling of sympathetic and respiratory activities in rats. *J Physiol* 592:2013–2033. [CrossRef Medline](#)
- Moraes DJ, Bonagamba LG, Zoccal DB, Machado BH (2011) Modulation of respiratory responses to chemoreflex activation by L-glutamate and ATP in the rostral ventrolateral medulla of awake rats. *Am J Physiol Regul Integr Comp Physiol* 300:R1476–R1486. [CrossRef Medline](#)
- Moraes DJ, Zoccal DB, Machado BH (2012) Sympathoexcitation during chemoreflex active expiration is mediated by L-glutamate in the RVLM/Bötzing complex of rats. *J Neurophysiol* 108:610–623. [CrossRef Medline](#)
- Moraes DJ, da Silva MP, Bonagamba LG, Mecawi AS, Zoccal DB, Antunes-Rodrigues J, Varanda WA, Machado BH (2013) Electrophysiological properties of rostral ventrolateral medulla presympathetic neurons modulated by the respiratory network in rats. *J Neurosci* 33:19223–19237. [CrossRef Medline](#)
- Naskar K, Stern JE (2014) A functional coupling between extrasynaptic NMDA receptors and A-type K $^{+}$ channels under astrocyte control regulates hypothalamic neurosecretory neuronal activity. *J Physiol* 592:2813–2827. [CrossRef Medline](#)
- Neher E, Sakaba T (2001) Estimating transmitter release rates from postsynaptic current fluctuations. *J Neurosci* 21:9638–9654. [Medline](#)
- Ortega-Sáenz P, Pardo R, Levitsky K, Villadiego J, Muñoz-Manchado AB, Durán R, Bonilla-Henao V, Arias-Mayenco I, Sobrino V, Ordóñez A, Oliver M, Toledo-Aral JJ, López-Barneo J (2013) Cellular properties and chemosensory responses of the human carotid body. *J Physiol* 591:6157–6173. [CrossRef Medline](#)
- Ortiz F, Iturriaga R, Varas R (2009) Sustained hypoxia enhances TASK-like current inhibition by acute hypoxia in rat carotid body type-I cells. *Adv Exp Med Biol* 648:83–88. [CrossRef Medline](#)
- Paton JF (1996) A working heart-brainstem preparation of the mouse. *J Neurosci Methods* 65:63–68. [CrossRef Medline](#)
- Paton JF, Foster WR, Schwaber JS (1993) Characteristic firing behavior of cell types in the cardiorespiratory region of the nucleus tractus solitarius of the rat. *Brain Res* 604:112–125. [CrossRef Medline](#)
- Paton JF, Boscan P, Pickering AE, Nalivaiko E (2005) The yin and yang of cardiac autonomic control: vago-sympathetic interactions revisited. *Brain Res Brain Res Rev* 49:555–565. [CrossRef Medline](#)
- Paton JF, Ratcliffe L, Hering D, Wolf J, Sobotka PA, Narkiewicz K (2013) Revelations about carotid body function through its pathological role in resistant hypertension. *Curr Hypertens Rep* 15:273–280. [CrossRef Medline](#)
- Pongs O (1999) Voltage-gated potassium channels: from hyperexcitability to excitement. *FEBS Lett* 452:31–35. [CrossRef Medline](#)
- Roach RC, Hackett PH (2001) Frontiers of hypoxia research: acute mountain sickness. *J Exp Biol* 204:3161–3170. [Medline](#)
- Scheuer DA, Zhang J, Toney GM, Mifflin SW (1996) Temporal processing of aortic nerve evoked activity in the nucleus of the solitary tract. *J Neurophysiol* 76:3750–3757. [Medline](#)
- Schild JH, Clark JW, Canavier CC, Kunze DL, Andresen MC (1995) Afferent synaptic drive of rat medial nucleus tractus solitarius neurons: dynamic simulation of graded vesicular mobilization, release, and non-NMDA receptor kinetics. *J Neurophysiol* 74:1529–1548. [Medline](#)
- Seiders EP, Stuesse SL (1984) A horseradish peroxidase investigation of carotid sinus nerve components in the rat. *Neurosci Lett* 46:13–18. [CrossRef Medline](#)
- Storm JF (1987) Action potential repolarization and a fast after-hyperpolarization in rat hippocampal pyramidal cells. *J Physiol* 385:733–759. [CrossRef Medline](#)
- Sundaram K, Johnson SM, Felder RB (1997) Altered expression of delayed excitation in medial NTS neurons of spontaneously hypertensive rats. *Neurosci Lett* 225:205–209. [CrossRef Medline](#)
- Suwabe T, Bradley RM (2009) Characteristics of rostral solitary tract nucleus neurons with identified afferent connections that project to the parabrachial nucleus in rats. *J Neurophysiol* 102:546–555. [CrossRef Medline](#)
- Tamisier R, Anand A, Nieto LM, Cunningham D, Weiss JW (2005) Arterial pressure and muscle sympathetic nerve activity are increased after two

- hours of sustained but not cyclic hypoxia in healthy humans. *J Appl Physiol* 98:343–349. [Medline](#)
- Tell F, Bradley RM (1994) Whole-cell analysis of ionic currents underlying the firing pattern of neurons in the gustatory zone of the nucleus tractus solitarius. *J Neurophysiol* 71:479–492. [Medline](#)
- Uteshev VV, Smith DV (2006) Cholinergic modulation of neurons in the gustatory region of the nucleus of the solitary tract. *Brain Res* 1084:38–53. [CrossRef Medline](#)
- Wang ZY, Baker TL, Keith IM, Mitchell GS, Bisgard GE (2000) Continuous, but not episodic hypoxia, induces CREB phosphorylation in rat carotid body type I cells. *Adv Exp Med Biol* 475:631–635. [Medline](#)
- Zhang W, Carreño FR, Cunningham JT, Mifflin SW (2008) Chronic sustained and intermittent hypoxia reduce function of ATP-sensitive potassium channels in nucleus of the solitary tract. *Am J Physiol Regul Integr Comp Physiol* 295:R1555–R1562. [CrossRef Medline](#)
- Zhang W, Carreño FR, Cunningham JT, Mifflin SW (2009) Chronic sustained hypoxia enhances both evoked EPSCs and norepinephrine inhibition of glutamatergic afferent inputs in the nucleus of the solitary tract. *J Neurosci* 29:3093–3102. [CrossRef Medline](#)
- Zoccal DB, Bonagamba LG, Paton JF, Machado BH (2009) Sympathetic-mediated hypertension of awake juvenile rats submitted to chronic intermittent hypoxia is not linked to baroreflex dysfunction. *Exp Physiol* 94: 972–983. [CrossRef Medline](#)
- Zucker RS, Regehr WG (2002) Short-term synaptic plasticity. *Annu Rev Physiol* 64:355–405. [CrossRef Medline](#)