Exercise training normalizes an increased neuronal excitability of NTS-projecting neurons of the hypothalamic paraventricular nucleus in hypertensive rats

Javier E. Stern1, Patrick M. Sonner 1*, Sook Jin Son1, Fabiana C. P. Silva1,2, Keshia Jackson1 and Lisete C. Michelini2

1Department of Physiology, Georgia Health Sciences University, Augusta GA
2Department of Physiology & Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, Brazil

* Current Address: Department of Neuroscience, Cell Biology and Physiology, Wright State University, Dayton, OH

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Corresponding author:
Javier E. Stern M.D., Ph.D.
Department of Physiology
Georgia Health Sciences University
Augusta GA 30912
Elevated sympathetic outflow and altered autonomic reflexes, including impaired baroreflex function, are common findings observed in hypertensive disorders. While a growing body of evidence supports a contribution of preautonomic neurons in the hypothalamic paraventricular nucleus (PVN) to altered autonomic control during hypertension, the precise underlying mechanisms remain unknown. Here, we aimed to determine whether the intrinsic excitability and repetitive firing properties of preautonomic PVN neurons that innervate the nucleus of the solitarii tract (PVN-NTS neurons) was altered in spontaneously hypertensive rats (SHRs). Moreover, given that exercise training is known to improve and/or correct autonomic deficits in hypertensive conditions, we evaluated whether exercise is an efficient behavioral approach to correct altered neuronal excitability in hypertensive rats. Patch-clamp recordings were obtained from retrogradely-labeled PVN-NTS neurons in hypothalamic slices obtained from sedentary (S) and trained (T) Wistar Kyoto (WKY) and SHR rats. Our results indicate an increased excitability of PVN-NTS neurons in SHR-S rats, reflected by an enhanced input-output function in response to depolarizing stimuli, a hyperpolarizing shift in Na⁺ spike threshold, and smaller hyperpolarizing afterpotentials (HAPs). Importantly, we found exercise training in SHRs to restore all these parameters back to those levels observed in WKY-S rats. In several cases, exercise evoked opposing effects in WKY-S rats, when compared to SHR-S rats, suggesting that exercise effects on PVN-NTS neurons are state-dependent. Taken together, our results suggest that elevated preautonomic PVN-NTS neuronal excitability may contribute to altered autonomic control in SHR rats, and that exercise training efficiently corrects these abnormalities.
INTRODUCTION

Elevated sympathetic outflow (Bergamaschi et al. 1995; Esler and Kaye 1998; Judy et al. 1976) and altered autonomic reflexes, including impaired baroreflex function (Grassi 2004; Judy and Farrell 1979) contribute to the development and maintenance of hypertensive disorders. Thus, elucidating the mechanisms underlying altered autonomic control in hypertension is of critical importance.

The hypothalamic paraventricular nucleus (PVN) is a major homeostatic center involved in the control of sympathetic outflow and regulation of blood pressure (Coote et al. 1998; Dampney et al. 2005; Swanson and Sawchenko 1983). PVN actions are mediated by preautonomic neurons that send descending projections to sympathetic preganglionic neurons in the intermediolateral column of the spinal cord (IML), as well as via projections to the rostral ventrolateral medulla (RVLM) and the nucleus of the solitarii tract (NTS) (Armstrong et al. 1980; Saper et al. 1976; Swanson and Kuypers 1980) in the medulla. Whereas PVN-RVLM and PVN-IML pathways have been shown to mediate sympathoexcitatory responses (Allen 2002; Tagawa and Dampney 1999; Yang and Coote 1998), PVN-NTS projections have been implicated in PVN-mediated suppression of baroreflex function (Chen et al. 1996; Duan et al. 1999; Hwang et al. 1998; Jin and Rockhold 1989; Michelini 1994; Pan et al. 2007; Patel and Schmid 1988), as well as in heart rate adaptation during exercise training (Higa et al. 2002; Michelini and Stern 2009). Importantly, a growing body of evidence supports overactivation of preautonomic PVN neurons and their descending pathways, as a major mechanism contributing to sympathoexcitation, altered reflex function and elevated blood pressure in hypertensive disorders (Allen 2002; Earle et al. 1992; Li and Pan 2007b; Martin and Haywood 1998). Several mechanisms have been proposed to mediate elevated preautonomic PVN neuronal activity in hypertension, including a shift in the balance of inhibitory/excitatory synaptic transmitters (Biancardi et al. 2010; Li and Pan 2007a; b; Osborn et al. 2007), as well as changes in intrinsic conductances, including altered $K^+$ and $Ca^{2+}$ channel function (Chen et al. 2010; Sonner et al. 2008; Sonner et al. 2010). For the most part however, these studies focused on PVN-RVLM and PVN-IML neurons. Thus, whether the excitability and/or activity of PVN-NTS neurons is also elevated in hypertensive rats, as a potential mechanism underlying altered baroreflex function in this condition, is at present unknown.
It is now widely recognized that physical activity reduces the risk of cardiovascular disease, promoting several beneficial cardiovascular adjustments, including remodeling of the heart and skeletal muscle circulation (Amaral et al. 2000; Melo et al. 2003), and improvement of autonomic control of the heart (Clausen 1977; Negrao et al. 1993). Importantly, exercise has been shown to improve and/or correct deficits associated with hypertension, both in humans and animal models, including diminished sympathetic activity and pressure (Amaral et al. 2000; Collins et al. 2000; Melo et al. 2003), improvement of vagal outflow, as well as baroreflex function (Brum et al. 2000; Pan et al. 2007).

Our knowledge on the central mechanisms underlying beneficial effects of the exercise on autonomic control, particularly during hypertension, is however, limited (see reviews of Waldrop et al. 1996; Potts, 2006; Raven et al, 2006; Michelini, 2007a, 2007b). Recent studies from our laboratories support the PVN as a likely brain region target mediating cardiovascular and autonomic effects of exercise. In this sense, we found exercise training to induce robust anatomical and functional plasticity within the PVN, including reorganization of afferent noradrenergic pathways, structural remodeling of dendritic trees, increased expression of peptidergic mRNA, and changes in intrinsic neuronal excitability (Higa-Taniguchi et al. 2007; Jackson et al. 2005; Martins et al. 2005; Michelini and Stern 2009), we proposed these actions to contribute to beneficial adaptive cardiovascular responses to exercise activity in normal rats (see (Michelini and Stern 2009) for review). In addition, we found exercise to diminish nor-adrenergic inputs to the PVN in spontaneously hypertensive rats (SHRs) (Higa-Taniguchi et al. 2007), and to partially correct a deficit in PVN and NTS oxytocinergic system in SHR (Martins et al. 2005). However, whether exercise training also affects and/or corrects altered neuronal excitability of preautonomic PVN neurons in hypertensive conditions is at present unknown.

Based on all this evidence, we aimed in the present study to (1) determine whether the intrinsic excitability of PVN-NTS neurons is altered in SHR rats, and (2) whether exercise training is an efficient approach to correct altered excitability in this important cardiovascular-associated pathway. To this end, patch-clamp electrophysiological recordings were obtained from retrogradely-labeled PVN-NTS neurons in four experimental groups: normotensive WKY sedentary (WKY-S), WKY trained (WKY-T), SHR sedentary (SHR-S) and SHR trained (SHR-T) rats. A detailed analysis of basic membrane properties, single action potential waveforms and repetitive firing properties were obtained from PVN-NTS neurons in these experimental groups.
Overall, our results indicate an increased excitability of PVN-NTS neurons in SHR-S rats, mostly reflected by an enhanced input-output function in response to depolarizing stimuli. Moreover, we found exercise training to restore the elevated PVN-NTS firing discharge during repetitive stimulation back to those levels observed in normotenstive WKY rats. The potential mechanisms underlying these effects are discussed in details.

METHODS

Animals and exercise training protocols

All protocols and surgical procedures used were approved the Georgia Health Sciences University Institutional Animal Care and Use Committee. Two months old male Wistar Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR), both based on a Wistar strain background (Harlan laboratories, USA) were housed in Plexiglas cages on a 12/12-hour light/dark schedule and allowed free access to food and water. Rats were pre-selected for their ability to walk on a treadmill (5-10 sessions, 0.3 up to 0.9 km/h, 0% grade, 10-15 min/day), and only active rats (16 WKY, 16 SHR) were used in this study. At week zero, before starting protocols, active rats were submitted to maximal exercise test (graded exercise on the treadmill, starting at 0.3 km/h with increments of 0.3 km/h every 3 min up to exhaustion) in order to determine maximal individual exercise capacities and to assign rats with equivalent capability to trained (T) or sedentary (S) groups. Half of the rats of each group were submitted to low-intensity training performed twice/day (1 hour each), 5 days/week over 6 weeks, as previously described (Jackson et al. 2005). Briefly, exercise intensity was increased progressively by a combination of time and speed to attain 50-60% of maximal exercise capacity, as determined by the maximal exercise tests on the treadmill. Maximal exercise tests were repeated at the 3rd and 6th week in order to adjust training intensity and to compare the efficacy of the training protocol, respectively. Rats allocated to S protocol were kept sedentary for a similar period of time and handled every day. Systolic blood pressure was measured using tail cuff method (RTBP 2000; Kent Scientific Corporation, CT) in conscious rats. Animals were restrained in a holder and the nose cone was adjusted that so the animal was comfortable but not able to move freely. The holder used was able to auto-monitor a pre set up temperature (36°C). Rats were allowed to acclimatize for 10 min before measurements were obtained (average of 10 measurements/rat). Blood pressure, as
well as body weight, were measured weekly during S and T protocols. Mean systolic pressure values obtained in each group at the end of the training protocol are summarized in Fig.1.

**Retrograde labeling of PVN-NTS projecting neurons**

At the end of the 6th week of the T protocol, rats from the four experimental groups (WKY-S, WKY-T, SHR-S and SHR-T) were anesthetized by intraperitoneal injection of ketamine/xylazine mixture (90 mg/kg and 5 mg/kg, respectively), placed in a stereotaxic frame, and injected with a fluorescent retrograde tracer in the area of the NTS, as previously described (Li et al. 2003). The dorsal medulla was exposed after retraction of overlying muscles and occipital membranes. A small part of the occipital bone was removed to increase the exposure of the medulla. Rhodamine-labeled microspheres (Lumaflor, Naples FL) were pressure-injected unilaterally (200 nl) into the NTS area at the level of the obex. The injection point was 1.0 mm lateral to the midline, and 0.8 mm below the dorsal surface. After the injection, muscles were sutured together and the wound was closed. The location and extension of the injection sites were confirmed histologically. Injections in the NTS area were mostly restricted to caudal aspects of the nucleus of the solitary tract although they also extended ventrally into the dorsal motor nucleus of the vagus (rostrocaudal extension: Bregma -16.2 to -15.6) (see also (Stern 2001)).

**Slice preparation**

Three days after the injection of the retrograde tracer, coronal hypothalamic slices (150-300 μm) containing the PVN were obtained for electrophysiological recordings using a vibroslicer (D.S.K. Microslicer, Ted Pella, Redding, CA), as previously described (Stern 2001). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg IP) and perfused through the heart with cold artificial cerebrospinal solution (aCSF) in which NaCl was replaced by an equiosmolar amount of sucrose, a procedure known to improve the viability of neurons in adult brain slices (Aghajanian and Rasmussen 1989). The standard aCSF solution contained (in mM): 120 NaCl, 2.5 KCl, 1.25 NaH2PO4, 1 MgSO4, 2 CaCl2, 26 NaHCO3, 20 glucose, and 0.4 ascorbic acid, pH 7.4 (297-300 mOsm). After the slicing procedure, hypothalamic slices were placed in a holding chamber containing standard oxygenated aCSF and stored at room temperature (22-24°C) until used.
Electrophysiology and data analysis

For electrophysiological recordings, a slice was transferred to a submersion-type recording chamber, continuously perfused (~2 ml/min) with a standard solution bubbled with a gas mixture of 95% O\textsubscript{2} and 5% CO\textsubscript{2}. All recordings were performed at 30-32°C. Patch pipettes (3 - 5 MΩ) were pulled from thin-wall (1.5 mm o.d., 1.17 mm i.d) borosilicate glass (GC150T-7.5, Clark, Reading, UK) on a horizontal electrode puller (P-97, Sutter Instruments, Novato, CA). The pipette internal solution contained (in mM) 135 K-gluconate, 20 KCl, 10 Hepes, 4 MgATP, 20 Phosphocreatine (Na\textsuperscript{+}), 0.3 NaGTP, and 0.2 EGTA, pH 7.3 (295 mOsm). Whole-cell recordings of PVN neurons were made under visual control using infrared differential interference contrast (IR-DIC) video microscopy in combination with epifluorescence illumination, as previously described (Jackson et al. 2005; Stern 2001). Recordings were obtained with a Multiclamp 700B (Axon Instruments, Foster City, CA) amplifier. No correction was made for the pipette liquid junction potential (measured to be ~10 mV). The series resistance was monitored throughout the experiment, and data was discarded if series resistance during recordings doubled from the one obtained at the beginning of the recording. The voltage output was digitized at 16-bit resolution in conjunction with pClamp 8 software (Digidata 1320, Axon Instruments, Foster City, CA). Data were digitized at 10 kHz and transferred to a disk. All neurons included in the analysis had membrane potentials of -40 mV or more negative, and action potentials of at least +55 mV. Cell input resistance and cell capacitance were calculated in voltage-clamp using a 5 mV pulse while holding the cells at -70 mV. To measure single action potential properties, neurons were current-clamped close to Na\textsuperscript{+} spike threshold and a 5 ms, 0.1 nA depolarizing pulse was applied. The action potential properties were analyzed using a peak detection program (MiniAnalysis, Synaptosoft, Leonia, NJ). Spike threshold was calculated based on the third derivative of the action potential waveform implemented by MiniAnalysis software (Botta et al. 2010; Meeks et al. 2005; Sonner and Stern 2007). Spike amplitude was then measured from the estimated threshold to the peak of the action potential, while spike width was measured at 50 % of the peak. (Sonner and Stern 2007) To study the input-output relationship of PVN neurons, repetitive firing was evoked by injecting 400 ms depolarizing current pulses of varying amplitudes, and plots of the number of evoked spikes as a function of the injected current were generated. Given the high degree of action potential dampening observed in some groups during repetitive firing (see Results) we used an arbitrary criteria to
define “spikeletes” as those waves whose amplitude were between 10-30% of the first spike in the train. Waves <10% were not considered for analysis. The amplitude of the hyperpolarizing after potential (HAP), a membrane hyperpolarization that follows the falling phase of individual action potential beyond resting Vm, was calculated and compared among groups. The amplitude of the after-hyperpolarizing potential (AHP), a prominent hyperpolarization that typically follows a train of action potentials in SON and PVN neurons (Armstrong et al. 1994; Chen and Toney 2009; Greffrath et al. 1998; Stern 2001), was measured following a 200 ms depolarizing step of 150 pA, and data corrected for the number of spikes in each evoked train (Andrew and Dudek 1984).

**Statistics**

Numerical data are presented as means ± SEM. The reported “n’s” represent the number of recorded neurons. In most cases, a two-way analysis of variance (ANOVA) was used, followed by a Bonferoni’s posthoc test. The two main factors were physical activity (sedentary vs. trained) and blood pressure (normotensive WKYs vs hypertensive SHRs). For convenience, in most cases F and related P values from the 2 way ANOVA are reported either in table or in figure legends, whereas results of post-hoc P values are reported in the main text. Differences in the incidence of functional properties across experimental groups were analysed using a Chi-square tests. Differences were considered statistically significant at P< 0.05.

**RESULTS**

**Efficacy of the exercise training protocol in normotensive and hypertensive rats**

At the beginning of protocols (week zero), peak velocity attained during graded exercise test was higher in SHR than WKY groups (1.58 ± 0.25 vs. 1.04 ± 0.29 km/h, P <0.05, **Fig. 1A**). Low intensity T was equally effective to improve treadmill performance in both groups: marked and parallel increases on the attained velocity were already observed at the 3rd week, with further increases on the 6th week of training. At the end of protocols T groups exhibited similar increases on treadmill performance (SHR= +1.63 ± 0.18; WKY= +1.65 ± 0.23 km/h from week zero to week 6, P <0.05 vs. respective S controls, **Fig.1B**) while S groups showed no significant changes during the 6 weeks period. All groups gained weight during T and S protocols, but body weight
gain was smaller in T groups compared to S controls (on average +143 ± 4 g and +166 ± 4 g for T and S groups, respectively, corresponding to a 14% decrease, Fig. 1C). At the end of protocols tail pressure was significantly increased in SHR-S vs. WKY-S, and significantly decreased by T only in the SHR group ( - 8.3%, Fig.1D). We have previously shown that SHR rats of similar age and strain were already in the established phase of the hypertension when the training protocol started (Amaral et al. 2000; Martins et al. 2005).

Figure 1 here

Exercise training differentially affects repetitive firing of PVN-NTS neurons in normotensive and hypertensive rats

Electrophysiological recordings were obtained from retrogradely-labeled, PVN-NTS projecting neurons (PVN-NTS) in four experimental groups: WKY sedentary (WKY-S, n= 24), WKY trained (WKY-T, n= 29), SHR sedentary (SHR-S, n= 28) and SHR trained (SHR-T, n= 16). As previously described (Stern 2001), recorded PVN-NTS neurons were characterized by the presence of a low-threshold spike (LTS), when depolarized from a hyperpolarized membrane potential (not shown). The majority of PVN-NTS recorded neurons in our conditions were spontaneously active (WKY-S: 20/24, 0.56 ± 0.14 Hz; WKY-T: 23/29, 0.62 ± 0.18 Hz; SHR-S: 19/28, 0.68 ± 0.24 Hz; and SHR-T: 10/16, 0.59 ± 0.26 Hz), and no differences in the incidence of spontaneous activity (P>0.3, Chi-square test) or firing rate (P> 0.9) were observed among groups.

Repetitive firing in PVN-NTS neurons was evoked by injecting depolarizing current pulses of incremental amplitude (10-200 pA). Representative examples of repetitive firing evoked in the four experimental groups are shown in Fig. 2.

Figure 2 here

To characterize the input-output (I/O) function of recorded neurons, a plot of the mean number of evoked action potentials (including spikelets), as a function of current injected was generated for each experimental group. As we previously reported in control rats (Jackson et al. 2005), the I/O function plot in WKY-S rats displayed a roughly parabolic relationship. The firing
discharge increased progressively at low intensity stimulation, reaching a peak at 79.1 ± 2.8 pA (~40% of maximum stimulation). Firing rate decreased thereafter, coinciding with a large degree of adaptation and/or dampening of action potential amplitude. As an index of dampening, we measured the number and proportion of “spikelets” recorded at a current step of 150 pA. In WKY-S rats, we measured 1.6 ± 0.3 “spikelets”, which represented 24.2 ± 3.5 % of the total number of spikes detected (see Fig.3). Exercise training in WKY rats significantly enhanced the I/O function, resulting in an overall increase in the number of action potentials evoked in response to depolarizing stimulation (F= 97.2, P< 0.0001, 2 Way ANOVA). Thus, in PVN-NTS neurons from WKY-T rats, the firing discharge increased progressively with stimulation amplitude, reaching on average a maximum firing frequency at 127.1 ± 11.9 pA (~65% of the maximum stimulation), a value significantly higher than that observed in WKY-S neurons (P< 0.0001). In this experimental group, the total number of “spikelets” was similar to WKY-S rats (1.7 ± 0.6, P>0.9), while their proportion was significantly diminished (14.0 ± 4.4%, P< 0.05) due to the total higher number of spikes in this group (see Fig.3). This indicates that the T-induced increase in firing discharge was not mainly due to an increase in the number of “spikelets”.

Interestingly, opposite results were observed in SHR rats. In SHR-S rats, the I/O function was similar to that observed in WKY-T rats. Thus, in this group the firing discharge increased progressively, showing little adaptation and/or dampening of action potential firing, reaching maximal firing frequency at 127.3 ± 10.2 pA (~65% of the maximum stimulation). At a current step of 150 pA, we measured 0.9 ± 0.3 “spikelets”, which represented 12.6 ± 3.9 % of the total number of spikes detected. In contrast to WKY, exercise training in SHR rats significantly diminished the gain of the I/O function, resulting in an overall lower number of action potentials in response to depolarizing stimulation (F= 46.7, P< 0.0001, 2 Way ANOVA). Thus, in PVN-NTS neurons from SHR-T rats, the firing discharge increased progressively at low stimulation, decreasing thereafter, as observed in WKY-S rats. The maximum firing discharge in SHR-T rats was attained on average at 85.6 ± 11.8 pA, a value significantly smaller than that observed in SHR-S neurons (P< 0.02). In SHR-T, the total number of “spikelets” (2.3 ± 0.4) as well as their relative proportion (38.4 ± 5.2%), increased significantly when compared to WKY-S rats (P< 0.05 and P< 0.01, respectively). This indicates that the T-induced decrease in firing discharge in SHR was partially due to an increase in the number of “spikelets”.
Trains of action potentials in PVN-NTS neurons were followed by a prominent after-hyperpolarizing potential (AHP, see examples in Fig. 4). Exercise training was found to diminish the magnitude of the AHP both in WKY and SHR rats (P< 0.05 in both cases). Similar results were observed when the AHP area was analysed (not shown).

Exercise training differentially affects action potential properties of PVN-NTS neurons in normotensive and hypertensive rats

To determine if the properties of Na\(^+\) action potentials varied among groups, individual action potentials were evoked using brief depolarizing pulses, while maintaining Vm at ~-50 mV. Representative examples and mean values are summarized in Fig. 5. Interestingly, exercise training affected spike threshold in opposing manners in WKY and SHR rats. Thus, while exercise significantly shifted spike threshold to a more hyperpolarized Vm in WKY rats (P< 0.05), a depolarizing shift was observed in SHR rats (P< 0.05).

As previously reported (Jackson et al. 2005), we found exercise training to increase the amplitude of action potentials in WKY rats (P< 0.05). However, no significant differences were observed in SHR rats (P > 0.5. Fig. 5). Exercise training did not affect the action potential width in either WKY or SHR rats (P > 0.5) (Fig. 5).

Na\(^+\) spikes in PVN-NTS neurons were followed by a large hyperpolarizing after potential (HAP). While exercise training significantly enhanced the peak amplitude of the HAP in WKY rats (P< 0.05), the opposite effect was observed in SHR rats (P< 0.05 in both cases, Fig. 5).

Basic intrinsic membrane properties of PVN-NTS neurons
Table 1 summarizes mean values for some of the basic membrane properties obtained from PVN-NTS neurons in the four experimental groups. Resting membrane potential (Vm) and input resistance were similar among all groups. Cell capacitance on the other hand, was significantly lower in PVN-NTS neurons from SHRs when compared to WKYs, both in sedentary and trained rats (P<0.01 and P<0.001, respectively, Bonferroni’s test).

DISCUSSION

In this work, we investigated the effects of exercise training on the intrinsic membrane excitability of NTS-projecting PVN neurons (PVN-NTS) in normotensive WKY and hypertensive SHR rats. A major finding of this work is that exercise training differentially affected neuronal excitability and repetitive firing properties of neurons from WKY and SHR rats. As we previously reported in normal Wistar rats (Jackson et al. 2005), exercise training enhanced the input-output (I/O) function of PVN-NTS neurons in WKY rats. Importantly, SHR rats under sedentary conditions already displayed an increased excitability and enhanced I/O function, as indicated by an increased number of action potentials fired in response to depolarizing stimuli, when compared to WKY-S rats. In this group, as opposed to what we observed in WKY rats, exercise training diminished the I/O function, restoring repetitive firing properties back to the levels observed in sedentary WKY rats.

In all tested groups, the firing discharge of PVN-NTS neurons increased progressively at low stimulation levels. However, neurons from WKY-S rats were only able to sustain continuous firing discharge up to ~40% of the maximal stimulation used. At higher stimulation, their firing discharge decreased progressively, and a high degree of adaptation and dampening of action potential amplitude became evident. This suggests that PVN-NTS neurons under normal conditions possess intrinsic mechanisms that prevent their excessive activation in response to incoming depolarizing stimuli. This adaptive response however, was blunted following exercise training, as shown by an enhanced firing responsiveness at high stimulation levels, with little or no action potential dampening. On the other hand, PVN-NTS neurons in SHR-S rats displayed an I/O function that was similar to that observed in WKY-T rats, displaying little or no adaptation to depolarizing stimuli, being thus able to sustain continuous repetitive firing throughout the stimuli applied. Interestingly, exercise training in this group restored adaptive
mechanisms, resulting in a similar I/O profile as that observed in WKY-S rats, i.e., diminished firing discharge and dampening of action potentials during sustained firing.

Action potential dampening was reflected as a progressive decrease in the magnitude of action potentials, leading occasionally to action potential failure. While we did observe exercise-mediated changes in the number of spikelets, particularly in the SHR rats, they did not account for the overall changes in firing properties reported among groups (i.e., full action potentials were also affected). The functional significance and precise mechanisms underlying the generation of these smaller amplitude action potentials, also known as spikelets is at present unknown. It was previously shown the spikelets can result not only from the progressive inactivation due to depolarization inactivation, but that they could also they can reflect electrical coupling between neurons, ectopic axonal spikes, or spikes originating at dendritic sites (Avoli et al. 1998; Galarreta and Hestrin 1999; MacVicar and Dudek 1981). Moreover, whether spikelets are capable or not of propagating down the axon is controversial, with some studies supporting propagation (Foust et al. 2010; Shu et al. 2007), while other do not (Khaliq and Raman 2005; Monsivais et al. 2005).

Taken together, our data suggest that exercise training differentially affects neuronal excitability of PVN-NTS neurons under physiological and pathological conditions. Thus, as further discussed below, one reasonable interpretation of these results is that exercise training in normal conditions increases neuronal excitability as an adaptive mechanism to cope with the increased cardiovascular demands related to exercise, whereas in pathological conditions, such as hypertension, exercise acts as a “corrective” factor, which normalizes the abnormally elevated neuronal excitability and repetitive firing properties observed in this condition.

Potential mechanisms underlying the differential effects of exercise training in PVN-NTS neuronal excitability in normotensive and hypertensive rats

The repetitive firing properties and the input-output function of a neuron could be influenced by a variety of intrinsic mechanisms, including changes in input resistance, action potential waveform and post-spike properties, such as hyperpolarizing-after potentials (HAPs) and after-hyperpolarizing potentials (AHPs).

In this study, we observed common differences among groups that shared similar repetitive firing properties. These could be mostly related to the degree of voltage-gated Na$^+$
channel inactivation, which in turn influences the degree of action potential dampen/failure during repetitive firing (Jackson et al. 2005). For example, neurons showing low ability to sustain continuous repetitive firing due to high degree of action potential dampening (WKY-S and SHR-T), displayed smaller HAPs than those showing enhanced ability to sustain repetitive firing (WKY-T and SHR-S). The membrane repolarization mediated by the HAP is an important mechanism that removes Na⁺ inactivation following an action potential, and differences in HAP magnitude would result in a differential degree of removal of Na⁺ channel inactivation during repetitive firing (i.e., larger HAPs lead to more efficiently removal Na⁺ channel inactivation, better supporting in turn, repetitive firing discharge). Comparable differences among groups sharing similar repetitive firing profiles were observed with respect to the Na⁺ action potential threshold, which is also largely dependent on the degree of Na⁺ channel availability. Thus, neurons showing low ability to sustain repetitive firing (WKY-S and SHR-T), displayed more depolarized spike thresholds than those showing enhanced ability to sustain repetitive firing (WKY-T and SHR-S). As a caveat however, despite differences in spike threshold, no major differences in firing properties among groups were observed with the lowest intensity current injections. Thus, it is tempting to speculate that differences in Na⁺ channel availability and/or channel properties (e.g., shift in the voltage-dependent properties of inactivation, changes in Na⁺ channel densities), may contribute to the differential ability of PVN-NTS neurons to sustain firing in response to strong depolarizing inputs in the different experimental conditions. However, other potential mechanisms should be considered, including altered resting K⁺ conductances, as well as altered spinning-evoked changes in intracellular Ca²⁺ levels. Elucidating the precise underlying mechanisms contributing to the altered repetitive firing properties reported in this study will be the focus of future studies.

The slow after-hyperpolarizing potential (AHP) that typically follows a train of action potentials in SON and PVN neurons (Armstrong et al. 1994; Chen and Toney 2009; Greffrath et al. 1998; Stern 2001) is another important membrane property known to influence repetitive firing. The AHP results from progressive accumulation of intracellular Ca²⁺ and subsequent activation of Ca²⁺-dependent SK potassium channels (Greffrath et al. 1998), resulting in spike frequency adaptation during repetitive firing. In a recent study, a reduced AHP was found to contribute to increased excitability of presympathetic RVLM-projecting PVN neurons in angiotensin II-high salt diet hypertensive rats (Chen et al. 2010). In our study, however, we
found no differences in the AHP magnitude in PVN-NTS neurons between WKY and SHR rats, indicating that changes in AHP during hypertension are either cell type-dependent, or depend on the experimental model of hypertension used. Moreover, we found exercise training to diminish the AHP magnitude both in WKY and SHR rats. Given that exercise training affected repetitive firing in an opposing manner in these two experimental groups, it is unlikely that differences or changes in AHP magnitude constitute an underlying mechanism mediating changes in repetitive firing discharge. Finally, the lack of significant differences in input resistance among groups also argues against this as an important factor contributing to differences in repetitive firing. Interestingly, as we previously reported in PVN-RVLM neurons (Sonner et al. 2008), we found the cell capacitance in PVN-NTS neurons to be diminished in hypertensive when compared to normotensive rats. Given that cell capacitance is a general indicator of neuronal surface membrane (Lindau and Neher 1988), these results may reflect somatodendritic structural changes in of PVN-NTS neurons during hypertension. While we previously showed this to be the case in PVN-RVLM neurons (Sonner et al. 2008), additional morphometric studies in intracellularly labeled PVN-NTS neurons would be needed to confirm hypertension-related structural plasticity in this PVN neuronal population.

Another caveat in our study is that the neurochemical identity of the recorded PVN-NTS neurons was not determined. Moreover, while our tracer injection was centered within caudal aspects of the NTS, the tracer also expanded into more rostral and ventral regions, involving thus multiple NTS subnuclei. Thus, it is likely that a heterogeneous population of PVN-NTS projecting neurons was sampled in this study. We recently showed that the majority (~65%) of the PVN-NTS neuronal population under basal conditions express either oxytocin (OT) or vasopressin (VP) peptides (Jackson et al. 2005). However other neurotransmitters were also implicated in this pathway, including enkephalin, somatostatin, galanin and glutamate (Chen and DiCarlo 1996; Kawabe et al. 2008; Sawchenko and Swanson 1982). Thus, it remains to be determined to what extent the reported changes in neuronal excitability induced by hypertension and exercise training affect discrete subpopulations of PVN-NTS neurons, or alternatively, whether PVN-NTS neurons are non-selectively affected.

Functional implications of exercise-mediated changes in PVN-NTS neuronal excitability in WKY and SHR rats
While the functional implications of the contrasting effects of exercise training in WKY and SHR rats are not straightforward, some insights may be obtained from previous work in the literature. Several reports support an enhanced PVN activation in different animal models of hypertension (Allen 2002; Li and Pan 2007a; Martin and Haywood 1998; Oliveira-Sales et al. 2009), contributing to sympathoexcitation and impaired baroreflex function, two characteristic findings in this disease state (Esler and Kaye 1998; Grassi 2004). Along these lines, PVN stimulation has been shown to diminish baroreflex responses (Chen et al. 1996; Hwang et al. 1998; Jin and Rockhold 1989; Pan et al. 2007; Patel and Schmid 1988) and to inhibit NTS barosensitive neurons (Duan et al. 1999; Kannan and Yamashita 1983), actions that could be mediated, at least in part, by neuropeptide-mediated modulation of presynaptic glutamate release from solitary tract primary afferent inputs (Bailey et al. 2006). While all the above studies were conducted in male rats, a suppression of baroreflex function following inhibition of the PVN was recently reported in females (Page et al. 2011), suggesting that PVN influence on baroreflex function could be gender-specific. In addition, VP and OT, two of the major peptides involved in descending PVN pathways (Sawchenko and Swanson 1982) and densely present in the NTS (Maley 1996; Sofroniew and Schrell 1981) have been shown to evoke pressor responses acting within the NTS (Matsuguchi and Schmid 1982; Matsuguchi et al. 1982; Pittman and Franklin 1985). Taken together, these data suggest that increased activity of descending peptidergic PVN-NTS pathways may contribute to altered autonomic function in hypertensive conditions. Results from the present study showing an enhanced excitability and firing responsiveness of PVN-NTS neurons in SHR rats further support this notion.

Accumulating evidence also supports enhanced activation of PVN-NTS projections during exercise training in normal rats. (Braga et al. 2000; Duflot et al. 1997; Jackson et al. 2005). Based on the evidence above, a blunted baroreflex function would be expected in exercise-trained rats. Indeed, several reports have shown blunted baroreflex function during exercise training, particularly reduced baroreflex-mediated sympathoexcitation (Alvarez et al. 2005; Bedford and Tipton 1987; Chen and DiCarlo 1996; DiCarlo and Bishop 1988; Negrao et al. 1993). However, it is important to acknowledge that opposite effects have been also reported (Ceroni et al. 2009; DiCarlo and Bishop 1990; Fadel et al. 2001). Thus, this remains a controversial topic.
In the present study we found exercise to diminish PVN-NTS neuronal excitability, correcting their repetitive firing profile back to that observed in normotensive rats. (Higa-Taniguchi et al. 2007). The “corrective” effect of exercise training on the repetitive firing profile of PVN-NTS neurons in SHR rats is in line with previous results from our laboratories showing that exercise training improved the blunted baroreflex function found in SHR rats (Ceroni et al. 2009). Similarly, exercise training was recently shown to normalize elevated PVN levels of AT1a receptors, NADPH oxidase subunit and superoxide in angiotensin II-treated rats, correcting as well the blunted baroreflex function observed in this condition (Pan et al. 2007). Finally, it is important to consider that the selective decrease in blood pressure evoked by exercise training in SHR rats likely involves mechanism other than those targeting central autonomic regulation, including arteriole structural remodeling. This is supported by our previous studies showing that exercise training normalized the wall/lumen ratio of skeletal muscle, heart and diaphragm arterioles, in SHR, but not in WKY rats (Amaral and Michelini 2011; Amaral et al. 2000; Melo et al. 2003).

In summary, our studies add to the growing notion that overactivation of descending preautonomic PVN pathways in hypertensive disorders involves not only changes in extrinsic factors, including altered efficacy (Li and Pan 2006) and redistribution of synaptic inputs (Biancardi et al. 2010), but also changes in intrinsic neuronal properties (see also (Chen et al. 2010; Sonner et al. 2008; Sonner et al. 2010). Moreover, our results indicate that exercise constitutes an efficient approach to correct abnormal hypothalamic neuronal function in hypertensive conditions, which in turn may contribute to the beneficial cardiovascular effects of exercise in hypertensive individuals.
Table 1- Comparison of the effect of exercise training on basic membrane properties of WKY and SHR rats in sedentary (S) and exercise trained (T) rats.

<table>
<thead>
<tr>
<th></th>
<th>Resting Vm (mV)</th>
<th>Input Resistance (MΩ)</th>
<th>Cell capacitance (pC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY-S</td>
<td>-42.7 ± 1.4</td>
<td>1.11 ± 0.09</td>
<td>36.7 ± 2.7</td>
</tr>
<tr>
<td>WKY-T</td>
<td>-42.2 ± 1.3</td>
<td>1.09 ± 0.12</td>
<td>46.1 ± 4.5</td>
</tr>
<tr>
<td>SHR-S</td>
<td>-45.5 ± 1.0</td>
<td>1.05 ± 0.09</td>
<td>25.6 ± 2.2*</td>
</tr>
<tr>
<td>SHR-T</td>
<td>-42.5 ± 1.5</td>
<td>0.89 ± 0.11</td>
<td>29.4 ± 2.4**</td>
</tr>
</tbody>
</table>

*P< 0.01 vs. WKY-S and **P< 0.001 vs. WKY-T.

Two-way ANOVA F results: Vm (blood pressure F= 1.6; exercise F= 2.0, interaction F= 0.5);
Input resistance (blood pressure F= 1.6; exercise F= 0.7, interaction F= 0.4); Cell capacitance:
(blood pressure F= 16.9, P< 0.0001; exercise F= 5.5, P< 0.05, interaction F= 1.8).
FIGURE LEGENDS

Figure 1. Efficacy of the exercise training protocol in WKY and SHR rats. A, Time course changes on treadmill performance in WKY and SHR groups submitted to sedentary (S) and training (T) protocols. Comparison of performance gain (B), body weight gain (C) and tail pressure (D) in the 4 groups at the end of S and T protocols. n=8 for each group. Significances (P<0.05) are * vs. WKY, + vs. S.

Figure 2. Exercise training differentially affects the input-output function of PVN-NTS neurons in WKY and SHR rats. A1, Plot of the mean number of spikes (including action potential and “spikelets”) as a function of current injection obtained in WKY-S and WKY-T rats. Neurons in WKY-T rats responded to current stimulation by generating a higher number of action potentials than in WKY-S rats. A2, Representative traces of repetitive firing evoked in PVN-NTS neurons from WKY-S and WKY-T rats in response to increasing depolarizing steps. B1, Plot of the mean number of spikes as a function of current injection obtained in SHR-S and SHR-T rats. Neurons in SHR-T rats responded to current stimulation by generating a lower number of action potentials than in SHR-S rats. Note that repetitive firing in SHR-S neurons was basally as high as WKY-T, and that exercise training normalized the I/O function back to control levels. B2, Representative traces of repetitive firing evoked in PVN-NTS neurons from SHR-S and SHR-T rats in response to increasing depolarizing steps. *P< 0.05; #P< 0.01 vs. respective current step in the S group. Filled arrow in A2 points to failed action potentials, whereas empty arrows in B2 point to representative “spikelets”.

Figure 3. Changes in the number and proportion of “spikelets” in WKY and SHR rats from sedentary and exercise –trained rats. The mean total number of action potentials (A), mean number of spikelets (B) and mean proportion of spikelets (C) following a depolarizing pulse of 150 pA for WKY-S, WKY-T, SHR-S and SHR-T are shown. Note that the data shown in A correspond to the same points displayed in Figure 2A1 and B1 for 150 pA. *P< 0.05 and **P< 0.01 vs respective S control.
Figure 4- Exercise training diminished the magnitude of the after-hyperpolarizing potential (AHP) both in WKY and SHR rats. A, Representative traces showing the effects of exercise training on evoked AHPs (arrows) in WKY rats (upper panel) and SHR rats (lower panel). Each trace represents an average of 8 sweeps. Action potentials were cropped. B, On average, the AHP magnitude in PVN-NTS neurons from both WKY and SHR rats was significantly diminished by T, when compared to S rats (Two way ANOVA results: blood pressure F=0.04, exercise F=13.5, P<0.0005, interaction F=0.1).

Figure 5- Exercise training affected the Na\(^+\) action potential waveform of PVN-NTS neurons in WKY and SHR rats. A, Representative traces showing the effects of exercise training on Na\(^+\) action potentials evoked in PVN-NTS neurons from WKY (left) and SHR (right) rats. The thin and thick traces correspond to sedentary (S) and exercise-trained (T) rats, respectively. Action potentials were aligned to better compare their waveform. Arrows point to hyperpolarizing afterpotentials (HAP) that followed each evoked action potential, while arrowheads point to spike threshold. Traces represent an average of 15 sweeps. B, Bar graphs depicting mean values for action potential amplitude (upper left), width (upper right), threshold (lower left) and HAP amplitude (lower right) in PVN-NTS neurons from WKY and SHR rats. Two way ANOVA results: (1) amplitude: blood pressure F=0.01, exercise F=5.9, P<0.02, interaction F=1.7); (2) width: blood pressure F=3.5, exercise F=0.7, interaction F=2.6); (3) threshold: blood pressure F=0.3, exercise F=0.3, interaction F=13.3, P<0.001); (4) HAP amplitude: blood pressure F=0.5, exercise F=0.4, interaction F=6.5, P<0.01).* and *P<0.05.
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A. Treadmill speed (km/h) over weeks.

B. Performance gain (km/h) for WKY and SHR.

C. Body weight gain (g) for WKY and SHR.

D. Tail pressure (mmHg) for WKY and SHR.

Symbols:
- *: Significant difference
- †: Significant difference compared to baseline

Graphs illustrate the progression of treadmill speed, performance gain, body weight gain, and tail pressure for WKY and SHR over weeks.