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Functional Interplay Between Subthreshold Ion Channels in Preautonomic Neurons of the Hypothalamic Paraventricular Nucleus in Health and Disease Conditions

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FUNCTIONAL INTERPLAY BETWEEN SUBTHRESHOLD ION CHANNELS
IN PREAUTONOMIC NEURONS OF THE HYPOTHALAMIC
PARAVENTRICULAR NUCLEUS IN HEALTH AND DISEASE CONDITIONS

A dissertation submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

By

Patrick M. Sonner
B.S., Ohio Northern University, 2000

2007
Wright State University
I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Patrick Michael Sonner ENTITLED Functional interplay between subthreshold ion channels in preautonomic neurons of the hypothalamic paraventricular nucleus in health and disease conditions BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

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ABSTRACT


Under normal conditions, blood pressure is tightly regulated through autonomic tonic and reflex mechanisms. However, when the set-point for blood pressure is chronically elevated, hypertension occurs. Hypertension if untreated can lead to further complications including heart failure, stroke and kidney failure. Elevated sympathetic outflow is known to contribute to the development and/or maintenance of hypertension, and while the hypothalamic paraventricular nucleus (PVN), a preautonomic center, has been implicated in the elevation of sympathetic activity during hypertension, the precise pathophysiological mechanisms underlying sympathoexcitation remain unclear.

Subthreshold ion channels, including the A-type K$^+$ ($I_A$) and the T-type Ca$^{2+}$ ($I_T$), are important mechanisms regulating the ability of neurons to generate firing activity, and changes in $I_A$ activity have been reported during hypertension. Thus, the first aim of the study focused on characterizing the basic biophysical and functional properties of $I_A$ in presympathetic PVN neurons projecting to the rostral ventrolateral medulla (PVN-RVLM). Our studies demonstrated the presence of a functionally relevant $I_A$ in PVN-RVLM neurons, which actively modulated the action potential waveform and firing activity. The second aim of the study was to determine whether alterations in the
biophysical properties of $I_A$ contributed to enhanced neuronal excitability of PVN-RVLM neurons during hypertension. Our studies indicated that diminished $I_A$ availability constituted a contributing mechanism underlying hyperexcitability in these neurons during hypertension. Previous studies have indicated an opposing balance between the subthreshold ion channels, $I_A$ and $I_T$. Thus, the final aim of the study assessed the biophysical competition between $I_A$ and $I_T$, and functionally addressed the influence of such balance on the activity of PVN-RVLM neurons under normal and hypertensive conditions. Our studies indicated that the balance between $I_A$ and $I_T$ was shifted during hypertension in favor of $I_T$ activity, resulting in increased $I_T$–dependent low threshold spikes, elevated intracellular calcium levels, and enhanced basal spontaneous firing activity during this condition. Taken together, this study confirms the importance of intrinsic factors, in particular the balance between opposing subthreshold conductances, in regulating the central control of cardiovascular output under normal and pathological conditions.
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DEDICATION

To Martha, your love, encouragement, and patience knows no bounds.

To my family, for support and laughter.
I. INTRODUCTION

1.1. Introduction

According to the American Heart Association, one in five Americans has hypertension, however, a third are unaware of their condition. Hypertension if untreated can lead to further complications including heart failure, stroke, and kidney failure. While elevated sympathetic outflow is known to contribute to the development and/or maintenance of hypertension, and is commonly observed in various forms of hypertensive disorders, both in humans and in experimental animal models [59, 81, 95], the precise pathophysiological mechanisms underlying sympathoexcitation in hypertensive individuals remain largely unknown. However, accumulating evidence supports altered central nervous system (CNS) control of autonomic function during hypertension [81], and implicates the hypothalamic paraventricular nucleus (PVN) as a major contributing neuronal substrate.

1.2. Neurogenic control of blood pressure

Neural regulation of blood pressure is controlled in both short and long-term time scales. The main short-term controller of blood pressure is the arterial baroreceptor reflex, in which peripheral baroreceptors sense changes in blood pressure and send afferent projections to central regions, resulting in rapid changes in cardiac output and
vascular resistance. These responses can be associated with substantial changes in blood pressure, and are physiologically adaptive, based upon behavioral and environmental demands [81]. It has been demonstrated, however, that surgical denervation of centrally projecting baroreceptors has no long-term effect on blood pressure [43,151], suggesting that a central role in the long-term control of blood pressure remains intact. While the sensor of neurally mediated long-term control of blood pressure remains unclear, it is believed to involve compounds associated with body fluid homeostasis and osmoreceptors, such as angiotensin II (AngII) and sodium [152]. Circumventricular organs, central regions lacking the blood-brain barrier, including the subfornical organ (SFO), are well known to be osmosensitive, as well as able to detect changes in circulating levels of hormones involved in fluid volume regulation [140]. It has been demonstrated that the SFO sends projections to a sympathetic preautonomic center located within the hypothalamus, the PVN [9,60]. Thus, these regions have been suggested to play an important role in the long-term control of blood pressure [47]. However, it has also been hypothesized that a central baroreceptor may exist within the rostral ventrolateral medulla (RVLM) [152].

1.3. Preautonomic cardiovascular control centers

The central autonomic nervous system provides control over peripheral functions necessary to maintain body homeostasis. This balance is achieved through the combined and balanced activity of the sympathetic and parasympathetic preganglionic neurons located in the intermediolateral column of the spinal cord (IML) as well as the nucleus ambiguus (NA) and dorsal motor nucleus of the vagus (DMX), respectively. Relatively
basic aspects of tonic and reflex autonomic functions, including the baroreflex, are mediated by spinal and bulbar regions. For example, when a drop in blood pressure occurs, the change is monitored peripherally by baroreceptors located in the aortic arch and carotid sinus providing afferent inputs to the nucleus of the solitary tract (NTS) via vagal and glossopharyngeal nerves, respectively [182]. The NTS, which is a major cardiovascular integrative center, then sends glutamatergic projections to the caudal ventrolateral medulla (CVLM), which inhibits the IML-projecting rostral ventrolateral medulla (RVLM) neurons through GABAergic projections, thus diminishing sympathetic output [49,81]. The NTS also directly innervates the NA and the DMX, through excitatory inputs [183] to regulate parasympathetic output. Thus, these preganglionic centers are capable of correcting changes in blood pressure through combined sympathetic and parasympathetic output. In addition to directly influencing sympathetic and parasympathetic outflows, these spinal/bulbar centers also send ascending projections to hierarchically higher centers including the major preautonomic centers. These are located in the hypothalamus and other forebrain regions, and participate in the generation of more complex and integrative autonomic responses, involving multiple territories and systems. It is the preautonomic centers, through direct projections to sympathetic and parasympathetic preganglionic neurons, which provide this complex integrative and behavioral regulation of tonic and reflex autonomic function [3,52,79,162,171,208].

There are numerous preautonomic centers, including the suprachiasmatic nucleus (SCN), ventrolateral medulla (VLM), A5, caudal raphe nucleus, NTS, RVLM and PVN [17,24,69,90,165], which connect bidirectionally with lower central regions in order to integrate a variety of behavioral and environmental signals. The NTS, as mentioned
above, acts as the major cardiovascular integrative center providing efferent projections to a variety of autonomic centers. However, while the NTS acts as the major afferent integrative center of the mechano- and chemoreceptors, it is the RVLM that primarily regulates the barosensitive sympathetic efferents [47]. Interestingly, the PVN, located within the forebrain, is a higher center which regulates both the RVLM and NTS [4,55,195], and thus allows for integrative and behavioral control over the cardiovascular system.

1.4. PVN descending pathways involved in cardiovascular control

As mentioned above, the PVN is an important homeostatic integrative center, able to regulate a variety of cardiovascular functions, such as blood volume [16,41,84,169], osmolality [32,61,62,196], and blood pressure [61,99,157,211,212], via a combination of neuroendocrine and preautonomic efferent projections [153,193]. The PVN is itself a preautonomic center with direct projections to preganglionic neurons in the intermediolateral column of the spinal cord [90,161,193], allowing for direct sympathetic control of cardiovascular function [131]. The PVN also mediates preautonomic output indirectly via the RVLM [34,48,83], a cardiovascular control center able to mediate tonic and reflex sympathetic outflow [49,104,108,162,170,171,188] via direct projections to preganglionic neurons in the IML [8,204]. RVLM-projecting PVN neurons (PVN-RVLM) are capable of eliciting sympathoexcitatory and pressor responses upon activation [4,42,110,195,205]. Interestingly, it has been shown that up to 30% of spinally projecting PVN neurons send collaterals to the RVLM, likely reflecting a more complex regulation of sympathetic nerve activity by the PVN [158,174].
The PVN also influences cardiovascular function through the regulation of parasympathetic output. A major target innervated by the PVN is the dorsal vagal complex (DVC) [55,72,98,178]. The DVC is an autonomic center comprising parasympathetic neurons known to regulate tonic and reflex cardiac activity [86,144,150,200]. Specifically, the DVC is comprised of multiple sub-nuclei that send direct projections to vagal preganglionic neurons, including the nucleus ambiguus and the dorsal motor nucleus [183], allowing for vagal motor control of the heart. The DVC also houses the NTS, a major visceroreceptive autonomic integrating center and key modulator of the baroreflex [69,111,138,154]. NTS-projecting PVN neurons (PVN-NTS), upon stimulation, have been shown to suppress the baroreflex through inhibition of the NTS [33,55,56,145], inducing an elevation in heart rate and cardiac output during the defense reaction or exercise. This phenomenon is also commonly observed during chronic hypertension [14,66,78].

Thus, through modulatory actions on the baroreceptor reflex, parasympathetic and sympathetic nerve activity, the PVN plays a major role in the short- and long-term control of the cardiovascular system [47].

1.5. Cellular organization of the PVN

The different modalities and pathways by which the PVN influences cardiovascular control are represented in discrete neuronal groups, including neuroendocrine and autonomic-related neurons. In general, neurons contained within the PVN are classified as magnocellular or parvocellular. The magnocellular neurons are contained within three distinct anatomical regions, and send neuroendocrine efferent
projections to the posterior pituitary. On the other hand, the parvocellular neurons are distributed within five distinct anatomical regions. One group of parvocellular neurons elicits neuroendocrine responses through projections to the median eminence resulting in the release of neurohormones that control anterior pituitary function. A second major group of parvocellular neurons mediate preautonomic functions via efferent projections to various autonomic-related centers in the brain stem and spinal cord [193], including the RVLM, NTS, intermediolateral column (IML) of the spinal cord, dorsal motor nucleus of the vagus and the locus coeruleus.

1.6. Role of PVN preautonomic neurons (PVN-PA) in hypertension

Accumulating evidence supports an important role for the PVN in the pathophysiology of hypertension. Elevated neuronal activation marker expression (e.g., hexokinase activity, FOS and Fra-like immunoreactivity) suggests increased neuronal activity in the PVN in a variety of experimental models of hypertension, including high Na\(^+\), renovascular, spontaneously hypertensive (SHR), and aortic depressor nerve transection [23,45,50,96,114,198]. This corresponds with an increased excitability of presympathetic PVN neurons, as recently shown [120]. In addition, lesions of the PVN diminish the mean arterial pressure and reduce sympathetic nerve activity in renal wrap, SHR, and high Na\(^+\) hypertensive rats [35,76,85,149]. More importantly, recent work indicates that the PVN directly contributes to the elevated blood pressure and sympathetic activity associated with spontaneous and renal wrap hypertension [3,4,85]. Furthermore, both the RVLM and NTS have been shown to be altered during hypertension resulting in sympathoexcitation [15,25,29,57,101,141,168]. Thus, altogether these data support that
enhanced PVN activity, and consequently altered RVLM and NTS function, contribute to enhanced sympathetic output and suppression of the baroreflex, respectively, during hypertension. Recent work in RVLM-projecting [4,120] and NTS-projecting PVN neurons [137] supports this idea, showing enhanced firing activity of PVN-RVLM neurons is mediated in part by a decrease and increase in GABA_A and GABA_B receptor function, respectively. As well, PVN-NTS neurons show reduced oxytocin mRNA levels and neurons in the NTS express less oxytocin receptor mRNA levels. Altogether, these data suggest that hyperactivation of PVN neurons contributes to maintenance of elevated blood pressure and sympathetic outflow during hypertension. Despite the pivotal role of the PVN during hypertension, the precise cellular mechanisms contributing to elevated PVN neuronal activity during hypertension remain largely unknown. This information is critical, because detailed knowledge on pathophysiological mechanisms underlying sympathoexcitation is needed for the development of novel and/or more efficient therapeutic approaches for the treatment of this prevalent disorder.

1.7. Potential mechanisms contributing to altered neuronal function during hypertension

Similar to other CNS neuronal populations, preautonomic PVN neuronal excitability and firing activity is fine-tuned by the combined actions of both intrinsic properties and synaptic inputs [26,117,118,120-122,124,180,185]. In this sense, most of the work in the field, so far, has focused on extrinsic, synaptic inputs. For example, alterations in various PVN neurotransmitter systems have been reported during hypertension, including diminished GABAergic activity [46,120], reduced nNOS levels
and elevated glutamatergic [121] and angiotensin II activity [80,96]. Thus, these studies indicate that a change in the balance of inhibitory and excitatory inputs, favoring the latter, contributes to increased PVN neuronal activity and autonomic outflow during hypertension. It is important to consider however, that some of these extrinsic mechanisms may in fact be acting through modulation of intrinsic conductances. For example, AngII has been shown to inhibit the transient K$^+$ current, $I_A$ [125,148,202], while simultaneously increasing the activity of the transient Ca$^{2+}$ current, $I_T$ [181] within the hypothalamus. This clearly emphasizes the importance of combined extrinsic/intrinsic mechanisms in controlling the overall activity of PVN neurons. Remarkably, the contribution of altered intrinsic membrane properties (i.e., number, distribution and functional properties of ion channels, neuronal structure and geometry, etc) to PVN neuronal hyperactivity during hypertension has not been thoroughly investigated. Thus, the work of this thesis focuses on elucidating how altered intrinsic mechanisms contribute to the maintenance of hypertension.

1.8. Major ionic mechanisms controlling neuronal activity of PVN neurons

In general, neuronal excitability is influenced by an extensive array of ion channels, including, among others, voltage-dependent sodium, chloride, calcium and potassium (both high-threshold and low-threshold, and calcium-dependent), many of which have been reported to be expressed in the PVN. Importantly, subthreshold currents, which are activated at membrane potentials below the threshold for activation of Na$^+$ channels, play a major role in controlling the ability of a neuron to generate action potentials. Thus, the present work focuses on this particular group of voltage-gated ion
channels. Among subthreshold ion channels, both the A-type K\(^+\) current (\(I_A\)) and the T-type Ca\(^{2+}\) current (\(I_T\)) have been found within non-identified PVN parvocellular neurons [133]. \(I_A\) has been identified as a rapidly activating and inactivating transient outward current. Activation of \(I_A\) decreases excitability, and is known to play an important role in the modulation of repetitive neuronal firing activity, by restricting the duration of the action potential waveform, and by increasing the interspike interval [164,166,172]. \(I_T\) is also a transient current, that when activated results conversely in an increased neuronal excitability. \(I_T\) has long been attributed to mediate bursting firing discharge [129,185]. However, \(I_T\) also has been shown to regulate continuous firing frequency [159] and the repolarization phase of the action potential waveform [112,139,147]. While the general properties of \(I_A\) and \(I_T\) have been previously studied in magnocellular neuroendocrine and non-identified parvocellular neurons [133], and have been shown to have overlapping voltage-dependent and kinetic properties, there is no information available on the biophysical and functional properties of \(I_A\) or \(I_T\) in identified populations of PVN-PA neurons.

Based on their general biophysical properties and function, it is clear that these two opposing conductances “compete” at subthreshold membrane potentials to influence neuronal firing discharge, as shown in thalamic and cerebellar neurons [27,142,146,155]. Thus, it is expected that if the balance between these conductances is shifted, one will predominate over the other, altering neuronal excitability and firing discharge. In this sense, roles for altered potassium and calcium currents in the pathophysiology of hypertension have been supported by recent work. Specifically, a diminished \(I_A\) activity has been observed in NTS neurons of renal wrap and spontaneously hypertensive rats.
[13,191], suggesting that an attenuation of \( I_A \) could contribute to increased neuronal excitability during hypertension. As well, an enhanced repetitive firing frequency in spontaneously hypertensive ganglionic sympathetic neurons has been attributed to an enhanced calcium conductance [207]. These results suggest that an altered balance between \( I_A \) and \( I_T \) contributes to neuronal hyper-excitability during hypertension. Whether a balanced \( I_A/I_T \) activity influences neuronal function in PVN-PA neurons, and whether a shift in this balance contributes to enhanced PVN neuronal activity during hypertension is at present unknown, and is a major focus of the present work.

1.9. Thesis focus and significance

This research has focused on understanding the contribution of the subthreshold ion channels, \( I_A \) and \( I_T \), in regulating the activity of preautonomic PVN neurons, under normal and pathological (i.e., hypertensive) conditions. To this end, a multidisciplinary experimental approach, involving a variety of complementary techniques was employed, including whole-cell patch-clamp electrophysiology, immunohistochemistry, real-time PCR and confocal calcium imaging. The work is organized in three major parts. The first part of the work focused on characterizing the basic biophysical and functional properties of \( I_A \) in PVN-RVLM neurons. These data demonstrate the presence of \( I_A \) in PVN-RVLM neurons, which actively modulates the action potential waveform and firing activity. This is significant in supporting intrinsic conductances as an important mechanism in controlling neuronal excitability in this presympathetic neuronal population. In the second part of this work, I explored how alterations in the biophysical properties of \( I_A \) contributed to enhanced neuronal excitability of PVN-RVLM neurons during
hypertension. The data indicate that diminished IA availability constitutes a contributing
mechanism underlying aberrant neuronal function and autonomic control during
hypertension. Finally, the last part of the study specifically assessed the biophysical
interplay between subthreshold ion channels, IA and IT, and functionally addressed the
influence of such balance on PVN neuronal activity. Moreover, I determined whether a
shift in this balance contributed to enhanced PVN-RVLM neuronal excitability in
hypertensive conditions. The data indicate that the balance between IA and IT is shifted
during hypertension in favor of IT activity, resulting in increased IT-dependent influences,
elevated intracellular calcium levels, and enhanced basal spontaneous firing activity.
Taken together, the data presented here confirm the importance of intrinsic factors in
regulating the central control of cardiovascular output under normal and pathological
conditions.
II. MATERIALS AND METHODS

Male Wistar rats (120-140g and 200-300g) were purchased from Harlan Laboratories (Indianapolis, IN, USA), and housed in a 12 h : 12 h light-dark cycle with access to food and water ad libitum. All procedures were carried out in agreement with both the University of Cincinnati and Wright State University Institutional Animal Care and Use Committees’ guidelines, and in compliance with NIH guidelines.

Renovascular surgery

Rats weighing between 150-180 g (approximately 5-6 weeks old) were used to induce the renovascular 2-kidney, 1-clip Goldblatt hypertension model, a well characterized and widely used model [15,136]. Rats were anesthetized with isoflurane (3 %) throughout the surgery. Following an abdominal incision, the left kidney was exposed, and a 0.2 mm clip was placed over the left renal artery, partially occluding it [25]. Sham rats were subjected to the same surgical procedure, although the artery was not occluded. Blood pressure was measured at the beginning of the sixth week post-surgery, using a tail-cuff method. All rats were used for experiments during the sixth-seventh week post-surgery.

Retrograde labeling of PVN-RVLM neurons

Preautonomic RVLM-projecting PVN neurons were identified by injecting rhodamine beads unilaterally into the brainstem region containing the RVLM, as
previously described [124]. Rats were anesthetized intraperitoneally with a ketamine-xylazine mixture (90 and 50 mg kg\(^{-1}\), respectively), the rat’s head was then placed in a stereotaxic apparatus, and 200 nl of rhodamine-labelled microspheres (Lumaflor, Naples, FL, USA) were pressure injected into the RVLM (starting from Bregma: 12 mm caudal along the lamina, 2 mm medial lateral, and 8 mm ventral). In general, injection sites were within the caudal pole of the facial nucleus to ~ 1 mm more caudal, and were ventrally located with respect to the nucleus ambiguus (see example in Fig. 1A). In a subset of experiments, injections were also performed in the area of the dorsal vagal complex (DVC) (at the level of the obex: 1 mm lateral to the midline and 0.8 mm below the dorsal surface) to label DVC-projecting PVN neurons [185] as a control neuronal population. The location of the tracer was verified histologically as previously described [124,185]. In a few instances, injections were located either rostrally or caudally to the RVLM, in which cases no PVN retrograde labeling was observed. If the injection site was not within the region of the RVLM, the experiment was discarded.

**Hypothalamic slices**

Two to seven days after the retrograde injection rats were anesthetized with nembutol (50 mg kg\(^{-1}\)) and perfused through the heart with a cold sucrose solution (containing in mM: 200 sucrose, 2.5 KCl, 3 MgSO\(_4\), 26 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 20 D-glucose, 0.4 ascorbic acid, 1 CaCl\(_2\) and 2 pyruvic acid (290-310 mosmol l\(^{-1}\)). This method has previously been shown to improve cell viability in slices obtained from adult rats [1]. Rats then were quickly decapitated, and brains dissected out. Slices were cut coronally (300 \(\mu\)m thick) utilizing a vibroslicer (D.S.K. Microslicer, Ted Pella, Redding, CA, USA). An oxygenated ice cold artificial cerebrospinal fluid (ACSF) was used
during slicing (containing in mM: 119 NaCl, 2.5 KCl, 1 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, 20 D-glucose, 0.4 ascorbic acid, 2 CaCl₂ and 2 pyruvic acid; pH 7.4; 290-310 mosmol l⁻¹). After sectioning, slices were placed in a holding chamber containing ACSF and then kept at room temperature until used.

**Electrophysiological recordings**

Slices were placed in a submersion style recording chamber, and bathed with solutions (~ 3.0 ml min⁻¹) that were bubbled continuously with a gas mix of 95% O₂-5% CO₂, and maintained at room temperature (~ 22 °C). Thin-walled (1.5 mm o.d., 1.17 mm i.d.) borosilicate glass (G150TF-3, Warner Instruments, Sarasota, FL, USA) was used to pull patch pipettes (3-6 MΩ) on a horizontal Flaming/Brown micropipette puller (P-97, Sutter Instruments, Novato, CA, USA). The internal solution contained (in mM): 140 potassium gluconate, 0.2 EGTA, 10 Hepes, 10 KCl, 0.9 MgCl₂, 4 MgATP, 0.3 NaGTP and 20 phosphocreatine (Na⁺); pH 7.2-7.3. Whole-cell recordings from PVN neurons were visually made using a combination of fluorescence illumination and infrared differential interference contrast (IR-DIC) video-microscopy. Recordings were obtained with a Multiclamp 700A amplifier (Axon Instruments, Union City, CA, USA). The voltage output was digitized at 16-bit resolution, 10 kHz (Digidata 1320A, Axon Instruments), and saved on a computer to be analyzed offline. The series resistance was monitored at the beginning and end of each experiment, and the experiment was discarded if the series resistance was not stable throughout the recording. The liquid junction potential (LJP, 6.5 mV) was experimentally determined using a 2 M KCl agar bridge. Data shown were corrected for the LJP.

**Voltage-clamp recordings of isolated voltage-gated K⁺ and Ca²⁺ currents**
For isolated A-type K$^+$ currents ($I_A$), slices were bathed in an ACSF with nominal Ca$^{2+}$ (0 mM) (containing in mM: 102 NaCl, 2.5 KCl, 3 MgSO$_4$, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 20 D-glucose, 0.4 ascorbic acid, 2 pyruvic acid, 3 EGTA, 200 μM CdCl$_2$, 30 TEA and 0.5 μM TTX; pH 7.4; 290-310 mosmol l$^{-1}$). The T-type Ca$^{2+}$ currents ($I_T$) were isolated by using an ACSF that enhances the signal to noise ratio of calcium currents (5 mM CaCl$_2$), blocks sodium channels, delayed rectifier potassium channels ($I_{K_{DR}}$), as well as the transient potassium channels, $I_A$ (containing in mM: 92 NaCl, 2.5 KCl, 1 MgSO$_4$, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 20 D-glucose, 0.4 ascorbic acid, 5 CaCl$_2$, 2 pyruvic acid, 0.5 μM TTX, 30 TEA and 5 4-AP; pH 7.4; 290-310 mosmol l$^{-1}$). Series resistance was electronically compensated for at least 60% throughout the recordings. The voltage error due to uncompensated series resistance at the half-activation and half-inactivation potentials for $I_A$ were calculated, however results were not corrected for them.

The quality of the space clamp was assessed as previously described [133]. Briefly, $I_A$ 10-90% rise time and time constant ($\tau$) of inactivation were measured following activation of the current by a test command (-10 mV), preceded by conditioning steps of varying amplitudes (-120 mV through -30 mV). Plots of 10-90% rise time and inactivation time constant ($\tau$) as a function of conditioning steps were then generated. Varying the conditioning step should affect only the amplitude of the current, without affecting its kinetic properties. Thus, only neurons showing unchanging 10-90% rise time and inactivation $\tau$ as a function of the conditioning pulse, as well as a lack of relationship between current amplitude and kinetics were included for analysis.

All protocols were run with an output gain of 2 and a Bessel filter of 2 kHz, and were leak subtracted using a P/4 protocol.
Voltage-dependence of activation of $I_A$ and $I_T$

In order to isolate $I_A$, a combination of electronic and pharmacological methods were used. Calcium channels were blocked using a 0 Ca$^{2+}$ ACSF containing EGTA and CdCl$_2$ (see above). TTX and tetraethyl ammonium (TEA) were also used to block voltage-dependent Na$^+$ channels and delayed rectifier K$^+$ channels (IK$_{DR}$), respectively. Since in many instances some TEA insensitive IK$_{DR}$ remained, two separate electrophysiological protocols were run in order to further isolate $I_A$ electronically. The first utilized a hyperpolarized conditioning pulse (-90 mV), which removed inactivation from $I_A$. This pulse was followed by depolarizing command pulses (-70 to +25 mV), which resulted in the activation of both $I_A$ and IK$_{DR}$. A second protocol was then run, in which a more depolarized (-40 mV) conditioning pulse was used to completely inactivate $I_A$. Thus, when the same command pulses as above were applied, only IK$_{DR}$ was activated. Currents recorded under these two protocols were then electronically subtracted offline using Clampfit 8.2 (Axon Instruments).

In order to isolate $I_T$, a combination of electronic and pharmacological methods also were used. TTX, TEA and 4-aminopyridine (4-AP) were used to block voltage-dependent Na$^+$ channels, IK$_{DR}$, and transient subthreshold potassium channels ($I_A$). To isolate $I_T$ from high-threshold calcium currents (HVA), the same protocol as above was used, however, the command pulses were only depolarized to -20 mV, limiting activation to that of $I_T$ only. The maximum peak amplitude and half-activation of $I_T$ were, therefore, unable to be measured. In a subset of recordings $I_A$ and $I_T$ were both activated in a single protocol using a modified ACSF (containing in mM: 97 NaCl, 2.5 KCl, 1 MgSO$_4$, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 20 D-glucose, 0.4 ascorbic acid, 5 CaCl$_2$, 2 pyruvic
acid, 0.5 μM TTX and 30 TEA; pH 7.4; 290-310 mosmol l⁻¹). The same protocol for the isolation of Iₜ (see above) was used.

For Iₐ, the chord conductance was calculated by measuring the peak amplitude of the evoked current at each command potential, divided then by the difference of the command potential and the reversal potential (calculated to be -104.2 mV from the Nernst equation). The chord conductance was then normalized to the maximum chord conductance obtained at +25 mV, and plotted as a function of the command potential. The plots were then fit with a Boltzmann function, and the half-activation potential (the Vₘ at which 50% of Iₐ currents are activated) was obtained.

The current densities for Iₐ and Iₜ were determined by dividing the current amplitude at each command potential by the cell capacitance, obtained by integrating the area under the transient capacitive phase of a 5 mV depolarizing step pulse, in the voltage-clamp mode. The rate of activation of Iₐ was determined by measuring the 10-90% rise time from the baseline to the peak of the current (command potential = -10 mV), while that of Iₜ was measured at a command potential of -40 mV.

Voltage dependence of inactivation of Iₐ

In order to determine the voltage-dependence of inactivation, neurons were voltage-clamped at -70 mV, and the membrane was subjected to conditioning pulses of varying amplitude (-120 to -35 mV, 50 ms), which removed varying amounts of inactivation from Iₐ. A command pulse to -10 mV was then used to activate Iₐ. In separate sets of experiments, the duration of the prepulses were extended to 115, 120 and 150 ms, as indicated. The mean normalized Iₐ peak amplitude was plotted as a function of the conditioning step potentials, and the I-V plots were fitted with a Boltzmann
function, to determine the half-inactivation potential (the Vm at which 50% of $I_A$ is inactivated). The voltage-dependence of $I_T$ was not determined in this study. The tau ($\tau$) of inactivation of $I_A$ and $I_T$ was determined by fitting a single exponential function to the decay phase of the current activated at -10 mV and -40 mV, respectively, following a conditioning step to -90 mV.

**Time-dependence of inactivation of $I_A$**

To determine the time-dependence of $I_A$ inactivation, neurons were voltage-clamped at -70 mV, and conditioning pulses to -45 mV or -50 mV of varying durations (10 to 200 ms, 10 ms increments) were followed by a command pulse to -10 mV (300 ms). Plots of the $I_A$ peak amplitude as a function of the duration of the conditioning pulse were then generated. Plots were fit by a monoexponential function, and the time constant of the decay was used for quantitative purposes.

**Kinetics of recovery from inactivation of $I_A$**

Once the A-type $K^+$ channel is inactivated following membrane depolarization, a sufficient amount of time, in which the membrane is hyperpolarized, must elapse before the channel can recover and be fully activated again. In order to determine the kinetics of recovery from the inactivated channel state, neurons were voltage-clamped at -50 mV, and a hyperpolarizing conditioning pulse (-100 mV) of increasing duration ($\Delta 10$ ms) was applied, followed by a depolarizing command pulse (-10 mV). The mean normalized peak amplitude was plotted against the conditioning pulse duration. A single exponential function was fit to the plot and the time constant ($\tau$) of recovery from inactivation was then calculated.

**Non-stationary fluctuation analysis of $I_A$**
For these studies, we followed procedures originally described by Sigworth [176,177] and subsequently used by others [5,53,163,197]. Traces of $I_A$ ($n=100-130$ traces) evoked with a command pulse to $+40$ mV were obtained and analyzed with Mini Analysis software (Synaptosoft, Fort Lee, NJ, USA). The mean evoked current was scaled to individual $I_A$ waveforms. The difference between the scaled mean and the single current resulted in difference currents with a variance that was higher than background levels. The variance of the individual $I_A$ current around the scaled average was then computed and variance-amplitude relationships were plotted and fit with a parabolic function. Values of unitary current, open probability and number of channels were provided by Mini Analysis algorithms.

*Current-clamp recordings of action potential waveform and firing activity*

For most current-clamp experiments, the ACSF used contained (in mM): 119 NaCl, 2.5 KCl, 1 MgSO$_4$, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 20 D-glucose, 0.4 ascorbic acid, 2 CaCl$_2$, 2 pyruvic acid; pH 7.4; 290-310 mosmol l$^{-1}$. In addition, the AMPA and NMDA glutamate receptor antagonists DNQX (10 $\mu$M) and AP-5 (100 $\mu$M), respectively, or the non-selective glutamate receptor antagonist kynurenic acid (2 mM), as well as the GABA$_A$ receptor antagonist bicuculline (40 $\mu$M), or picrotoxinin (0.3 mM) were added to this solution (see below). All protocols run used an output gain of 10 and a Bessel filter of 10kHz.

To test for the influence of $I_A$ on PVN-RVLM firing properties, the K$^+$ channel blocker 4-aminopyridine (4-AP) was used. Since 4-AP facilitates pre-synaptic release of neurotransmitter [67], direct effects of 4-AP on intrinsic properties could be masked by this presynaptic effect. Thus, all current-clamp experiments were performed in the
presence of receptor blockers of the main excitatory and inhibitory neurotransmitters in this system: GABA and glutamate (see above). Supporting the efficacy of this approach, we found that in the absence of these receptor blockers, 4-AP induced a significant decrease in PVN-RVLM input resistance (control = 1414 ± 462.7 MΩ; 4-AP = 829.4 ± 244.3 MΩ; n=7; P= 0.05), an effect likely due to overall increased neuronal conductance following robust release of neurotransmitters. Conversely, an increased input resistance was observed when 4-AP was applied in the presence of the receptor blockers listed above (control = 886.1 ± 127.0 MΩ; 4-AP = 1167 ± 147.5 MΩ; n= 23; P< 0.05). When needed, 4-AP was bath applied using a peristaltic pump (Gilson, Middleton, WI, USA; flow ~ 2 ml/min) for a period of 5 minutes before recording its effects. In most cases, in our hands, a complete washout of 4-AP and its effects were not accomplished within the period that we were able to maintain a good quality recording (see, however, Fig. 6A). Thus, values corresponding to the washout period are not reported.

Low-threshold spikes

Evoked low-threshold spikes (LTS) were simultaneously recorded with changes in intracellular calcium levels, before and after bath application of 5 mM 4-AP. The ACSF used, contained (in mM): 110 NaCl, 2.5 KCl, 1 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, 20 D-glucose, 0.4 ascorbic acid, 5 CaCl₂, 2 pyruvic acid, 0.5 μM TTX, 300 μM picrotoxinin and 2 mM kynurenic acid; pH 7.4; 290-310 mosmol l⁻¹). Thus, isolated LTS (no Na⁺ spikes) were evoked, from a Vm held at ~ -90 mV, by injecting depolarizing pulses (20-100 pA, 220ms). The LTS threshold was obtained by fitting a single monoexponential function to the trace and determining at what Vm the function no longer fits the trace. From threshold the LTS trace was then baselined to zero. The LTS
area (under the curve) and peak amplitude were then measured. For LTS experiments combined with calcium imaging, recordings were obtained with an Axopatch 200B amplifier (Axon Instruments). Those without calcium imaging were obtained as mentioned above.

**Evoked Action Potentials**

To elicit individual action potentials, PVN-RVLM neurons were current-clamped at either -80 mV or -50 mV, and subjected to depolarizing pulses (5 ms; 0.5-1.0 nA). At least ten sweeps of evoked action potentials were averaged together, and the mean action potential half-width and 90-10% decay time were analyzed and compared before and after addition of the A-type K\(^{+}\) channel blocker 4-AP, using algorithms provided by Mini Analysis software (Synaptosoft, Fort Lee, NJ, USA).

**Repetitive firing activity**

Spontaneous or evoked (DC current injection) firing discharge was recorded from PVN-RVLM neurons in continuous mode. The mean firing frequency obtained before and after addition of 4-AP or the voltage-dependent calcium channel inhibitor, NiCl\(_2\) (100 \(\mu\)M) (2 min period) were calculated and compared using Mini Analysis software (Synaptosoft). Neurons were arbitrarily considered responsive to 4-AP or NiCl\(_2\) if a change in firing rate >5% was observed.

In addition, all action potentials from the control and treated group were averaged into a single spike waveform, and the half-width and 90-10% decay time of the action potential were compared. In addition, parameters related to the hyperpolarizing afterpotential (HAP), including peak amplitude, area and kinetics, were calculated and compared before and after 4-AP addition. Action potential threshold was measured at the
abrupt transition from the pre-spike depolarizing ramp to the up-stroke of the action potential, as determined by algorithms provided by Mini Analysis software (Synaptosoft). HAP properties (e.g., amplitude, area and decay time course) were also determined by algorithms provided by Mini Analysis software (Synaptosoft).

To study spike broadening during repetitive firing, neurons were held at a $V_m \approx -90$ mV and depolarizing pulses (110-130 pA, 180 ms) were used to evoke firing discharge. The half-widths of the evoked action potentials were calculated. The degree of spike broadening was quantified as the ratio of the half-width of the third spike to that of the first spike (Shao et al., 1999; Stern, 2001).

Confocal calcium imaging

Dye-loading into identified retrogradely-labeled PVN-RVLM neurons was achieved by addition of fluo-5F pentapotassium salt (100 μM; Molecular Probes, Carlsbad, CA, USA) into the internal pipette solution. Once whole cell was established, the dye was allowed to diffuse into the cell for at least 20 minutes before the initiation of the recordings. Calcium imaging was conducted using the Yokogawa real time live cell laser confocal system (CSU-10) combined with a highly-sensitive EMCCD camera (iXon+885, Andor Technology, South Windsor, CT, USA). Fluorescence images were obtained using diode-pumped solid-state laser (Melles Griot, Carlsbad, CA, USA), and fluorescence emission was collected at >495 nm. Images were acquired at a rate of 40 - 50 Hz. The fractional fluorescence ($F/F_0$) was determined by dividing the fluorescence intensity ($F$) within a region of interest (ROI; 6 x 6 pixels $\approx 4.9 \times 4.9 \, \mu m$) by a baseline fluorescence value ($F_0$) determined from 30 images before single action potentials were evoked (a period showing no change in intracellular calcium levels) [63]. Between 8-10
traces of calcium transients were averaged together in order to increase the signal to noise ratio. Data was analyzed using Andor IQ software (Andor Technology).

Single cell real time, reverse transcription-polymerase chain reaction

The cytoplasm of a single neuron was gently pulled into a pipette with negative pressure, taking care not to contain the nucleus. The cytoplasm in the pipette was dissipated into a prepared tube containing (in μl): 18 of nuclease free water, 3 of 7x genomic DNA Wipeout Buffer and stored at -70 °C. After finishing all recordings, the tubes were heated to 42 °C for 2 min and then incubated on ice for at least 1 min. The mixture of (in μl) 6 of 5x Quantiscript RT Buffer, 1 of RT Primer Mix, 1 of Quantiscript Reverse Transcriptase was subsequently added and incubated at 42 °C for 15 min. The reaction was terminated by heating at 95 °C for 3 min and stored at -20 °C. All reagents for reverse transcription were purchased from Qiagen (Valencia, CA, USA). Real time PCR amplification was induced by using a fraction of the single cell cDNA as a template. The cDNA of the single neuron was split by 4 μl for three primer sets of GAPDH, Kv4.3 and Cav3.1. The reaction mixture (20 μl total volume) contained (in μl): 1 of 25 μM each forward and reverse primer, 4 of nuclease free water and 4 of the cDNA template, 10 of 2x SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The annealing temperature in the thermal cycler was 60 °C and 50 cycles were performed using an ABI Prism 7700 sequence detector (Applied Biosystems). Quantification was conducted by determining the relative changes in gene expression, to a chosen reference gene, using the \(2^{-\Delta\Delta Ct}\) method [128]. Statistical significance was determined by comparing raw ΔCt values. The standard deviations were used to calculate range values
(2−(ΔΔCT ± S.D. of ΔCT)) for graphs. We compared the efficiency between the target gene (Kv4.3 or Cav3.1) and GAPDH by plotting the delta Ct versus the log of the dilution ratio. The primers were considered efficient if the absolute value of the slope was less than 1. The slopes of Kv4.3 and Cav3.1 were determined to be 0.07 and 0.005, respectively. All primers except GAPDH used in this study were designed by Primer 3 (Whitehead Institute, Cambridge, MA, USA) and synthesized by Bioneer (Alameda, CA, USA). The primer set used in this study is presented in Table #1. The product size was adjusted around 150 bp that is suitable for the real time PCR reaction. The primer for GADPH was purchased from Qiagen.

**Neuronal morphometry**

In a subset of recordings, cells were intracellularly filled with biocytin (0.2%) and then stained with the avidin-biotin complex (ABC)-diaminobenzidine tetrahydrochloride (DAB) as previously described [185]. Briefly, after recordings were completed, slices were placed in a 4% paraformaldehyde and 0.2% picric acid solution, dissolved in 0.3 M PBS (pH~ 7.3) overnight and then thoroughly rinsed with 0.01 M phosphate buffered saline (PBS). Slices then were incubated at 4 °C for 1 hour in 10% normal horse serum with 0.01 M PBS and 0.5% Triton X-100. Slices were again thoroughly rinsed with 0.01 M PBS and incubated overnight in ABC (Vector Laboratories, Burlingame, CA, USA) diluted 1:100 in 0.01 M PBS containing 0.5% Triton X-100. Slices were then reacted with DAB (60 mg/100 ml) in 0.01 M PBS containing 0.5% Triton X-100, 0.05% nickel sulfate, and 0.006% H2O2, for approximately 2-3 minutes. Sections then were rinsed in 0.01 M PBS, mounted, and dried for 24 hours [185]. For morphometric analysis, the
### Real Time PCR Primers

<table>
<thead>
<tr>
<th>Gene (Accession No)</th>
<th>Sequence (forward/reverse)</th>
<th>Tm (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv4.3 (U42975)</td>
<td>5’-AGGCTTCTTCATTCGCTCT-3’</td>
<td>60</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>5’-GTGTCCAGGCAGAAAGAC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cav3.1 (AF027984)</td>
<td>5’-CAGTGTGTGGAGAATTGTGG-3’</td>
<td>60</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>5’-TGTCATGTGCTCTCAGTC-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. List of primers for A-type and T-type channel subunits used in the single cell real time PCR reactions.
entire somatic and dendritic compartments were reconstructed three-dimensionally using a tracing system (Neurolucida, Microbrightfield). Reconstructions were performed using a x60 oil objective, and the course of each dendrite was traced by digitizing the X, Y, and Z coordinates as well as its width. Several parameters, including dendritic length, surface area and volume were calculated by algorithms provided by Neurolucida. Axons were identified by their thinner diameter and beaded appearance, and were cut at the slice surface [185]. Damaged (e.g., somatic swelling, missing or cut dendritic trees) or weakly labeled neurons were not included in the analysis [185]. Data were not corrected for tissue shrinkage.

Immunohistochemistry

In order to determine which A-type channel subunits are expressed in PVN-RVLM neurons, these neurons were retrogradely labeled using cholera toxin B (CTB; 1%, List Biological Laboratories), using the same stereotaxic procedure as described above. Two to seven days after surgery, rats were anesthetized with nembutol (50 mg kg$^{-1}$) and perfused transcardially in 4% paraformaldehyde in 0.01 M phosphate buffer saline (PBS). Brains were then removed, postfixed for 2-4 hours, cryoprotected in 30% sucrose in 0.01 M PBS (4 °C, 3 days), and then stored at -80 °C until further use.

Coronal slices (30 μm) containing the PVN were cut and collected in 0.01 M PBS. Slices then were incubated in 0.01 M PBS with 0.1% Triton X-100, 0.04% NaN$_3$ (PBS$_{TXNaN_3}$), and 5% normal horse serum for 1 hour at room temperature. Slices were then rinsed thoroughly with 0.01 M PBS, followed by incubation with one of the following primary antibodies for A-type K$^+$ channel subunits (Kv1.4 1:100, Kv4.2 1:500, and Kv4.3 1:10000; Alomone Labs, Jerusalem, Israel), along with an anti-CTB antibody.
(goat anti-CTB 1:2500; List Biological Laboratories) for 2 days at 4 °C in PBS\textsubscript{TXNaN}_3. Slices were again thoroughly rinsed. Secondary antibodies were then applied for 4 hours at 4 °C in PBS\textsubscript{TXNaN}_3 (donkey anti-goat Cy-5: 1:50 and donkey anti-rabbit FITC: 1:250; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). Slices were then rinsed thoroughly, mounted, and visualized using confocal microscopy (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA); 63x oil immersion, zoomed x2; single optical plane = 0.5 μm thick).

Pre-adsorption controls were run in order to test the specificity of the Kv primary antibodies. The antigens were used at concentrations 5x those of the primary antibodies (Kv1.4 15 μg/ml, Kv4.2 8 μg/ml, and Kv4.3 0.4 μg/ml; Alomone Labs) and incubated in PBS\textsubscript{TXNaN}_3 for 2 hours at room temperature, with or without the primary antibodies. The solutions were then centrifuged for 5 minutes at 10000xg, and the supernatants applied to the tissue, following the same procedure described above.

In a subpopulation of recordings, in order to determine whether PVN-RVLM neurons expressed vasopressin and/or oxytocin, neurons were intracellularly filled with biocytin (0.2%). Slices were briefly fixed overnight in a 4% paraformaldehyde-0.2% picric acid solution, dissolved in 0.3 M phosphate buffered saline (PBS; pH~7.3) and then thoroughly rinsed with 0.01 M PBS. Slices were then taken through a dehydration procedure, in which slices were incubated in increasing concentrations of ethyl alcohol (60-100%; 10% increments; 10 min. each step except 100% for 20 min.). Slices were then incubated in xylene for 10 min. followed by the reverse ethyl alcohol procedure (100-60%). Slices were then rinsed in 0.01 M PBS with 0.5% Triton X-100 (TX) for 10 minutes. Slices were then incubated for 45 minutes in 10% normal horse serum with
0.01 M PBS, 0.5% TX and 0.04% NaN₃. Slices were then thoroughly rinsed with 0.01 M PBS, 0.5% TX and 0.04% NaN₃, followed by incubation with the primary antibodies for 2 days (1 day at room temperature and 1 day at 4°C): OT<sub>gp</sub> and VP<sub>gp</sub> (both 1:50,000; Bachem, Torrance, CA, USA) in 0.01 M PBS, 0.5% TX and 0.04% NaN₃. Slices were then rinsed in 0.01 M PBS, 0.5% TX and 0.04% NaN₃ for 30 minutes. Slices were then incubated with the secondary antibodies for 1 day (room temperature): CY5-streptavidin (1:10,000) and FITCgp (1:400; both from Jackson ImmunoResearch Laboratories) in 0.01 M PBS, 0.5% TX and 0.04% NaN₃. Slices were then thoroughly rinsed in 0.01 M PBS for 20 minutes, mounted, and visualized using fluorescence microscopy (20x; Olympus America Inc., Melville, NY, USA).

**Chemicals**

All chemicals were obtained from Sigma-Aldrich (St Louis, MO), with the exceptions of pyruvic acid (MP Biomedicals, Aurora, OH) and tetrodotoxin (Alomone Labs, Jerusalem, Israel).

**Statistical analysis**

All values are expressed as means ± S.E.M. Student’s paired t test was used to compare differences in various physiological parameters, as indicated in the text. Unpaired t test was used to compare between group differences, as indicated. A one- or two-way ANOVA with Bonferroni’s post hoc test were used when appropriate. Fisher’s exact test was used to determine differences in the incidence of a particular effect, and Pearson’s correlation test was used to determine if correlations existed between two parameters. Differences were considered statistically significant at a P< 0.05. All
statistical analyses were conducted using the same statistical software, GraphPad Prism (GraphPad Software, San Diego, CA, USA).
III. RESULTS

3.1. Functional Role of A-type Potassium Currents in Rat Presympathetic PVN Neurons

3.1.1. Intrinsic properties of PVN-RVLM neurons

Whole-cell patch clamp recordings were obtained from 119 retrogradely-labeled PVN-RVLM neurons, located in the three main PVN subnuclei known to contain long-descending preautonomic neurons: the ventromedial, dorsal cap and posterior subnuclei [185,192,194]. In many instances, neurons were efficiently loaded intracellularly with biocytin, and identified following an ABC-DAB procedure (see Fig 1B). Overall, 40, 16 and 24 neurons were found to be located within the ventromedial, dorsal cap and posterior subnuclei, respectively. Mean input resistance and cell capacitance of recorded neurons were 992.7 ± 49.3 MΩ, and 35.2 ± 1.4 pF, respectively. In current-clamp mode, most PVN-RVLM neurons displayed low threshold spikes (LTS) in response to positive current injection from hyperpolarized membrane potentials (~ -90 mV). Similarly to our previous study in PVN neurons innervating the dorsal vagal complex [185], LTSs in PVN-RVLM neurons varied greatly in shape and amplitude, including fast spikes, small humps, and long lasting plateaus (Fig 1C).

In voltage-clamp mode, using solutions that allowed isolation of K⁺ currents (see Methods), membrane depolarization evoked both transient (A-type, Iₐ) and sustained outward (IK_DR) components. To pharmacologically isolate the transient component, 30
Figure 1. PVN-RVLM projecting neurons are identified following microinjections of a fluorescent retrograde tracer in the RVLM. A- Representative example of a retrograde tracer injection site in the RVLM at three rostrocaudal brainstem levels (A1: Bregma – 11.30, A2: Bregma – 11.96; A3, Bregma – 14.08). Sections in the right panels show the rhodamine beads injection site (arrowheads). Bright light and fluorescent images were superimposed to better depict the injection site. Images on the left panels were obtained from similar rostrocaudal levels, and were counterstained to better depict the anatomy of the region. Arrows in A1, A2 and A3 point to the facial nucleus, the nucleus ambiguus and the area postrema, respectively. CC, central canal. B- A representative example of an intracellularly labeled PVN neuron located in the posterior subnucleus is shown in B1. The same neuron is shown at an expanded scale in B2, along with a following an ABC-DAB staining. C- PVN-RVLM neurons displayed low threshold spikes (LTS) (arrows) of varying shapes and magnitudes.
mM TEA was used [130,166]. However, since a remaining, TEA-insensitive sustained component was still present at relatively depolarized membrane potentials, \( I_A \) was further isolated electronically (see Methods and Fig. 2). Since the focus of the present study was on \( I_A \), the properties of \( I_{K_{DR}} \) were not further studied herein.

3.1.2. Activation properties of \( I_A \) in PVN-RVLM neurons

As previously described in other neuronal types [12,94], A-type \( K^+ \) currents (\( I_A \)) in PVN-RVLM neurons were characterized by strong voltage-dependency and rapid activation and inactivation kinetics, resulting in a transient outward current.

The voltage-dependent activation properties of \( I_A \) were studied in 26 PVN-RVLM neurons. Depolarizing steps of increasing amplitudes (-70 mV to +25 mV, in 5 mV increments) were used to activate \( I_A \) (Fig. 2A). The mean \( I_A \) peak amplitude, current density, and chord conductance at +25 mV were 933.8 ± 110.8 pA, 31.7 ± 4.0 pA•pF^{-1}, and 8.3 ± 1.4 nS, respectively (see Methods). The mean \( I_A \) 10-90% rise time (see Methods) was determined to be 6.1 ± 0.4 ms at a command potential of -10 mV (Fig. 2B). Plots of chord conductance vs. command potential were generated (see Methods), and the voltage-dependent properties of activation of \( I_A \) were calculated using a Boltzmann fit (Fig. 2C). The mean \( I_A \) activation threshold was -48.9 ± 1.4 mV, and the half-activation voltage was -24.5 ± 1.6 mV, with a slope factor of 11.7 ± 0.6 mV.

3.1.3. Inactivation properties of \( I_A \) in PVN-RVLM neurons

The voltage and time-dependent inactivation properties of \( I_A \) were studied in 35 PVN-RVLM neurons. The time-dependence of inactivation of \( I_A \) was studied using a command pulse to -10 mV (300 ms) from a conditioning step of either -50 mV or -45 mV of successively longer durations (10-200 ms, 10 ms increments). Plots of the evoked \( I_A \)
Figure 2. Isolation and voltage-dependent activation of $I_A$ in PVN-RVLM neurons.

A- Outward currents were activated in the presence of 30 mM TEA using depolarizing steps (from -70 to +25 mV in 5 mV increments, 400 ms) following a conditioning step to -90 mV (340 ms). Responses included a transient ($I_A$, arrow) and a TEA-insensitive sustained component ($I_{K_{DR}}$, arrowheads) (A1). Using a conditioning step to -40 mV, the same depolarizing steps resulted only in the activation of $I_{K_{DR}}$ (A2). $I_A$ was then electronically isolated by digitally subtracting traces in A1 and A2 (A3). B-

Representative example of the rapid rate of activation of $I_A$ following a command potential to -10 mV. C- Plot of the normalized chord conductance versus the command potential was created, and a Boltzmann function was fit to the I-V plot. The mean half-activation potential was -24.5 ± 1.6 mV (n= 23), with a slope factor of 11.7 ± 0.6 mV. Data were corrected for LJP.
amplitude as a function of the conditioning step duration were generated (see Fig. 3A). As shown, the amplitude of the evoked $I_A$ current rapidly decreased as a function of duration of the $-45$ mV conditioning step, reaching steady-state within a range of 80-110 ms ($n=6$). Plots were fit with a monoexponential function, and a mean time constant of $28.3 \pm 3.8$ ms was obtained. Similar results were observed with conditioning steps of $-50$ mV (results not shown).

The voltage-dependence of inactivation was then studied. To remove variable amounts of $I_A$ inactivation, neurons were depolarized using a command pulse to $-10$ mV, from a range of conditioning steps ($-120$ to $-35$ mV, $5$ mV increments, $50$ ms duration), preceded by a fixed pulse to $-40$ mV (65 ms) (Fig. 3B). I-V plots were generated and fit with a Boltzmann function, and the voltage-dependent inactivation properties were then calculated (see Methods) (Fig. 3C). The mean $I_A$ half-inactivation potential was $-87.4 \pm 3.1$ mV, with a slope factor of $13.1 \pm 0.5$ mV. Similar values were obtained when conditioning steps were prolonged in duration from $50$ ms to $115$, $120$ and $150$ ms (results not shown).

The mean inactivation $\tau$ of $I_A$ using a command potential to $-10$ mV was $33.9 \pm 3.0$ ms (Fig. 3C inset), and was found to be independent of the command potential ($F=0.41; P=0.8$; one-way ANOVA, data not shown).

When the voltage-dependent activation and inactivation curves of $I_A$ were plotted together, a small region of overlap between these two curves (i.e., “window current”) at potentials between $-55$ mV to $-40$ mV, was observed (Fig 3D). Despite its small amplitude ($1.25\%$ of maximal $I_A=\sim12$ pA at a membrane potential of $-51.5$ mV this window current may contribute substantially to subthreshold changes in membrane
Figure 3. Voltage-dependent and kinetics of inactivation of $I_A$. A- Time-dependence of inactivation of $I_A$ (n= 6). A command pulse to -10 mV (300 ms) was applied from a conditioning step of -45 mV of successively longer durations (10-200 ms, 10 ms increments). Note that the amplitude of the evoked $I_A$ rapidly decreased as a function of the duration of the conditioning step. The inset shows a plot of the evoked $I_A$ peak amplitude as a function of the conditioning step duration. The plot was fit with a monoexponential function, and a time constant ($\tau$) of 27.0 ms was obtained. B- Representative example of $I_A$ currents evoked by voltage steps to -10 mV from conditioning step to between -120 and -35 mV in 5 mV increments (50 ms). C- The mean normalized current amplitude was plotted against the conditioning potential and a Boltzmann function was fit to the I-V plot. The mean half-inactivation potential was -87.4 ± 3.1 mV (n=21), with a slope factor of 13.1 ± 0.5 mV. Data were corrected for LJP. Inset, a single exponential function (thick line) was fit to the $I_A$ decay phase. The mean time constant of inactivation (t) was 33.9 ± 3.0 ms at a command potential of -10 mV. D- When the mean normalized $I_A$ amplitude obtained with the voltage-dependent activation (triangles) and inactivation (squares) protocols are plotted together as a function of the command potential, a region of overlap between -55 mV and -40 mV is observed (gray area). Note that the plots were expanded to better depict the overlapping region. Data were corrected for LJP. Inset, summary data showing that blockade of $I_A$ with 5 mM 4-AP induced a significant membrane depolarization (*P<0.005, n=4). Neurons were current-clamped at ~ -50 mV.
potential, due to the relatively high input resistance of PVN-RVLM neurons. This is in fact supported by our results showing that pharmacological blockade of $I_A$ with 5 mM 4-AP (see below) induced a significant membrane depolarization (in 4/5 cells tested), when neurons were current-clamped at ~ -50 mV in the presence of TTX ($\Delta V_m$: 5.5 ± 0.5 mV, $P< 0.005$, paired t-test, n= 4, see Fig 3D inset).

3.1.4. Recovery of $I_A$ from inactivation

The time-course of recovery of $I_A$ from inactivation was studied in 16 PVN-RVLM neurons. To vary the amount of $I_A$ available for activation, neurons were hyperpolarized to -100 mV using conditioning steps of increasing duration (10 to 250 ms, in 10 ms increments). $I_A$ was then activated using a depolarizing command potential to -10 mV (Fig. 4A). The normalized $I_A$ peak amplitude at each command was plotted as a function of the duration of the respective conditioning step (Fig. 4A). The plots were best fit by a single exponential function. As depicted in the example of Fig. 4B, $I_A$ recovery from inactivation in PVN-RVLM neurons was strongly time-dependent, with a mean recovery time constant ($\tau$) of 65.7 ± 4.5 ms.

3.1.5. 4-AP inhibits $I_A$ in PVN-RVLM neurons

The sensitivity of $I_A$ to the K$^+$ channel blocker 4-AP was studied in 17 PVN-RVLM neurons. Similar protocols as those used to study activation of $I_A$ were used here. Currents were recorded before and after bath application of 1 or 5 mM 4-AP (Fig. 5A). Using a command step to -10 mV, we found $I_A$ to be significantly inhibited by both concentrations used (1 mM 4-AP: 20.3 ± 2.8% inhibition, n= 5; 5 mM 4-AP: 44.3 ± 2.3% inhibition, n= 12; $P< 0.01$ and $P< 0.0001$, compared to control, respectively). The larger inhibition observed with 5 mM 4-AP ($P< 0.0001$, when compared to 1 mM 4-AP),
Figure 4. Time course of recovery from inactivation of $I_A$.  

A - Representative example of $I_A$ currents evoked with command steps to -10 mV (200 ms), from conditioning steps to -100 mV of increasing duration (10 ms increments). Note that longer conditioning steps removed increasing amounts of inactivation of $I_A$, allowing for larger $I_A$ amplitudes at the command step.  

B - The mean normalized current amplitude evoked at the command test was plotted against the conditioning step duration. A single exponential function was fit to the plot, and the mean time course of recovery from inactivation ($\tau$) of $I_A$ in PVN-RVLM neurons was calculated to be $65.7 \pm 4.5$ ms ($n = 16$).
Figure 5. Inhibition of $I_A$ by 4-AP is both concentration and voltage-dependent.  

A- Representative trace of isolated $I_A$ before and after 5 mM 4-AP, at a command potential of -10 mV.  

B- Summary data showing partial block of $I_A$ with 1 and 5 mM 4-AP ($n=17$). Note the larger inhibition induced by the larger 4-AP concentration.  

C- The mean current amplitude of $I_A$ in control ACSF and in the presence of 5 mM 4-AP was plotted versus the command potential ($n=3$). Data was corrected for LJP. $\# p<0.01$ 'p<0.05 and $^* p<0.0001$ vs. control ACSF.
supports a concentration-dependent sensitivity of $I_A$ to 4-AP in PVN-RVLM neurons, as previously described in other neuronal types [18,179].

In a subset of neurons (n=3), the effect of 5 mM 4-AP was tested at a wide range of command steps (-70 mV to +25 mV) (Fig. 5C). As shown in the mean I-V plot generated from these recordings, $I_A$ sensitivity to 4-AP was found to be voltage-dependent ($F=63.3; n=3; P<0.0001$, 2-way ANOVA), with larger inhibition observed at more depolarized membrane potentials.

3.1.6. $I_A$ shapes the \textit{Na}⁺ action potential waveform in PVN-RVLM neurons

To determine whether $I_A$ modulates action potential waveform in PVN-RVLM neurons, recordings were obtained in the current-clamp mode (n=15). Action potential amplitudes in all recorded neurons were $\geq +50$ mV. Individual action potentials were evoked using short (5 ms) depolarizing pulses, while clamping the neurons at two different membrane potentials (-80 mV and -50 mV, see Methods), in order to obtain different degrees of $I_A$ inactivation. The effects of 4-AP (5 mM) on various action potential parameters were then determined. Results are summarized in Fig. 6.

At a holding potential of -80 mV, evoked action potential half width, and 90-10% decay time were $2.0 \pm 0.1$ ms and $1.9 \pm 0.2$ ms, respectively. Bath application of 4-AP at this holding membrane potential prolonged spike duration by $94.2 \pm 16.7\%$ ($P<0.0001$ vs control ACSF), and slowed down its decaying phase by $166.7 \pm 27.5\%$ ($P<0.0001$, vs control ACSF) (see Fig. 6).

Interestingly, similar changes in action potential waveform to those induced by 4-AP were observed when neurons were clamped at a more depolarized $V_m$. Thus, at a
Figure 6. Effects of 4-AP on evoked Na\(^+\) action potential waveforms. 

A- Representative examples of evoked (500 pA, 5 ms pulse) single action potentials before and after 5 mM 4-AP, at holding potentials of -80 (left) or -50 mV (right). 

B- Summary data showing the effects of 4-AP on action potential width and decay time at these two holding potentials (n= 15). Note that 4-AP significantly prolonged spike width and decay times, effects that were significantly larger when neurons were held at -80 mV.

***P<0.0001 vs. control within same holding potential group.  #P< 0.05, ##P< 0.01 and ###P< 0.0005 compared to same treatment at -80 mV.
holding potential of -50 mV, spikes were broader (half width: 3.2 ± 0.2 ms; P< 0.0005, vs. -80 mV), and displayed slower 90-10% decay times (2.6 ± 0.3 ms; P< 0.01, vs -80 mV). These changes were likely due to a higher degree of $I_A$ inactivation at the more depolarized $V_m$.

At this depolarized $V_m$ (-50 mV), 4-AP still induced similar changes in action potential waveform as those observed when neurons were clamped at -80 mV. However, these effects were significantly reduced. Thus, at a holding potential of -50 mV, 4-AP prolonged spike duration by 54.5 ± 12.4% (P< 0.05, vs. % change in 4-AP at -80 mV) and slowed down the decaying phase of the action potential by 95.1 ± 16.6% (P< 0.05, vs. % change in 4-AP at -80 mV). Results are summarized in Fig. 6. Altogether, these results suggest that the Na$^+$ action potential waveform in PVN-RVLM neurons is regulated by $I_A$, an effect found to be likely dependent on its voltage-dependent availability.

3.1.7. $I_A$ differentially regulates repetitive firing activity of PVN-RVLM neurons

The effects of 4-AP on firing activity were studied in 21 PVN-RVLM neurons. About 62% (13 out of 21) of recorded neurons were spontaneously active. In the remainder, firing activity was induced by injecting depolarizing DC current (+0.6 pA - +36.7 pA). In the majority of recorded cells (~62%, 13/21), 5 mM 4-AP resulted in an increased firing discharge. In the remainder (~38%, 8/21), a diminished firing discharge was observed.

Interestingly, PVN-RVLM neurons that were differentially affected by 4-AP also differed in some basic intrinsic properties. Neurons whose firing activity was enhanced by 4-AP had a more hyperpolarized resting $V_m$ (-51.7 ± 1.7 mV vs. -42.8 ± 2.2 mV, P<
and displayed a lower incidence of spontaneous activity than those inhibited by 4-AP (38.5% vs. 100%, respectively, \( P < 0.01 \), Fisher’s exact test). No differences in input resistance between the two groups were observed (842.6 ± 154.2 MΩ vs. 896.8 ± 157.4 MΩ in 13 and 8 neurons, respectively, \( P > 0.5 \)). Furthermore, as summarized in Table 2, significant differences in the Na\(^+\) action potential waveform were observed between the two groups of PVN-RVLM neurons. For example, neurons whose firing activity was increased by 4-AP displayed narrower (32% \( P < 0.0005 \)) and faster (44% \( P < 0.001 \)) decaying action potentials than those inhibited by 4-AP.

For simplistic purposes, further results obtained from these two differently responsive PVN-RVLM neurons are presented below in separate sections.

3.1.8. \( I_A \) restrains firing activity in a subset of PVN-RVLM neurons

Among the neurons whose firing activity were enhanced by 4-AP, ~69% (9/13) and ~31% (4/13) displayed continuous or bursting firing patterns, respectively. The relative low incidence of the latter group precluded us from obtaining a detailed analysis of the effects of 4-AP on bursting properties. Thus, all these neurons were pooled and the effect of 4-AP on their mean firing discharge was analyzed (see Methods). Results are summarized in Fig. 7. Within this group, 4-AP resulted in ~ 90% increase in firing rate (control: 1.0 ± 0.2 Hz; 4-AP: 1.9 ± 0.4 Hz; \( n = 13 \); Fig. 7A).

To determine whether \( I_A \) also modulates action potential waveform during repetitive firing activity, action potentials recorded in periods before and during 4-AP application were analyzed (Methods). Similarly to the effects observed on single evoked spikes (see above), bath application of 4-AP prolonged action potential duration (~70%) and 90-10% decay time (~93%), and slightly increased action potential amplitude (~9%).
### Action Potential Waveform Parameters

<table>
<thead>
<tr>
<th></th>
<th>4-AP Stimulated Group</th>
<th>4-AP Inhibited Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACSF</td>
<td>5mM 4-AP</td>
</tr>
<tr>
<td><strong>Peak Amplitude (mV)</strong></td>
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<td>70.3 ± 1.3 *</td>
</tr>
<tr>
<td><strong>Half-width (ms)</strong></td>
<td>3.0 ± 0.09</td>
<td>5.1 ± 0.3 *</td>
</tr>
<tr>
<td><strong>90-10% Decay Time (ms)</strong></td>
<td>1.5 ± 0.06</td>
<td>2.9 ± 0.2 *</td>
</tr>
<tr>
<td><strong>Threshold (mV)</strong></td>
<td>-31.2 ± 0.8</td>
<td>-32.8 ± 0.8 *</td>
</tr>
</tbody>
</table>

Table 2. The effects of 4-AP upon action potential waveform parameters. *P < 0.05 within same group; #P < 0.05 between different groups (same drug treatment).
Figure 7. 4-AP increases firing discharge in a subset of PVN-RVLM neurons.  

A- Representative examples of firing discharge in a continuously (upper trace) and bursting (lower trace) firing neurons before (left), during (middle) and after (right) bath application of 5 mM 4-AP. As summarized in the bar graphs, the firing frequency was significantly increased by 4-AP (n= 13).  

B- Representative examples of averaged spontaneous action potential waveform before (thin line) and after (thick line) application of 5 mM 4-AP, obtained from a neuron whose firing activity was increased by 4-AP. Inset, the HAP peaks were normalized and overlapped in order to more clearly depict the effects of 4-AP on the HAP slope. The mean effects of 4-AP on HAP properties are summarized in the bar graphs. *P<0.05 vs control.  

C- Another example of a 4-AP-induced increment in firing discharge in a PVN-RVLM neuron. In this case, negative current was injected in the presence of 4-AP, in order to diminish firing rate back to control levels. A plot of firing frequency as a function of time (10 s binning) is shown in the middle panel. In the right panel, a plot of action potential half-width as a function of time (10 s binning) is shown. Note that in the presence of 4-AP, the width of the action potentials were still prolonged, even when firing rate was similar to control levels.
Importantly, action potential threshold was shifted (~ 2 mV) to a more hyperpolarized membrane potential in the presence of 4-AP. Results are summarized in Table 2.

In order to rule out that changes in action potential waveform induced by 4-AP were secondary to membrane depolarization and increased firing rate *per se*, we performed a subset of recordings (n= 3) in which the membrane potential in the presence of 4-AP was hyperpolarized with DC current injection, in order to bring the firing frequency of the recorded cell back to control levels. As shown in Fig. 7C, the action potential half-width was still prolonged in the presence of 4-AP (92.8 ± 7.1% increase in half width, compared to control, P< 0.05, paired t-test), even when the firing rate was decreased near to control levels.

In addition, we analyzed the effects of 4-AP on hyperpolarizing afterpotentials (HAPs) during repetitive firing (Fig. 7B). Neither the HAP peak amplitude (measured as the difference from threshold to peak) nor its area were affected by 4-AP (P> 0.05 in both cases). Nonetheless, the absolute HAP peak potential reached a significantly more hyperpolarized membrane potential in the presence of 4-AP (control: -55.5 ± 0.9 mV; 4-AP: -58.2 ± 1.2 mV; n= 13, P< 0.05, paired t-test), likely due to the hyperpolarizing shift in spike threshold. Finally, the HAP decay time course was found to be steeper (~ 25%, P< 0.05) in the presence of 4-AP.

Multiple correlation analysis within this subset of PVN-RVLM neurons failed to identify any significant correlation between the degree of 4-AP-induced changes in firing activity, intrinsic membrane properties and/or action potential properties (R² values: 0.009 – 0.1).

3.1.9. Iₐ contributes to ongoing firing activity in a subset of PVN-RVLM neurons
Among the neurons whose firing activity were inhibited by 4-AP, 75% (6/8) fired in continuous mode, while the rest (2/8) displayed a bursting pattern. In 1 case, 4-AP switched the firing pattern from continuous to bursting mode (Fig. 8A). On average, 4-AP decreased the firing rate of this subgroup of PVN-RVLM neurons by ~ 40% (control ACSF: 2.3 ± 0.5 Hz; 4-AP: 1.4 ± 0.3 Hz; Fig. 8A).

Similarly to the other subset of PVN-RVLM neurons, bath application of 4-AP in the subset of PVN-RVLM neurons inhibited by 4-AP, increased the amplitude of the action potential (~ 10%), and prolonged its duration (~ 80%) and 90-10% decay time (160%). On the other hand, action potential threshold was not affected by 4-AP (results are summarized in Table 2).

In a subset of recordings (n= 4) the membrane potential in the presence of 4-AP was depolarized with DC current injection, in order to bring the firing frequency of the recorded neurons back to control levels. In these cases, action potential width was still prolonged by 4-AP (78.7 ± 25.8% increase in half width, compared to control, P< 0.05 paired t-test).

Interestingly, opposing effects on various HAP parameters within the subset of 4-AP inhibited PVN-RVLM neurons were observed when compared to the 4-AP-enhanced group. For example, 4-AP slowed down the HAP decay slope by ~ 24% (P< 0.05), and despite a slight decrease in HAP peak amplitude (~ 14%, P< 0.05), the overall HAP area was increased by ~ 27% (P< 0.01), likely due to the slower HAP decay time course. No differences in the absolute HAP peak potential were observed between control ACSF and 5 mM 4-AP (control: -53.4 ± 1.3 mV; 4-AP: -51.0 ± 3.0 mV; n= 8, P= 0.2). Results are summarized in Fig. 8B.
Figure 8. 4-AP diminished firing discharge in a subset of PVN-RVLM neurons.  
A- Representative examples of firing discharge in a continuously (upper trace) and bursting (lower trace) firing neurons before (left) and during (right) bath application of 5 mM 4-AP. As summarized in the bar graphs, the firing frequency was significantly diminished by 4-AP (n= 8).  
B- Representative examples of averaged spontaneous action potential waveforms before (thin line) and after (thick line) application of 5 mM 4-AP, obtained from a neuron whose firing discharge was diminished by 4-AP. Inset, the HAP peaks were normalized and overlapped in order to more clearly depict the effects of 4-AP on the HAP slope. The mean effects of 4-AP on HAP properties are summarized in the bar graphs. *P< 0.05 and **P< 0.01 vs control.
In this subset of neurons, a significant correlation between percent changes
induced by 4-AP on firing activity and HAP decay slope was observed ($R^2$: 0.6, $P<0.02$).
No other significant correlations between 4-AP-induced changes in firing activity,
intrinsic membrane properties and/or action potential properties were observed ($R^2$
values: 0.009 – 0.3).

3.1.10. Effects of TEA on action potential waveform and firing activity in PVN-RVLM
neurons

In addition to preferentially blocking $I_A$, 4-AP at low millimolar concentrations
may also partially block $I_{KD}$ [133,166]. In an attempt to further explore this possibility,
we tested the effects of TEA, which preferentially blocks $I_{KD}$ over $I_A$ [126,143], on
action potential waveform and firing activity in PVN-RVLM neurons. Bath application of
30 mM TEA robustly diminished $I_{KD}$ (94.9 ± 2.2%; n= 8) while only slightly inhibiting
$I_A$ (17.6 ± 3.5%; n= 8, $P<0.0001$, unpaired t-test; Fig. 9A). Differently from 4-AP, long
lasting plateau potentials were observed in the presence of TEA, resulting in all cases in a
robust inhibition of firing discharge (control: 6.0 ± 1.2 Hz; TEA: 1.0 ± 0.2 Hz; n= 4, $P<
0.05$ (paired t-test; Fig. 9C). Since TEA *per se* induced robust changes in action potential
waveform and firing activity, testing the effects of 4-AP in the presence of TEA in
current clamp recordings was not feasible. Thus, while we cannot completely rule out
that 4-AP effects on firing activity are in part due to blockade of $I_{KD}$, the disparity in the
effects observed between 4-AP and TEA treatments, along with the voltage-dependency
of 4-AP effects on some of the measured parameters (e.g., duration of action potential)
would suggest that these compounds act through different mechanisms.
Figure 9. Effects of TEA on outward $K^+$ currents and action potential waveform in PVN-RVLM neurons. A- Representative traces of outward currents evoked by a depolarizing step to -10 mV, from a conditioning step to -100 mV (500 ms), before and during bath application of 30 mM TEA. Note that TEA completely abolished the sustained outward component, while having very little effect on the transient A-type $K^+$ component (n= 8). B- Representative examples of spontaneous action potentials before and during bath application of 30 mM TEA. Note that TEA induced a long lasting plateau potential on the spontaneous spike. C- Summary data showing a significant decrease in the firing frequency of PVN-RVLM neurons in the presence of 30 mM TEA (n= 4). *P<0.05 vs control.
3.1.11. The effects of 4-AP on action potential waveform and firing activity in PVN-RVLM neurons are Ca\(^{2+}\)-dependent

By prolonging action potentials, blockade of I\(_A\) could result in an enhanced Ca\(^{2+}\) entry per spike [31,89], leading in turn to activation of various Ca\(^{2+}\) dependent mechanisms, such as Ca\(^{2+}\)-dependent K\(^+\) channels [167]. Depending on the complement of voltage-gated Ca\(^{2+}\), and Ca\(^{2+}\)-dependent conductances available within each particular neuronal type, 4-AP-induced changes in Ca\(^{2+}\) entry could either increase or decrease membrane excitability. Thus, to determine to what extent 4-AP-induced changes in firing discharge in PVN-RVLM neurons were Ca\(^{2+}\)-dependent, experiments were repeated in the presence of the broad spectrum Ca\(^{2+}\) channel blocker Cd\(^{2+}\) (200 μM; n= 12). Results are summarized in Fig. 10. In the presence of Cd\(^{2+}\), after-hyperpolarizing potentials (AHPs) following trains of spikes, a well-characterized Ca\(^{2+}\)-dependent property [20,91,187], were blocked, supporting the efficacy of Cd\(^{2+}\) to block Ca\(^{2+}\)-dependent membrane properties in these neurons (Fig. 10A).

In the presence of Cd\(^{2+}\), 4-AP was still able to prolong the duration of the action potential. However, the magnitude of this effect was smaller (though the difference did not reach statistical significance) as compared to control conditions (4-AP-ACSF= 77.2 ± 8.7%, 4-AP-Cd\(^{2+}\)= 55.5 ± 14.1%, P= 0.09). These results indicate that 4-AP-induced spike broadening likely results from both the slower action potential decaying phase, as well as increased Ca\(^{2+}\) influx during the depolarizing phase of the action potential prolonging its duration, as previously shown in magnocellular neurosecretory neurons [20]. In fact, the 90-10% decay time course of the action potential was still significantly slowed down by 4-AP (P< 0.05) in the presence of Cd\(^{2+}\), to a similar extent as that
Figure 10. 4-AP effects on PVN-RVLM firing discharge are Ca$^{2+}$-dependent. A-

The afterhyperpolarizing potential (AHP), a Ca$^{2+}$-dependent process, is blocked by bath application of 200 µM Cd$^{2+}$. B- In the presence of 200 µM Cd$^{2+}$, 4-AP failed to affect the firing discharge of PVN-RVLM neurons. Representative traces and the summary data are shown in the upper and lower panels, respectively (n= 12).
observed in control ACSF (4-AP-ACSF = 115.5 ± 15.2%, 4-AP-Cd\(^{2+}\) = 97.7 ± 26.7%, P= 0.3).

Importantly, 4-AP failed to affect the firing activity of all recorded neurons in slices pre-incubated (~ 5 min.) with Cd\(^{2+}\) (control: 1.2 ± 0.2 Hz; 4-AP: 1.4 ± 0.2 Hz; n= 12, P= 0.3, paired t-test; Fig. 10B). Furthermore, 4-AP failed to affect the action potential peak amplitude (p= 0.3), threshold (p= 0.4), HAP area (p= 0.3), or HAP slope (p= 0.1) in the presence of Cd\(^{2+}\).

3.1.12. Possible potassium channel subunits underlying A-type potassium currents in PVN-RVLM neurons

To gain insights into the possible Kv subunits underlying I\(_A\) in PVN-RVLM neurons, we combined immunohistochemical identification of Kv1.4, 4.2 and 4.3 subunits, (known to underlie A-type K\(^+\) currents in other CNS neurons, [37]) with neuronal tracing techniques (see Methods). Representative confocal photomicrographs of Kv1.4, 4.2 and 4.3 immunoreactivities in retrogradely-labeled PVN-RVLM neurons are shown in Fig. 11. Kv1.4 and 4.3 subunits were found to be widely expressed and distributed within the PVN (Fig. 11A). On the other hand, Kv4.2 immunoreactivity was very weak and/or undetectable in the PVN. Conversely, a robust Kv4.2 immunoreactivity was observed in ependymal cells lining the third ventricle, and in glial-like processes in the median eminence (Fig. 11A2), supporting the efficacy of our approach to detect this immunoreactivity. All Kv immunoreactive reactions were blocked following pre-adsorption of antibodies with their respective peptides (results not shown).

Immunoreactivities for these Kv subunits were sampled in a total of 68 retrogradely-labeled PVN-RVLM neurons at higher magnification. Strong
Figure 11. Kv1.4, 4.2 and 4.3 immunoreactivity in retrogradely labeled PVN-RVLM neurons. A- Low magnification confocal images of the PVN (10X) showing Kv1.4 (A1), Kv4.2 (A2) and Kv4.3 (A3) immunoreactivities. The inset in A2 shows Kv4.2 immunoreactivity in the median eminence. B- High magnification confocal images of retrogradely labeled PVN-RVLM neurons (B1) and Kv 1.4 immunoreactivity (B2). Both images were superimposed in B3 to better depict colocalization of the two signals. Arrowheads in B3 point to representative Kv1.4 immunoreactive clusters located at the surface of the retrogradely labeled neuron. A magnified image is shown in the inset. C- High magnification confocal images of retrogradely labeled PVN-RVLM neurons (C1) and Kv 4.2 immunoreactivity (C2). Both images were superimposed in C3. Note the lack of Kv4.2 immunoreactivity in the neurons displayed. D- High magnification confocal images of retrogradely labeled PVN-RVLM neurons (D1) and Kv 4.3 immunoreactivity (D2). Both images were superimposed in C3 to better depict colocalization of the two signals. Arrows in B-D point to the location of PVN-RVLM retrogradely labeled neurones (n= 68).
immunoreactivities for Kv1.4 and Kv4.3 subunits were clearly present in PVN-RVLM neurons (32/33 and 15/16, respectively), as well as in nearby, unlabeled neurons. Representative examples are shown in Fig. 11B1-B3 (for Kv1.4) and Fig. 11D1-D3 (for Kv4.3). Immunoreactivities for these subunits were highly punctate in nature, with immunoreactive clusters located within the cytoplasm as well as near the surface membrane, both in somatic and dendritic compartments. In the case of Kv4.3 subunits, nuclear immunoreactivity was also evident. On the other hand, weak or undetectable Kv4.2 immunoreactivity was observed in PVN-RVLM, or other PVN neurons (Fig. 11C1-C3).

3.2. Altered A-type Potassium Current and Firing Properties in Rat Sympathetic Preautonomic PVN Neurons During Hypertension

3.2.1. Intrinsic properties of sham and hypertensive PVN-RVLM neurons

Whole-cell patch clamp recordings were obtained from retrogradely labeled PVN-RVLM neurons in sham (n= 41) and hypertensive (n= 43) rats. The mean input resistance was 1226.0 ± 170.8 MΩ and 1178.0 ± 109.4 MΩ for sham and hypertensive PVN-RVLM neurons, respectively (P> 0.8). Neuronal cell capacitance was significantly reduced in PVN-RVLM neurons during hypertension (sham: 27.92 ± 1.37 pF; hypertensive: 23.05 ± 1.31 pF, P= 0.01).

3.2.2. Changes in PVN-RVLM neuronal morphometry during hypertension

Cell capacitance is a general indicator of neuronal membrane surface area [82,127]. Thus, changes in cell capacitance during hypertension may be indicative of somatodendritic structural changes during this condition. To determine if this was the
case, a subset of recorded neurons that were properly filled with biocytin (n= 24 and 27 in sham and hypertensive) were reconstructed in three-dimension to compare for differences and/or changes in somatic/dendritic surface area among groups (Methods). Results are summarized in Fig.12. Overall neuronal surface area was significantly reduced in PVN-RVLM neurons in hypertensive rats (P< 0.05). While a tendency for reduced somatic area was observed (P= 0.07), our results suggest that the reduced neuronal size was mostly due to a significant reduction in dendritic surface area (P= 0.02).

Importantly, significant correlations were observed between cell capacitance and somatic area, as well as cell capacitance and dendritic surface area (r= 0.6, P< 0.0001 in both cases, Pearson’s correlation test), supporting cell capacitance measurements in our studies as reliable indicators of neuronal surface area (see below).

3.2.3. $I_A$ voltage-dependent activation properties

In a first set of studies (voltage-clamp mode), a combination of pharmacological and digital methods were used to isolate and compare the magnitude and voltage-dependent properties of the A-type $K^+$ current ($I_A$) between the two experimental groups (n= 27 and 20 in sham and hypertensive rats). Depolarizing steps of increasing amplitudes (-70 mV to +25 mV, in 5 mV increments) were used to activate $I_A$ (Figure 13A), and I/V plots were generated and fit with a Boltzmann function (Fig. 13B-C). $I_A$ amplitude/density, activation threshold, half-activation potential, and 10-90% rise time were analyzed and compared between groups.

We first compared the $I_A$ activation curves. As shown in Fig. 13B, $I_A$ activation was significantly reduced in PVN-RVLM neurons from hypertensive rats ($F= 37.2, P<
Figure 12. Changes in PVN-RVLM neuronal morphometry during hypertension.

A- Representative examples of reconstructed PVN-RVLM neurons in sham and hypertensive rats. Axons are identified with arrows. B- Summary data of overall neuronal, somatic and dendritic surface areas (n= 24 and 27 sham and hypertensive neurons, respectively). Note the reduction in the overall neuronal and dendritic surface area in PVN-RVLM neurons during hypertension. *P< 0.05.
Figure 13. Changes in $I_A$ voltage-dependent activation properties during hypertension.  

A- Representative example of $I_A$ voltage-dependent activation in PVN-RVLM neurons obtained from a sham and a hypertensive rat. Note the smaller maximum current amplitude in the latter.  

B- Mean plots of current amplitude versus the command potential fit with Boltzmann functions. Squares and triangles represent sham and hypertensive rats, respectively (n= 27 and 20, respectively). Note the significant reduction of $I_A$ magnitude during hypertension.  

C- Mean plots of $I_A$ current density versus the command potential fit with Boltzmann functions. Note the lack in reduction of $I_A$ current density in PVN-RVLM neurons during hypertension.  

D- Summary data of $I_A$ activation threshold and half-activation potential. No significant differences were observed among the experimental groups. *P< 0.05, **P< 0.01.
0.0001, 2 way ANOVA). Based on the differences found in cell size/morphometry between groups (see above), we addressed whether changes in current amplitude during hypertension persisted after being normalized by cell size (i.e., current density, Fig. 13C). Our results indicate no differences in PVN-RVLM $I_A$ current density between sham and hypertensive rats ($F=1.4, P>0.2$, 2 way ANOVA). No differences in $I_A$ activation threshold or in the half-activation potential were observed between sham and hypertensive rats ($P>0.5$ in both cases) (Fig. 13D). Similarly, no differences in the slope factor (k) of the voltage-dependent activation curve, which describes the steepness of the activation curve (voltage sensitivity), were observed between groups ($P>0.5$). Finally, no differences in the rate of activation of $I_A$, calculated as the 10-90% rise time following a command potential to -10 mV, were observed between the two groups ($P=0.9$).

To determine whether changes in $I_A$ properties during hypertension were specific to PVN-RVLM neurons, or alternatively, whether they affected other preautonomic cell populations, we obtained recordings from identified PVN neurons that innervate the dorsal vagal complex (DVC), as a control group. PVN-DVC neurons comprise a heterogeneous population that innervates both the nucleus of the solitarii tract (NTS), as well as the dorsal motor nucleus of the vagus [55,72,98,178]. Results are summarized in Fig. 14. Differently from PVN-RVLM neurons, no structural morphometric changes were observed during hypertension in PVN-DVC neurons (Fig. 14B). Nonetheless, and similar to PVN-RVLM neurons, $I_A$ activation was significantly reduced in PVN-DVC neurons from hypertensive rats ($F=87.6, P<0.0001$, 2 way ANOVA). Since no differences in cell size were observed between sham and hypertensive rats, $I_A$ current
Figure 14. Morphometric and state-dependent differences in $I_A$ voltage-dependent activation properties of PVN-DVC neurons.  

A- Representative examples of reconstructed PVN-DVC neurons in sham and hypertensive rats. Axons are identified with arrows.  

B- Summary data of somatic and dendritic surface area. Note the lack of changes during hypertension.  

C- Mean plots of the current amplitude and current density versus the command potential fit with Boltzmann functions. Squares and triangles represent sham and hypertensive rats, respectively (n= 18 and 14, respectively). Note the significant reduction of $I_A$ amplitude and current density during hypertension. *P< 0.05, **P< 0.01, ***P<0.0001.
density was also diminished in PVN-DVC neurons during hypertension (F = 61.0, P < 0.0001, 2 way ANOVA). Due to the more homogenous nature, and more established relevance in cardiovascular control, the rest of our studies focused on PVN-RVLM neurons.

3.2.4. $I_A$ voltage-dependent inactivation properties

The voltage-dependent and kinetic properties of inactivation of $I_A$ were studied in 25 sham and 18 hypertensive PVN-RVLM neurons. Results are summarized in Fig.15. Overall, $I_A$ steady state inactivation was found to be significantly increased in PVN-RVLM neurons from hypertensive rats (F = 37.8, P < 0.0001, 2 way ANOVA). Moreover, the mean half-inactivation potentials ($V_{1/2\text{inact}}$, i.e., the Vm at which 50% of the maximum current is inactivated) were significantly shifted to a more hyperpolarized membrane potential (~ 6 mV) in PVN-RVLM neurons from hypertensive rats (P < 0.05). The slope factor (k) of the inactivation curve was slightly though significantly increased in PVN-RVLM neurons during hypertension (sham: 12.1 ± 0.4 mV; hypertensive: 14.5 ± 0.6 mV (P < 0.01). Finally, no differences in $I_A$ rate of inactivation ($\tau$) were observed between groups (P > 0.5).

3.2.5. Recovery of $I_A$ from inactivation

The rate of $I_A$ recovery from inactivation was determined in 20 sham and 12 hypertensive PVN-RVLM neurons. A progressively larger amount of $I_A$ activation was achieved following hyperpolarizing pulses of increasing durations (-100 mV, 10-250 ms, in 10 ms increments), followed by a command potential to -10 mV (Fig. 16A), supporting hyperpolarization-dependent removal of $I_A$ inactivation. Plots of the normalized $I_A$ peak
Figure 15. Changes in I_A voltage-dependent inactivation properties in PVN-RVLM neurons during hypertension. A- Representative traces of the voltage dependence of inactivation of I_A in sham and hypertensive rats. The gray traces (arrow) represent the current evoked at the half-inactivation potential (values listed under the traces). B- Plots of mean normalized current amplitude versus the conditioning potential fit by Boltzmann functions. Note the hyperpolarizing shift in PVN-RVLM neurons, during hypertension. Squares and triangles represent sham and hypertensive rats, respectively (n= 25 and 18, respectively). C- Summary data of I_A half-inactivation potential. *P< 0.05.
Figure 16. Time course of recovery from inactivation of $I_A$ in PVN-RVLM neurons in sham and hypertensive rats. A- Representative traces of $I_A$ currents evoked from a command pulse of -10 mV with a conditioning pulse (-100 mV) of increasing duration (10-250 ms, Δ10 ms increments). Lower panels depict the normalized current amplitude evoked at the command test, plotted against the conditioning step duration. A single exponential function was fit to the plot, and the time course of recovery from inactivation ($\tau$) of $I_A$ was calculated. The time constant ($\tau$) of recovery from inactivation is listed below each trace. B- Summary data of mean $\tau$ of recovery from inactivation (n= 20 sham and 12 hypertensive neurons). No significant differences were observed between groups.
amplitude as a function of the duration of the hyperpolarizing conditioning steps were built, and fit with a monoexponential function (Fig. 16B), to determine the $\tau$ of recovery from inactivation. No significant differences in the mean $\tau$ of recovery from inactivation were observed between PVN-RVLM neurons in sham and hypertensive rats ($P > 0.5$).

3.2.6. Non-stationary fluctuation analysis of $I_A$ in PVN-RVLM neurons: Changes during hypertension

To determine whether changes in single channel properties contributed to the diminished $I_A$ current observed during hypertension (see above), we used non-stationary fluctuation analysis, a method originally described by Sigworth [176,177]. This approach enables the extraction of single-channel information from macroscopic currents, including unitary current, open probability and number of channels. $I_A$ evoked ($n = 100-130$ traces) with a command pulse to $+40$ mV were obtained. The mean evoked current was scaled to individual $I_A$ waveforms, and the variance of the individual $I_A$ current around the scaled average was computed. Variance-amplitude relationships were plotted and fit with a parabolic function (Methods). A representative example is shown in Fig. 17. In most cases, experimental points over the right side of the parabola were missing, indicative that channel open probability never reached a value of 1.0, likely due to channel inactivation [5,176,177]. Under this condition, we were limited to estimate the unitary current ($i$), determined by the slope of the parabola, shown to be highly accurate even at limited levels of open probability. On the other hand, the number of channels can only be accurately estimated at an open channel probability near 1 [5,176,177]. As shown in Figure 17C, our results indicate a significantly diminished $I_A$ single channel conductance in PVN-RVLM neurons during hypertension ($P < 0.05$).
Figure 17. Non-stationary noise analysis of $I_A$ in PVN-RVLM neurons of sham and hypertensive rats. 

**A**- Representative traces of $I_A$ evoked with a command potential to +40 mV. The grey trace is the mean evoked current.  

**B**- Plot of the variance from the mean trace as a function of the current amplitude, fit with a parabolic function.  

**C**- Summary data of mean single channel conductance in sham and hypertensive rats (n= 12 and 9, respectively). Note the significantly reduced single channel conductance in the hypertensive neurons. *P<0.05.
3.2.7. The Na$^+$ action potential waveform is affected in PVN-RVLM neurons during hypertension

Abundant evidence supports a major role for I$_A$ in the regulation of single and repetitive neuronal firing behavior [39,105,166,173]. Thus, we evaluated whether action potential properties known to be regulated by I$_A$ are altered in PVN-RVLM neurons during hypertension. In a first series of studies, we evaluated differences in the single action potential waveform. Individual action potentials were evoked using short (5 ms) depolarizing pulses, while injecting current to maintain Vm at approximately two different membrane potentials (~ -80 mV and ~ -50 mV, see Methods), in order to obtain different degrees of I$_A$ inactivation. In PVN-RVLM neurons from sham rats, action potentials evoked from a more depolarized Vm (~ -50 mV) were wider (by ~ 177.5 ± 16.3%, P< 0.001) and displayed a slower decay time course when compared to spikes evoked at a Vm of ~ -80 mV (by 160.0 ± 23.3%, P< 0.05 for width and decay time, respectively). These differences were likely due to a higher degree of I$_A$ inactivation at the more depolarized Vm, as we recently reported [180]. At a membrane potential of ~ -80 mV, action potentials in PVN-RVLM neurons from hypertensive rats were wider and decayed slower than those evoked in sham rats at a similar Vm (P< 0.05 in both cases). Spike width in hypertensive rats was further increased at a Vm ~ -50 mV (151.3 ± 11.0%, P< 0.05), though to a lesser extent than that observed in sham rats (see above). Moreover, the decay time course at ~ -50 mV was not significantly different than that observed at ~ -80 mV (P> 0.5). Results are summarized in Fig. 18A.

As we previously reported [180], 4-AP, a K$^+$ channel blocker that more selectively blocks I$_A$ over other voltage-dependent K$^+$ channels (e.g., IK$_{DR}$) prolonged the
Figure 18. Changes in Na\textsuperscript{+} action potential waveform of PVN-RVLM neurons during hypertension. A1- Representative traces of evoked action potentials at a membrane potential ~ -80 mV (black trace) and ~ -50 mV (grey trace) in a PVN-RVLM neuron from a sham rat. Action potentials were aligned to better depict differences in their waveforms. A2- Summary data of evoked action potential half-widths and decay-times at membrane potentials of ~ -80 mV and ~ -50 mV. *P<0.05, **P<0.01. B1- Representative traces of spontaneous firing activity in PVN-RVLM neurons from a sham and a hypertensive rat. The averaged spontaneous action potential from the sham (black trace) and the hypertensive (grey) rat were superimposed and shown in the right panel. B2- Summary data of basal spontaneous firing activity, half-width, decay-time, and HAP decay slope from the averaged spontaneous single spikes (n= 9 sham and 12 hypertensive neurons). *P<0.05, **P<0.01.
action potential width and slowed down the decay time course in PVN-RVLM neurons, effects that were significantly diminished in hypertensive rats (spike width: sham: 243.4 ± 17.2%; hypertensive: 176.4 ± 16.9%; decay time: sham: 285.0 ± 27.4%; hypertensive: 190.9 ± 24.7 %, P< 0.05 in both cases, paired-t test).

To determine whether the action potential waveform during repetitive firing activity in PVN-RVLM neurons also was altered in hypertensive rats, action potentials from spontaneously active neurons (n=9 and 12 in sham and hypertensive rats, respectively) were grouped and compared between experimental groups (Methods). Representative examples are shown in Fig. 18B. Spontaneous firing activity was significantly higher in PVN-RVLM neurons from hypertensive rats (P< 0.01). Similarly to the differences observed on single evoked spikes, spontaneous action potentials in hypertensive rats were wider (P< 0.05) and displayed a slower decay time course (P< 0.005) than those recorded from sham rats. Moreover, the slope of the hyperpolarizing afterpotential (HAP) following each spike was significantly steeper in PVN-RVLM neurons from hypertensive rats (P< 0.01). Altogether, these data support a diminished I_A influence in the regulation of the action potential waveform in PVN-RVLM neurons during hypertension.

3.2.8. Action potential-dependent increase in intracellular Ca^{2+} is enhanced in PVN-RVLM neurons during hypertension

Since an increase in spike duration is known to be associated with an increase in intracellular calcium levels [106], we combined simultaneous patch clamp recordings and confocal Ca^{2+} imaging in order to determine if intracellular calcium levels ([Ca^{2+}]_c) were altered during hypertension. Retrogradely-labeled PVN-RVLM neurons were
intracellularly filled with Fluo-5F (100 μM), and relative changes in \([\text{Ca}^{2+}]_{\text{ic}}\) in response to single evoked action potentials were measured (Methods, Fig. 19A,B), and between 8-10 traces of calcium transients were averaged together in order to increase the signal to noise ratio. The peak of the \([\text{Ca}^{2+}]_{\text{ic}}\) transient evoked from a single spike was measured before and after 5 mM 4-AP application in PVN-RVLM neurons from sham (n=9) and hypertensive (n=11) rats. Results are summarized in Fig. 19C. Action potential-evoked \([\text{Ca}^{2+}]_{\text{ic}}\) transients in PVN-RVLM neurons from hypertensive rats were significantly larger to those evoked in sham rats (P< 0.05). Moreover, while 4-AP significantly enhanced the \(\text{Ca}^{2+}\) transient in the sham group (78.1 ± 19.3%, P<0.05), it failed to do so in the hypertensive group (P>0.05) (Fig 19C).

3.2.9. Spike broadening during repetitive firing is enhanced in PVN-RVLM neurons during hypertension

Repetitive firing activity of PVN neurons, including identified preautonomic neurons, is characterized by a progressive increment in action potential duration (spike broadening) [10,185], a phenomenon shown to be dependent on progressive increment in \([\text{Ca}^{2+}]_{\text{ic}}\) and steady-state inactivation of \(I_A\) [88]. Thus, we explored whether spike broadening in PVN-RVLM neurons was also affected during hypertension. Repetitive firing was evoked with depolarizing pulses (110-130 pA, 180 ms), and the spike width of the evoked action potentials was measured and compared between groups. The degree of spike broadening was quantified as the ratio of the duration of the third spike to that of the first spike during the train [175,185]. Representative examples and summary data are shown in Fig. 20. While spike broadening was observed in both groups, the degree of
Figure 19. Changes in action potential-evoked $[\text{Ca}^{2+}]_{ic}$ levels in PVN-RVLM neurons during hypertension.  

A- Representative traces of an evoked action potential (grey) and the resultant change in $[\text{Ca}^{2+}]_{ic}$ levels (black) in a sham rat.  

B- Representative confocal images showing a Fluo-5 loaded PVN-RVLM neuron (left panel), as well as basal and peak action potential-evoked $[\text{Ca}^{2+}]_{ic}$ levels (pseudocolor images) in the absence and presence of 5 mM 4-AP (scale units are F/F$_0$). Note the increase in the amplitude of $[\text{Ca}^{2+}]_{ic}$ levels upon 4-AP application. The lower traces show the time course of changes in $[\text{Ca}^{2+}]_{ic}$ in control (black trace) and in the presence of 4-AP (grey trace).  

C- Summary data of the peak of the $[\text{Ca}^{2+}]_{ic}$ transient, before and after 4-AP, in sham and hypertensive neurons (n= 9 and 11, respectively). *P<0.05.
Figure 20. Changes in spike broadening in PVN-RVLM neurons during hypertension. 

A- Representative trace of an evoked burst of spikes (110 pA, 180 ms) in a PVN-RVLM neuron from a sham rat (left panel). In the right panel, the evoked spikes were scaled and superimposed to better depict the progressive increase in width. 

B- Mean ratio of the half-width of the third spike to that of the first spike. Note the significantly increased ratio in the hypertensive group (n= 22 and 15 in sham and hypertensive rats, respectively). *P<0.05.
broadening was significantly larger in PVN-RVLM neurons from hypertensive rats (P<0.05).

3.3. Contribution of Subthreshold Ion Channels, to Hypertension, in Preautonomic PVN Neurons

3.3.1. Intrinsic Properties of sham and hypertensive PVN-RVLM neurons

Whole-cell patch clamp recordings were obtained from retrogradely labeled PVN-RVLM neurons in sham (n= 80) and hypertensive (n= 90) rats. The mean input resistance was 1003 ± 84.0 MΩ and 1034 ± 79.2 MΩ for sham and hypertensive PVN-RVLM neurons, respectively (P= 0.8). Neuronal cell capacitance was significantly reduced in PVN-RVLM neurons during hypertension (sham: 20.6 ± 1.0 pF; hypertensive: 17.6 ± 0.9 pF, P< 0.05), as previously reported (see 3.2.1.).

3.3.2. \( I_A \) and \( I_T \) are expressed and compete within individual PVN-RVLM neurons

In response to depolarizing steps from a hyperpolarized \( V_m \), and under conditions where \( K^+ \) and \( Ca^{2+} \) currents were pharmacologically isolated (Methods), both a transient outward \( K^+ \) current \( (I_A) \) and a transient inward \( Ca^{2+} \) current \( (I_T) \) were observed, respectively, in PVN-RVLM neurons (Fig. 21A). As we previously reported, \( I_A \) and \( I_T \) were sensitive to block by 4-AP (2-5 mM) and NiCl\(_2\) (100 µM), respectively (not shown, [180,185]). \( I_T \) rise-time (10-90%) and \( \tau_{\text{inactivation}} \) ranged from 3.2-25.4 ms (mean rise time= 14.6 ± 3.1 ms) and from 8.4-50.6 ms (mean \( \tau_{\text{inactivation}} = 31.9 ± 5.5 \) ms), respectively. These values were similar to those we recently reported for \( I_A \) in these neurons [180]. While no differences in current density between \( I_A \) and \( I_T \) were observed over the voltage range tested (P= 0.12, 2-way ANOVA, Fig. 21B), \( I_T \) activated at a
Figure 21. $I_A$ and $I_T$ are expressed within individual PVN-RVLM neurons. 

A- Representative examples of isolated $I_A$ and $I_T$ in different PVN-RVLM neurons.  

B- Mean plots of $I_A$ (squares) and $I_T$ (triangles) current densities versus the command potential.  

C- Representative example of a PVN-RVLM neuron in which both $I_A$ and $I_T$ were recorded at the same command potential. Note that when the $I_A$ inhibitor 4-AP (5 mM) was applied (right), $I_A$ was blocked and $I_T$ became larger in amplitude.  

D- Plot of current amplitude versus command potential of example trace in C ($I_A$ control= squares, $I_T$ control= triangle, $I_T$ in 4-AP= upside down triangle).  

E- Summary data of the mean $I_T$ current amplitude measured at a command potential of -20 mV before and after 4-AP application (n= 9). Note the more robust $I_T$ amplitude upon $I_A$ inhibition. *P< 0.05.
significantly more hyperpolarized Vm than I_A (I_A= -46.3 ± 1.2 mV and I_T= -54.6 ± 1.6 mV, P= 0.001).

Based on their similar magnitude and voltage-dependent properties, we investigated whether both opposing currents “competed” at similar membrane potentials [27,142,146,155]. To this end, I_A and I_T were simultaneously recorded in the same PVN-RVLM neurons (Methods) (Fig. 21C). In all cases (n= 16, both sham and hypertensive), both currents were observed within individual neurons. In most cases (13/16 neurons), and as shown in the representative example in Fig. 21D, I_T became apparent at a significantly more hyperpolarized Vm than I_A (I_T= -51.8 ± 1.9 mV and I_A= -36.2 ± 2.3 mV; P< 0.0005). Under this condition, the activation threshold of I_A was more depolarized than that observed when I_A was pharmacologically isolated (see above). As the Vm became more depolarized (-45 through -20 mV), I_A grew in amplitude while opposing changes were observed in I_T (Fig. 21D). These results suggest an active competition/balance between the two opposing currents, with I_T and I_A predominating at relatively hyperpolarized and depolarized Vms, respectively. This was further supported by our results showing that pharmacological inhibition of I_A with 5 mM 4-AP “unmasked” a more robust I_T (P< 0.05, n= 9; Fig. 21D,E).

In recent studies, we demonstrated that the Kv4.3 and Cav3.1 K^+ and Ca^{2+} subunits are predominantly expressed in PVN-RVLM neurons [115,180], likely mediating I_A and I_T, respectively. To determine whether the relative expression of these subunits influenced the balance of these two subthreshold conductances, we recorded I_A and I_T in a subset of neurons (n= 15), in which the cytoplasmic content was subsequently aspirated, and quantitative single-cell RT-PCR was performed (Methods). The ratio of
the amplitude of the evoked $I_T$ and $I_A$ at a $V_{m}$ of -20 mV was plotted as a function of the Cav3.1/Kv4.3 subunit expression for that particular neuron (Fig. 22). A significant correlation between the two ratios was observed ($r^2 = 0.61; P< 0.001$).

3.3.3. $I_A/I_T$ balance influences the expression and magnitude of LTS

It is well-established from studies in numerous neuronal types that while $I_T$ mediates low threshold spikes (LTS) [129,185], $I_A$ on the other hand, underlies a transient outward rectification (TOR) [19,65,133]. Thus, it is unclear within a given cell that contains both $I_A$ and $I_T$, such as PVN neurons, what dictates which membrane property is observed. Therefore, we simultaneously recorded $I_A$ and $I_T$ under voltage-clamp conditions, and determined whether an LTS or a TOR was observed in current-clamp mode (Methods). We observed that in all cases where $I_T$ was evident at a command potential more hyperpolarized than $I_A$, an LTS would be observed (n= 40/49). Conversely, in the few cases where $I_A$ was evident at a command potential more hyperpolarized than $I_T$, a TOR was observed (P< 0.0001, Fisher’s exact test) (Fig. 23A).

We then investigated whether pharmacologically altering the $I_T/I_A$ balance towards an inward current predominance affected the magnitude of the LTS. LTS properties were analyzed before and after application of 5 mM 4-AP (n= 22). While the LTS threshold was not significantly affected by $I_A$ blockade ($\Delta = 0.62 \pm 0.65$ mV, $P= 0.9$), both the LTS peak amplitude and area were significantly enhanced during $I_A$ blockade ($100.1 \pm 18.1\%$, $P< 0.0005$ and $44.9 \pm 16.2\%$, $P= 0.01$, respectively) (Fig. 23B).
Figure 22. The relative expression of mRNA for A-type and T-type channel subunits correlates with the balance of these two subthreshold conductances. A- Representative example of a single-cell real-time RT-PCR amplification plot obtained from an identified PVN-RVLM neuron, in which the A-type channel subunit Kv4.3 (dashed line) and the T-type channel subunit Cav3.1 (dotted line) was tested against the reference gene, GAPDH (whole line). The cycle numbers that the respective lines crossed the x-axis are their threshold values (Ct). Note Cav3.1 mRNA crossed the x-axis at a later cycle number than did Kv4.3, indicating less Cav3.1 mRNA expression compared to Kv4.3 within the sampled neuron. B- Plot of the $I_T/I_A$ amplitude ratio measured at a command potential of -20 mV vs. the relative expression of Cav3.1/Kv4.3 mRNA ($r^2 = 0.61$, $P<0.001$, n=15). Note that the lower the $\Delta Ct$ ratio value is, the larger the expression of Cav3.1 relative to that of Kv4.3 is, and the larger the $I_T$ amplitude is relative to $I_A$. 
Figure 23. The balance of $I_A$ and $I_T$ influences the expression and magnitude of LTS. **A**- PVN-RVLM neurons were categorized in subgroups, depending on whether $I_T$ or $I_A$ activated at a more hyperpolarized membrane potential, and whether an LTS or TOR was observed upon membrane depolarization ($n=49$). Note that when $I_T$ activated first, an LTS was observed. Conversely, when $I_A$ activated first, a TOR was observed. **Right**- Representative traces in which $I_A$ or $I_T$ activated at a more hyperpolarized potential and the resultant TOR or LTS, respectively. **B**- Representative example of an LTS before (left, black) and after (center, gray) application of the $I_A$ inhibitor, 4-AP (5 mM). Note the increased peak and area of the LTS in 4-AP, when the two traces are overlapped (right). **C**- Summary data of LTS area in sham and hypertensive PVN-RVLM neurons, before (white) and after application of 4-AP (black). Note the LTS area was significantly increased in the sham group upon 4-AP application. Also, note the more robust basal LTS area in the hypertensive group ($n=24$) compared to sham ($n=22$), and the lack of a further effect of 4-AP in this group of PVN-RVLM neurons. *$P<0.05$*
3.3.4. The balance of $I_A/I_T$ is neurochemical-dependent

We wanted to investigate more on potential factors influencing the expression of $I_T$-LTS vs $I_A$-TOR in PVN-RVLM neurons. Long descending PVN neurons, including PVN-RVLM, are neurochemically heterogeneous, a proportion of which express the neurohormones vasopressin (VP) and/or oxytocin (OT) [134,194,204]. Since magnocellular OT and VP neuroendocrine neurons of the PVN and SON, express large $I_A$ and TOR [65,133], we hypothesized that PVN-RVLM neurons in which $I_A$ overcomes $I_T$ at more hyperpolarized Vms and expresses TOR, may also express VP and/or OT. Therefore, in a subset of recordings (n= 12), we tested for the presence of OT/VP immunoreactivity in recorded PVN-RVLM neurons (Methods). As shown in Fig. 24, the majority of neurons (n= 6/7) in which $I_T$ was evident at a more hyperpolarized potential than $I_A$ were VP/OT immunonegative, whereas all of those in which $I_A$ activated at a more hyperpolarized potential than $I_T$ were VP/OT immunoreactive (n= 5/5) (P= 0.01, Fisher’s exact test).

3.3.5. LTS-dependent increase in intracellular calcium in PVN-RVLM neurons

In addition to an increase in membrane conductance and induction of LTS, activation of T-type Ca$^{2+}$ channels could also lead to changes in intracellular Ca$^{2+}$ levels ([Ca$^{2+}$]$_{ic}$), and activation of downstream Ca$^{2+}$-dependent signaling, as previously shown [58,74,156]. Thus, in order to determine whether LTS in PVN-RVLM neurons evoked an increase in [Ca$^{2+}$]$_{ic}$ and whether this was dependent on the $I_T/I_A$ balance, we performed simultaneous patch clamp recordings and confocal Ca$^{2+}$ imaging. Our results indicate that LTS in PVN-RVLM neurons consistently evoked transient increases in somatic [Ca$^{2+}$]$_{ic}$ (n= 13, Fig. 25). The peak of the Ca$^{2+}$ transient was delayed with respect to the LTS peak.
Figure 24. The neurochemical identity of PVN-RVLM neurons influences the balance of $I_A$ and $I_T$. A- Representative low magnification image (20x) of a PVN-RVLM neuron displaying vasopressin and/or oxytocin (VP/OT) immunoreactivity. Right- Summary data of numbers of PVN-RVLM neurons expressing VP/OT, segregated into those in which either $I_A$ or $I_T$ activated at a more hyperpolarized command potential ($P=0.01$, Fisher’s exact test, $n=12$). These cells also expressed LTS or TOR, based upon which current activated at a more hyperpolarized $V_m$ (as explained in Figure 23A). Note the larger proportion of neurons expressing VP/OT were those in which $I_A$ activated at a more hyperpolarized potential than $I_T$. 
Figure 25. LTS-evoked changes in intracellular calcium levels in PVN-RVLM neurons. A- Representative confocal images of LTS-evoked changes in intracellular calcium levels (pseudocolor) in a PVN-RVLM neuron, before and after 4-AP application. Images of the basal intracellular calcium levels (black/white and #1), maximum LTS-evoked peaks of intracellular calcium (#2), and subsequent return to basal levels (#3), are displayed (scale units are F/F₀). B- Traces depicting the time course of the LTS-evoked relative changes in intracellular calcium levels obtained from the pseudocolor images in A. Note the increase in the amplitude of intracellular calcium levels upon 4-AP addition (gray) compared to control (black). C- Traces of the evoked LTS (grey) and resultant change in intracellular calcium levels (black) are overlapped, to better depict the time-course relationship between them. The upper and lower traces correspond to before and after 4-AP application, respectively. Right- Expanded view of the LTS and peak of the calcium transient. Note that the peak of the LTS occurs before the peak of the calcium transient. D- Summary data of the peak of the LTS-evoked calcium transient in sham (n= 13) and hypertensive (n= 11) PVN-RVLM neurons, before and after 4-AP application. Note the LTS-evoked calcium transient was significantly increased in the sham group upon 4-AP application. Also, note the more robust LTS-evoked calcium response in the hypertensive group compared to sham, and the lack of a further effect of 4-AP in this group of PVN-RVLM neurons. E- Plot of the LTS-evoked calcium transient as a function of the area of the LTS, depicting a positive correlation between the two parameters (r² = 0.66, P< 0.005). Sham and hypertensive control groups were plotted together. *P< 0.05, **P< 0.01
(time to peak LTS: 184.3 ± 24.3 ms; time to peak [Ca\(^{2+}\)]\(_{ic}\): 305.0 ± 49.4 ms, P= 0.01), P< 0.0001), and in all cases, the evoked change in [Ca\(^{2+}\)]\(_{ic}\) persisted beyond the duration of the LTS. The LTS-evoked change in [Ca\(^{2+}\)]\(_{ic}\) was blocked in nominal 0 Ca\(^{2+}\) ACSF, and subthreshold depolarizations failed to evoke a change in [Ca\(^{2+}\)]\(_{ic}\) (not shown).

When the I\(_T\)/I\(_A\) balance was shifted in the presence of 4-AP, the LTS-evoked change in [Ca\(^{2+}\)]\(_{ic}\) was significantly enhanced (P<0.01, Fig. 25D). In general, a strong correlation between the LTS area and the peak of the Ca\(^{2+}\) transient was observed (r= 0.66, P< 0.005, Pearson’s correlation test, Fig. 25E). On the other hand, the kinetics of the changes in [Ca\(^{2+}\)]\(_{ic}\) (i.e., rise and decay time courses) were not affected by 4-AP (not shown).

In a few instances, relatively long dendritic processes contained within the same focal plane were efficiently loaded with Fluo-5 (n= 3). In those cases, we found the somatic-induced LTS to efficiently increase [Ca\(^{2+}\)]\(_{ic}\) along the dendritic process (Fig. 26). As shown in the representative example in Fig. 26, the magnitude (i.e. area) of the Ca\(^{2+}\) transient increased as a function the distance from the soma (P< 0.0001, 2-way ANOVA), and was significantly enhanced in the presence of 4-AP (P< 0.001, 2-way ANOVA) (Fig. 26C).

3.3.6. Changes during hypertension

Our studies indicate that the subthreshold balance between I\(_T\)/I\(_A\) influences both membrane properties and activity-dependent Ca\(^{2+}\) dynamics in PVN-RVLM neurons. Moreover, a pharmacological shift in this balance towards a predominance of I\(_T\) resulted in enhanced LTS and somatodendritic evoked Ca\(^{2+}\) transients. Finally, we wanted to determine whether similar changes were observed during a pathophysiological condition
Figure 26. LTS-evoked changes in intracellular calcium in PVN-RVLM dendrites.

A- Representative confocal images (pseudocolor) of LTS-evoked changes in intracellular calcium, in a PVN-RVLM neuron in which the dendrites were efficiently filled with Fluo-5. Images represent periods corresponding to basal levels (left), and the peak of the LTS-evoked calcium transient before (middle) and after 4-AP application. Note that the evoked changes in dendritic intracellular calcium levels were enhanced upon 4-AP addition (scale is a colorimetric range of the relative levels of intracellular calcium compared to background; dark- no change from background, red- maximal change from background).  

B- Representative traces of calcium transients from the cell in A at various dendritic distances from the soma, under control conditions. Note that the farther dendritic distance from the soma, the larger the relative change in intracellular calcium was (dark blue- smallest change, light blue- largest change).  

C- Summary plot of the mean area of the calcium transient along the dendritic process, before and after addition of 4-AP, obtained from 3 neurons. Note the overall increase in the area of the intracellular calcium levels after 4-AP application, and how the evoked calcium transient linearly increased as a function of the dendritic distance (control $r^2=0.67$, 4-AP $r^2=0.66$, binning= 10 µm).
in which the $I_T/I_A$ balance is intrinsically changed. We have recently reported a diminished $I_A$ availability in PVN-RVLM neurons during chronic hypertension (see 3.2). Further supporting a change in the $I_T/I_A$ balance during hypertension, we found, in this study, an enhanced $I_T$ current amplitude and density in PVN-RVLM neurons during hypertension ($P<0.0005$ in both cases, 2-way ANOVA, Fig. 27A). No differences in activation threshold and kinetic properties (rise time and $\tau_{\text{inactivation}}$) were observed between sham and hypertensive rats (not shown).

In agreement with a diminished $I_A$ and enhanced $I_T$, results from single-cell real-time PCR studies in identified PVN-RVLM neurons from control and hypertensive rats ($n=20$ and $31$ neurons, respectively) indicated a 57% reduction and a 294% increase in the relative expression levels of Kv4.3 mRNA Cav3.1 mRNA, respectively, during hypertension ($P<0.05$ in both cases) (Fig. 27C). In fact, a nearly 10 fold decrease in the relative expression of Kv4.3 to Cav3.1 mRNA within individual PVN-RVLM neurons was observed during hypertension ($P<0.005$).

The magnitude of the evoked LTS was significantly larger in PVN-RVLM neurons from hypertensive ($n=24$) when compared to control rats ($P<0.05$, Fig. 23C). Moreover, blockade of $I_A$ in hypertensive rats, unlike what we observed in controls, failed to significantly increase LTS magnitude ($3.3 \pm 8.1\%$, $P=0.4$) (Fig. 23C). Likewise, the magnitude of the LTS-evoked change in $[\text{Ca}^{2+}]_{\text{ic}}$ was significantly larger in PVN-RVLM neurons from hypertensive ($n=11$) when compared to control rats ($P<0.05$, Fig. 25D), and blockade of $I_A$ in hypertensive rats failed to significantly increase the magnitude of the evoked $\text{Ca}^{2+}$ transient ($P>0.1$, not shown).
Figure 27. Shift in the balance of $I_A$ and $I_T$ during hypertension. **A**- Mean plots of $I_T$ current densities obtained in PVN-RVLM neurons from sham (*squares*, $n=9$) and hypertensive (*triangles*, $n=7$) rats. Note the significantly larger $I_T$ current density in the hypertensive group. **B**- Representative examples of single-cell RT-PCR amplification plots for Kv4.3 (dashed line), Cav3.1 (dotted line) and GAPDH (whole line) obtained from PVN-RVLM neurons in a sham (black) and a hypertensive (gray) rat. **C**- Summary data of the mean normalized expression ± range of Kv4.3 mRNA (left), Cav3.1 mRNA (middle), and the Kv4.3 mRNA/Cav3.1 mRNA ratio (right) ($n=20$ sham and 31 hypertensive neurons). Note the expression of Kv4.3 mRNA was reduced during hypertension, while Cav3.1 mRNA expression was enhanced. Also, note the significant reduction in the relative expression of Kv4.3 to Cav3.1, during hypertension. *$P<0.05$, **$P<0.01$.**
Altogether, these results support an imbalanced $I_T/I_A$ relationship during hypertension, resulting in an enhanced LTS and $Ca^{2+}$ entry. Since $I_T$ has been shown to regulate spontaneous activity in a variety of CNS neurons [94,159], we addressed whether the subthreshold $I_T/I_A$ imbalance resulted and/or contributed to increased ongoing firing activity in PVN-RVLM neurons during hypertension. PVN-RVLM basal spontaneous firing frequency was significantly higher in hypertensive when compared to control rats (n= 12 and 8, respectively, $P< 0.05$). Bath application of NiCl$_2$ (100 μM), which at this concentration more efficiently blocks $I_T$ over HVAs [68,77] failed to affect firing rate in control rats ($P= 0.4$). On the other hand, the firing rate in hypertensive neurons was significantly reduced in the presence of NiCl$_2$ ($P< 0.005$) (Fig. 28), supporting an enhanced $I_T$ contribution to spontaneous firing activity during hypertension.
Figure 28. $I_T$ contributes to enhanced spontaneous firing activity in PVN-RVLM neurons during hypertension. A- Representative traces of spontaneous firing activity in a hypertensive PVN-RVLM neuron, before and after addition of 100 μM NiCl$_2$. B- Summary data of the mean spontaneous firing activity in sham (n= 8) and hypertensive (n= 12) neurons before and after NiCl$_2$ application. Note the significantly elevated basal firing rate in the hypertensive group compared to sham. Also note that while NiCl$_2$ significantly reduced the firing activity in the hypertensive group, it failed to do so in the sham group. *P< 0.05.
IV. DISCUSSION

The PVN is a highly heterogeneous region containing both magnocellular neurosecretory (type I) and parvocellular (types II-III) neurons, the latter including both neurosecretory and preautonomic neurons [193,194]. Previous studies indicate that these two major PVN populations can be distinguished based on their electrophysiological properties. Thus, while magnocellular neurosecretory neurons are characterized by the expression of a robust transient outward rectification (TOR) [21,133,186], parvocellular neurons, including preautonomic ones, are characterized by their ability to generate a Ca\(^{2+}\)-dependent low-threshold spike (LTS) [133,185]. Elegant work by Tasker and colleagues showed that while both I\(_A\) and I\(_T\) are in fact present in both types of PVN neurons, differences in their relative expression and voltage-dependent properties determine their relative contribution to the general membrane properties characteristic of these two neuronal populations (i.e., TOR and LTS, respectively) [133]. However, whether and how these two opposing conductances compete with each other, and what the impact of such competition is on the membrane excitability in PVN neurons, has not been established. In this study, we focused on a specific group of parvocellular preautonomic neurons, those that innervate the RVLM and are known to play an important role in sympathetic control [4,42,110,195,205]. While most previous electrophysiological studies on parvocellular neurons have focused on I\(_T\) and the role of
the LTS \([133,185]\), little is known about the characteristics and functional role of \(I_A\) in this neuronal population. Thus, **major goals** of this study included: a) to characterize the basic biophysical and functional properties of \(I_A\) in PVN-RVLM neurons, b) to determine whether interactions between \(I_A\) and \(I_T\) influence membrane excitability in these neurons, and c) to evaluate whether changes in the properties of \(I_A\) and its interaction with \(I_T\) contribute to enhanced excitability of PVN-RVLM neurons during hypertension. Our **major hypothesis** is that a balanced interaction between the subthreshold currents, \(I_A\) and \(I_T\), plays an important role in maintaining physiological neuronal excitability/activity. Moreover, we hypothesize that during hypertension, a disbalanced interaction favoring \(I_T\) results in enhanced neuronal excitability, constituting thus an important underlying mechanism contributing to the characteristic sympathoexcitation observed in this disease. This hypothesis is supported by results from our work. Thus, our data indicate that in addition to \(I_T\) \([185]\), preautonomic PVN neurons also express a functionally relevant \(I_A\), which shapes the \(Na^+\) action potential waveform and modulates their firing activity. In addition, our results support an important balance between \(I_A\) and \(I_T\), which regulates action potential-evoked changes in intracellular calcium levels, the magnitude of LTS and overall firing activity. Finally, our results support a disbalance between \(I_A\) and \(I_T\) during hypertension, favoring \(I_T\), resulting in more robust LTS, enhanced intracellular calcium entry during LTS and single action potentials, and increased neuronal excitability.

**4.1. A-type Potassium Currents in PVN-RVLM Neurons**

4.1.1. Biophysical, pharmacological and molecular properties of \(I_A\) in PVN-RVLM neurons
While the biophysical and pharmacological properties of $I_A$ in PVN-RVLM neurons reported herein are generally consistent with those previously described in other CNS neuronal types [22,201], some interesting differences were observed. For example, the activation threshold, half-activation and half-inactivation $V_m$ of $I_A$ in PVN-RVLM neurons were all found to be more hyperpolarized than those previously reported in non-identified parvocellular PVN neurons, and very similar in fact to those reported in Type 1 PVN magnocellular neurons [125,133].

In agreement with previous reports on other CNS regions, including the hypothalamus [64,87,125,133], we found $I_A$ in PVN-RVLM neurons to consistently show high sensitivity to the traditional A-type blocker 4-AP, and low sensitivity to the delayed-rectifier $K^+$ channel blocker TEA (Fig. 9A) [166]. The sensitivity to 5 mM 4-AP block in PVN-RVLM neurons was relatively consistent across neurons (32-54% inhibition). This differs from the previously reported large variability in type II parvocellular PVN neurons (10-69% inhibition) [133], likely due to the diverse neuronal types (e.g, neurosecretory and/or alternative preautonomic ones) included in the type II category. Thus, while $I_A$ seems to be ubiquitously present in the hypothalamus, some of their major properties appear to be regulated in a cell type-dependent manner. In this sense, both the biophysical and pharmacological properties of $I_A$ have been shown to be dependent upon specific A-type $K^+$ channels subunit composition, as well as the auxiliary subunits and Kv channel-interacting proteins (KChIPs) associated with them [6,11,37]. Thus, differences in A-type properties between PVN-RVLM and other PVN neuronal populations could rely on the differential expression of molecularly diverse channels. A-type $K^+$ channels have been shown to consist of Kv1.4 or Kv4.1-3 subunits,
either homomerically, or heteromerically assembled with other subunits from the same subfamily [189]. Despite the fact that the presence and functional relevance of A-type K$^+$ currents in various hypothalamic neuronal types has been long recognized, the subunit composition of the underlying channels remains at present unknown. Using tract-tracing techniques in combination with immunohistochemistry, we found here that PVN-RVLM neurons consistently expressed high immunoreactive levels for the Kv1.4 and 4.3 subunits, with almost no immunoreactivity for the Kv4.2 subunit. These results suggest that A-type K$^+$ channels in these neurons likely comprise one or both of the former two subunits. As a caveat, the lack of commercially available Kv4.1 antibody prevented us from assessing its expression in PVN-RVLM neurons. Moreover, the presence of immunoreactivity for these subunits does not necessarily imply their incorporation into functional channels. In this sense, previous studies indicate that Kv1- and Kv4-containing channels recover from inactivation with time constants of several seconds or milliseconds, respectively [37,173]. Thus, our findings, showing a recovery from inactivation time constant of ~70 ms, suggest that functional channels in PVN-RVLM neurons, at least those more likely activated during our recordings (i.e., those located in somatic and proximal dendritic compartments), are probably comprised of Kv4.3 subunits. Supporting this, our single-cell RT-PCR studies in identified PVN-RVLM neurons confirmed the presence of Kv4.3 mRNA. Further studies using subunit-selective pharmacological tools for these Kv subunits would be needed to further clarify this issue.

In summary, an A-type K$^+$ current is present within PVN-RVLM preautonomic neurons; it is likely mediated by channels containing the Kv4.3 subunit, and has biophysical
properties similar to those previously reported centrally, yet unique to those reported in
the PVN.

4.1.2. $I_A$ influences excitability and repetitive firing properties of PVN-RVLM neurons

In agreement with previous reports in other neuronal types [107,201], our data support the presence of a tonically active $I_A$ (i.e., “window current) in PVN-RVLM neurons. This “window” current was found to be available between a narrow, though apparently physiologically relevant range of membrane potentials. However, it is important to take into account that due to differences in our recording conditions between voltage- and current-clamp studies, and our limitation to examine the divalent cation sensitivity of $I_A$ in these neurons (see Appendix B), the voltage-dependent availability of the “window” current may not represent the physiological condition. Nonetheless, our results showing membrane depolarization and increased input resistance following pharmacological blockade of $I_A$, suggest that $I_A$ may play a role in setting resting membrane potential and/or in the regulation of subthreshold variations of membrane potential in PVN-RVLM neurons.

By regulating action potential waveform and interspike intervals [135,164,166], $I_A$ has been shown to play important roles in shaping temporal firing patterns of various neuronal types. Our present studies indicate this to be the case in PVN presympathetic neurons as well: pharmacological blockade of $I_A$, and/or its voltage-dependent inactivation, prolonged the duration of the action potential waveform. Importantly, the firing activity of these neurons was differentially affected by $I_A$ blockade: while the majority of the neurons (60%) responded to 4-AP with an increased firing rate, opposite effects were observed in the remainder, suggesting a differential role of $I_A$ within PVN-
RVLM neurons. The precise mechanisms underlying such disparate roles are at present unknown. However, some insights could be drawn from the present results. Worth noting is the fact that some basic intrinsic properties differed between the two subpopulations of 4-AP sensitive PVN-RVLM neurons (e.g., resting Vm, degree of spontaneous activity, action potential duration), suggesting the presence of distinct subsets of PVN-RVLM neurons. This is in agreement with previous observations indicating a high degree of functional and neurochemical heterogeneity among preautonomic PVN neurons, even within those innervating a common target [185,192,194]. Alternatively, differences in 4-AP sensitivity could represent variability within a single population of PVN-RVLM neurons. Future studies comparing for instance the neurochemical identity of these subsets of PVN-RVLM neurons will provide more insights into this important phenomenon. Nonetheless, the overall lack of significant correlations between 4-AP induced changes in firing activity and any of the parameters that differed between the two subpopulations of PVN-RVLM neurons, indicates that the opposing effects of 4-AP on firing discharge were not due to differences in these parameters per se. Similarly, both 4-AP sensitive subgroups of PVN-RVLM neurons displayed either continuous or bursting firing patterns, indicating that initial firing pattern was not a key factor influencing the type of response induced by I_A blockade. Moreover, the general properties of individual action potential waveforms in the two subsets of PVN-RVLM neurons were similarly affected by 4-AP (i.e., spikes were increased in amplitude and prolonged in duration) suggesting that changes in these action potential parameters per se also did not contribute to the differential effect of 4-AP on the firing discharge of these neurons.
Conversely, other neuronal properties were differentially affected by 4-AP in these two subsets of PVN-RVLM neurons. As previously shown in SON and PVN magnocellular neurosecretory neurons [7,20], the repolarizing phase of the action potential in PVN-RVLM neurons was followed by a prominent HAP. In those neurons in which an increase in firing rate was observed, the HAP peak reached a more hyperpolarized membrane potential in 4-AP, an effect expected to result in a more efficient removal of Na$^+$ channel inactivation following each action potential. This in turn may contribute to the more hyperpolarized Na$^+$ spike threshold observed in this subset of PVN-RVLM neurons. In fact, previous studies have shown that modulation of the degree of Na$^+$ channel inactivation is an effective mechanism by which I_A modulates firing properties [38,89]. Moreover, a steeper HAP decay slope, with concomitant shorter interspike intervals, was also observed in this group in the presence of 4-AP. Thus, these combined actions of 4-AP (i.e., hyperpolarized HAP peak, faster HAP decay time course, shorter interspike intervals, and hyperpolarized Na$^+$ spike threshold) likely contribute to the enhanced firing discharge found in this subset of PVN-RVLM neurons.

On the other hand, in those neurons in which 4-AP decreased their firing discharge, these parameters were either unchanged, or affected in opposite ways by 4-AP. For example, a slower HAP decay time course, leading in turn to an overall enhanced HAP area, was induced by 4-AP, effects that likely contributed to the decreased interspike interval found in this subgroup of PVN-RVLM neurons.

In summary, our results indicate that I_A differentially modulates firing discharge in PVN-RVLM neurons, actions that seem to be in part mediated by differential and/or
opposing modulatory effects on HAP parameters. These results raise the intriguing question of how such disparate actions can be mediated by $I_A$.

4.1.3. The effects of $I_A$ on PVN-RVLM firing activity are $Ca^{2+}$-dependent

An important finding of the present work is that 4-AP effects on the firing activity of PVN-RVLM neurons were blocked by the broad spectrum $Ca^{2+}$ channel blocker $Cd^{2+}$. Several mechanisms could be proposed to underlie this $Ca^{2+}$-dependent effect. For example, it could be argued that these effects are due to $Ca^{2+}$-dependent inhibition of $I_A$, as previously shown in magnocellular supraoptic neurons [19]. However, the fact that a large $I_A$ component was always present in a $0 Ca^{2+}$/EGTA/Cd$^{2+}$ containing ACSF (see Figs 2-5), along with lack of evidence for a diminished $I_A$ amplitude before and after addition of $Cd^{2+}$ to control ACSF (data not shown), argue against this possibility. Alternatively, and due to the mostly overlapping voltage-dependent properties between $I_A$ and $I_T$, it is possible that blockade of $I_A$ results in amplification and/or prolongation of the $Ca^{2+}$-dependent LTS typically found in preautonomic PVN neurons (Fig. 1, [185]), and as previously shown in other neuronal populations [27,156]. Thus, we hypothesized that $I_A$ and $I_T$ act in a concerted manner to regulate subthreshold membrane excitability in PVN-RVLM neurons, and that a diminished availability of $I_A$ (i.e., pharmacological blockade or during a pathological condition, see below) could lead to increased influence of $I_T$ and a $Ca^{2+}$-dependent effect on neuronal firing discharge.

4.2. Balance between $I_A$ and $I_T$ in PVN-RVLM Neurons

Based on the results described above, we aimed in our study to characterize the opposing competition between subthreshold ion channels, $I_A$ and $I_T$, and to determine
whether a balance between these two conductances influenced neuronal excitability in PVN-RVLM neurons.

4.2.1. Interplay between the subthreshold currents, $I_A$ and $I_T$, in PVN-RVLM neurons

An opposing competition between the subthreshold ion currents, $I_A$ and $I_T$, has been well documented centrally \[27,142,146,155\], and our data, for the first time, supports that this competition also plays an important role in modulating neuronal excitability in PVN-RVLM neurons. Previously, it was widely believed that $I_A$ and $I_T$ were functionally segregated among PVN neurons, with $I_A$ more abundantly expressed and predominantly influencing type 1 magnocellular neurons, whereas $I_T$ was more abundantly expressed and predominantly influencing type 2 parvocellular neurons \[133\]. However, our data supports the presence of overlapping $I_A$ and $I_T$ conductances within individual PVN-RVLM neurons, at similar membrane potentials.

As mentioned above, differences in the relative expression and voltage-dependent properties of $I_A$ and $I_T$ have been shown to determine their relative contribution to general membrane properties (i.e., TOR and LTS, respectively) \[133\]. While the TOR is a membrane property that delays excitation in response to membrane depolarization, decreasing overall membrane excitability, the LTS facilitates and boosts membrane depolarization, increasing overall membrane excitability. In the majority of PVN-RVLM neurons, $I_T$ activated at a more hyperpolarized membrane potential than $I_A$, corresponding with the expression of an LTS in this group of neurons. Conversely, in a subpopulation of PVN-RVLM neurons, $I_A$ activated at a more hyperpolarized membrane potential than $I_T$, corresponding with the expression of a TOR in this group of neurons.
Thus, the relative predominance of $I_T$ over $I_A$ seems to determine whether an LTS or TOR will be expressed in PVN-RVLM neurons. Numerous factors could influence the balance and predominance of one conductance over the other one. For example, it has been suggested that differences in activation threshold can shift the balance between $I_A$ and $I_T$ [155]. In this sense, our data shows that in the majority of cells, $I_T$ activates at a more hyperpolarized potential than $I_A$. This may account for the larger proportion of cells expressing LTS that we observed, and has previously been shown in preautonomic PVN neurons [185]. In addition, our data also demonstrated a positive correlation between the relative expression of mRNA for Cav3.1/Kv4.3 subunits and $I_T/I_A$ amplitude within single cells. Thus, it is also possible that differences in the relative expression of $I_T$ and $I_A$ channels may influence the ability of PVN-RVLM neurons to express an excitatory (LTS) or inhibitory (TOR) membrane behavior. This will need to be determined in future studies.

Interestingly, the balance between $I_A$ and $I_T$, and the membrane properties observed in PVN-RVLM neurones, seemed to be in part dependent on their neurochemical phenotype. As has previously been demonstrated, OT and VP neuroendocrine neurons of the PVN and SON express large $I_A$ and TOR [65,133]. However, a proportion of preautonomic PVN-RVLM neurons also express VP [204]. Surprisingly, our present studies indicate that the majority of PVN-RVLM neurons in which $I_A$ activated at a more hyperpolarized $V_{\text{m}}$ than $I_T$, and expressed TOR, were immunoreactive for VP/OT, while the majority of neurons in which $I_T$ activated at a more hyperpolarized $V_{\text{m}}$ were not.
In summary, I_A and I_T are both expressed within individual preautonomic PVN-RVLM neurons, and opposingly interact at similar membrane potentials. Their relative expression, will dictate the predominance of excitatory (LTS) or inhibitory (TOR) membrane behavior, a phenomenon that seems to be in part dependent on the neurochemical phenotype of PVN-RVLM neurons (I_A= VP/OT+, I_T= VP/OT-),

4.2.2. I_A/I_T balance influences neuronal activity

As we have mentioned above, the balance of I_A and I_T dictates whether an LTS or TOR is expressed. However, it has also been demonstrated that subtle changes in this balance may affect the magnitude of these membrane properties. For example, pharmacological blockade of I_A has been shown to shift the balance towards a predominance of I_T, resulting in a more robust LTS [155]. Our data support this phenomenon in PVN-RVLM neurons as well, as inhibition of I_A with 4-AP resulted in a significant increase in the peak and area of the LTS. Concomitantly, as the LTS magnitude was enhanced, so too was the LTS-mediated change in intracellular calcium levels, in both somatic and dendritic compartments. Thus, a decrease in I_A magnitude can lead to secondary increases in intracellular calcium levels. Altogether, this data indicate that the Ca^{2+}-dependent effect of I_A on firing activity (see above) could be due to an enhanced LTS-dependent Ca^{2+} conductance. As calcium is increased intracellularly, a cascade of downstream Ca^{2+}-dependent mechanisms could be activated, resulting in a differential influence on firing activity, depending on the complement of Ca^{2+}-dependent mechanisms present in specific sets of neurons. It is currently unknown what calcium dependent mechanisms may be activated within subpopulations of PVN-RVLM neurons, and whether these are differentially distributed within subpopulations of PVN-RVLM
neurons. However, it is likely that a variety of voltage-gated Ca\(^{2+}\) channels, Ca\(^{2+}\)-dependent conductances, and Ca\(^{2+}\)-dependent second messenger systems are present in PVN-RVLM neurons. One possible mechanism that has been suggested to differentially regulate neuronal excitability is the presence of two second-messenger systems within a given cell, calcium/calmodulin-dependent protein kinase II (CamKII) and protein kinase A (PKA) [206,213]. Phosphorylation is well known to regulate ion channel activity [116], and CAMKII and PKA have been shown to increase and decrease I\(_A\), [199,206], resulting in decreased and increased firing activity, respectively [206]. PKA has also recently been shown to increase I\(_T\) [30], the opposite effect as that of I\(_A\). Thus, the relative presence and activity of these second messenger systems may contribute to the differential Ca\(^{2+}\)-dependent effect of I\(_A\) blockade in subsets of PVN-RVLM neurons. In summary, the balance of I\(_A\) and I\(_T\) influence PVN-RVLM neuronal activity, by modulating the magnitude of LTS, intracellular calcium levels and likely activation of downstream Ca\(^{2+}\)-dependent mechanisms.

4.3. Altered Contribution of Subthreshold Ion Channels to Increased PVN-RVLM Neuronal Excitability During Hypertension

Sympathoexcitation of a central origin is characteristic of hypertension [81,95], and mounting evidence supports an important role for the PVN in the pathophysiology of this disease state [4,35,76,85,149]. Increased neuronal excitability, associated with elevated blood pressure and sympathetic activity, has recently been demonstrated in PVN-RVLM neurons [4,120]. However, while alterations in various PVN neurotransmitter systems have been reported during hypertension [46,54,80,96,120,121], the contribution of altered intrinsic membrane properties to PVN neuronal hyperactivity
during hypertension remains unclear. As summarized above, results from our work demonstrate that the $I_A/I_T$ balance in PVN-RVLM neurons is a key factor influencing their overall activity. Thus, another major goal of this work was to determine whether a disbalance in the interplay between these opposing subthreshold conductances during hypertension may contribute to increased neuronal excitability during this condition. To this end, we used a renovascular hypertensive rat model, 2-kidney 1-clip, at a chronic stage of hypertension, characterized by enhanced sympathetic nerve activity [136].

4.3.1. Altered balance between $I_A$ and $I_T$ in PVN-RVLM neurons, during hypertension

Our results indicate an overall reduction in the magnitude of $I_A$ through a wide range of membrane potentials in PVN-RVLM neurons during hypertension. In addition to changes in current amplitude, some critical voltage-dependent properties of $I_A$ were also altered during hypertension. For example, the degree of $I_A$ steady-state inactivation was enhanced in PVN-RVLM neurons during hypertension, and a hyperpolarizing shift in the $V_{1/2}$ inactivation was also observed in this condition. An increased degree of steady-state inactivation during hypertension indicates that at a particular membrane potential, fewer channels will be available for activation. Thus, the larger degree of voltage-dependent inactivation of $I_A$ may be a contributing factor to the reduced magnitude of the evoked $I_A$ during hypertension.

Another potential mechanism contributing to diminished $I_A$ availability during hypertension is a reduction in single channel conductance. Our results using non-stationary noise analysis of whole cell currents, a well-established approach [5,163,176,177,197], do in fact support a diminished single channel conductance of A-type $K^+$ channels during hypertension. The values for A-type $K^+$ single channel
conductance obtained in our studies are in general agreement with previous reports based on single-channel recordings [40,97,100,202], further supporting the validity of this approach.

Concomitant with a decreased $I_A$ magnitude during hypertension, both the current amplitude and density of $I_T$ were found to be enhanced during hypertension. Thus, a diminished $I_A$ along with an increased $I_T$ magnitude strongly supports a shift in the $I_A/I_T$ balance towards an inward current predominance during hypertension. Several lines of evidence support such an imbalance during hypertension. Firstly, while 4-AP increased the $I_T$ current amplitude in sham cells, 4-AP failed to do so in hypertensive cells. Single-cell RT-PCR data also lends credence to this, as the mRNA levels of Kv4.3 and Cav3.1 were significantly decreased and increased, respectively, during hypertension. As well, the relative expression of Kv4.3 to Cav3.1 mRNA in a given cell was significantly reduced, during hypertension, indicating that the relative level of Cav3.1 mRNA expression was nearly identical to that of Kv4.3. *In summary*, a shift in the balance between $I_A$ and $I_T$, favoring $I_T$, as supported by current density and mRNA data, occurs in PVN-RVLM neurons during hypertension.

4.3.2. Altered $I_A/I_T$ balance during hypertension influences PVN-RVLM neuronal activity

Consistent with a diminished $I_A$ availability during hypertension, our data also demonstrated wider and slower-decaying action potentials in PVN-RVLM neurons during hypertension. This is also supported by the fact that during hypertension, the effects on action potential waveform of two procedures that diminish $I_A$ availability (i.e., membrane depolarization and the A-type $K^+$ channel blocker 4-AP [180]) were blunted.
Prolongation of action potential duration due to diminished \( I_A \) availability during hypertension resulted in enhanced action potential-dependent \( \text{Ca}^{2+} \) entry. As previously reported in other neuronal types [75], 4-AP enhanced the action-potential evoked \( \text{Ca}^{2+} \) transient, an effect that was diminished in hypertensive rats. It is unclear whether this change in calcium entry is mediated in part by \( I_T \). However, since we demonstrated that \( I_T \) amplitude is enhanced by 4-AP application, at similar potentials that \( I_A \) is active, it is likely that \( I_T \) contributed in part to the elevated action potential-dependent \( \text{Ca}^{2+} \) transient observed during hypertension.

As stated above, the diminished \( I_A \) availability, along with an increased availability of \( I_T \), resulted in a shift in the \( I_A/I_T \) balance towards an inward predominance. Our data indicates that this subthreshold disbalance had additional impacts on the excitability of PVN-RVLM neurons during hypertension. In this sense, the magnitude of the \( I_T \) – mediated LTS (peak and area), as well as the LTS-mediated increase in intracellular calcium levels, were significantly elevated during hypertension. The blunted effect of 4-AP on the LTS and LTS-mediated changes in intracellular calcium levels further supports a contribution of the diminished \( I_A \) to the \( I_T \) predominance.

Since \( I_T \) has been shown to regulate spontaneous firing activity in a variety of CNS neurons [93,159], we wanted to determine if the \( I_A/I_T \) disbalance also contributed to an enhanced ongoing firing discharge of PVN-RVLM neurons during hypertension [4,120,180]. Our studies indicated that the basal spontaneous firing activity was significantly increased in hypertensive cells compared to control, and that a low concentration of \( \text{NiCl}_2 \) (100 \( \mu \text{M} \)), significantly reduced the firing activity in the hypertensive cells, but had no effect on control cells. This data supports an enhanced \( I_T \)
contribution to the elevated spontaneous firing activity observed during hypertension in PVN-RVLM neurons.

**In summary**, the work of this thesis indicates that there is an opposing competition between $I_A$ and $I_T$ in PVN-RVLM neurons, which is shifted in favor of $I_T$ activity during hypertension, resulting in an increased magnitude of low-threshold spikes, elevated activity-dependent levels of intracellular calcium, as well as enhanced firing activity during hypertension.

### 4.4. Source of Disbalanced Interplay between $I_A$ and $I_T$ During Hypertension

Our studies indicate that the disbalanced $I_A$ and $I_T$ interplay observed in PVN-RVLM neurons, during hypertension, could be in part due to changes in the relative expression of ion channel subunits underlying $I_A$ and $I_T$ (i.e. the diminished Kv4.3 subunit mRNA and the enhanced Cav3.1 subunit mRNA, respectively). It is currently unclear what the source causing these observed changes is, although a possible contributor is the neurotransmitter angiotensin II (AngII). ANGII is a key neurotransmitter involved in the central control of cardiovascular function [184]. Within the PVN, ANGII has been shown to increase sympathetic activity [214], and abundant evidence supports enhanced PVN AngII activity during hypertension[80,96]. Numerous studies indicate that ANGII can mediate its actions by modulating the expression/activity of $I_A$ and $I_T$. For example, AngII has been shown to diminish $I_A$ current amplitude and diminish Kv4.3 mRNA in the RVLM of rats with congestive heart failure [71]. As well, there is evidence indicating that AngII inhibits $I_A$ [125,148,202] and enhances the $I_T$-dependent LTS activity [181] within the hypothalamus. Furthermore, it has been suggested that an AngII induced increase of the duration of the action potential waveform
is mediated by block of K⁺ conductances that contribute to the resting membrane potential [181]. Results from our studies indicate that I_A contributes to maintaining the resting membrane potential, as well as suppressing the action potential duration in PVN-RVLM neurons[180], and that I_A influences are blunted during hypertension. Taken together, it is reasonable to speculate that an enhanced AngII activity within the PVN during hypertension, may contribute to the subthreshold shift between I_A and I_T, unveiled by our studies in PVN-RVLM neurons during hypertension.

4.5. Future Studies

Certainly, existing evidence about AngII effects on the subthreshold ion channels, I_A and I_T, and how this interaction may contribute to hypertension, would warrant further studies on this important subject. AngII receptors have been shown to be densely distributed throughout the PVN [2,73,117], and more specifically, has been shown to acutely increase firing activity in PVN-RVLM neuron, in part through disinhibition of GABAergic inhibitory currents [118] or through activation of a mixed cationic conductance [26]. However, it is unclear whether AngII may also influence activity of PVN-RVLM neurons through interactions with intrinsic mechanisms, such as I_A and I_T, as has been reported in other hypothalamic regions [125,148,181,202]. AngII activity has been reported to be elevated in the PVN, during hypertension, and since AngII has been demonstrated to increase firing activity in PVN-RVLM neurons (see above), it would be important to test in future studies the hypothesis that enhanced AngII activity in PVN-RVLM neurons contributes to increased firing activity and sympathetic output, during hypertension, by disbalancing I_A and I_T, in favor of I_T.
If this mechanism is proven, understanding the underlying mechanism(s) by which AngII may differentially act upon I_A and I_T would be important. It is well known that AngII acts through AT1 receptors in the PVN [26,113,125,202], and that AT1 receptors are G-protein-coupled receptors [44]. In the hypothalamus, AngII elicits G-protein-mediated stimulation of phosphoinositide hydrolysis, through AT1 receptors, and subsequent activation of protein kinase C (PKC), resulting in a decrease in voltage-dependent K^+ currents and an increase in voltage-dependent Ca^{2+} currents [190]. Thus, evaluating the role of PKC in the altered I_A/I_T balance during hypertension would be important.

Finally, it would be extremely relevant to confirm at the whole animal level whether the disbalance of I_A/I_T reported in our studies contributes to enhanced blood pressure and sympathoexcitation during hypertension. This could be performed by simultaneously recording blood pressure and renal sympathetic nerve activity in anesthetized rats, while addressing the effects of local microinjections of compounds within the PVN (e.g., 4-AP, NiCl2). Alternatively, instead of transiently affecting the I_A/I_T balance pharmacologically, we could molecularly affect the balance for a prolonged period of time using short interference RNA (siRNA) technology.

Another route that would be worthwhile to continue would be study of the PVN-DVC neurons. As mentioned above, the PVN can influence cardiovascular function through the regulation of both sympathetic and parasympathetic output. A major target innervated by the PVN is the dorsal vagal complex (DVC) [55,72,98,178], an autonomic center comprising parasympathetic neurons known to regulate tonic and reflex cardiac activity [86,144,150,200]. One of the subnuclei that the DVC houses is the
NTS, a major visceroreceptive autonomic integrating center and key modulator of the baroreflex [69,111,138,154]. NTS-projecting PVN neurons (PVN-NTS), upon stimulation, have been shown to suppress the baroreflex through inhibition of the NTS [33,55,56,145], inducing an elevation in heart rate and cardiac output during the defense reaction or exercise. This phenomenon is also commonly observed during chronic hypertension [14,66,78]. Our data indicated that both $I_A$ amplitude and current density were significantly reduced in these neurons during hypertension. Thus, it would be interesting to continue this line of study, and to determine the contribution of such change to blunted baroreflex during hypertension.

4.6. General Conclusions

Hypertension is a highly deleterious disease, causing a huge health and economic burden to the public health system. Sympathoexcitation has been identified as a key pathophysiological mechanism during hypertension, contributing to morbidity and mortality in this disorder. Despite its importance, little is known about the precise underlying mechanisms contributing to hypertension-related sympathoexcitation. Results from our work identified altered interactions among subthreshold membrane conductances as a key mechanism contributing to exacerbated neuronal activity in presympathetic hypothalamic neurons known to contribute to sympathoexcitation during hypertension. It is expected that this information will help in the device of improved and/or novel therapeutic approaches for the treatment of this prevalent cardiovascular disorder.
## Appendix A
### Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>ABC</td>
<td>avidin-biotin complex</td>
</tr>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AHP</td>
<td>after-hyperpolarizing potential</td>
</tr>
<tr>
<td>AngII</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>([\text{Ca}^{2+}]_\text{i})</td>
<td>intracellular calcium levels</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CVLM</td>
<td>caudal ventrolateral medulla</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DMX</td>
<td>dorsal motor nucleus of the vagus</td>
</tr>
<tr>
<td>DVC</td>
<td>dorsal vagal complex</td>
</tr>
<tr>
<td>F/F₀</td>
<td>fractional fluorescence</td>
</tr>
<tr>
<td>HAP</td>
<td>hyperpolarizing afterpotential</td>
</tr>
<tr>
<td>HVA</td>
<td>high threshold calcium current</td>
</tr>
<tr>
<td>Iₐ</td>
<td>A-type potassium current</td>
</tr>
<tr>
<td>IᴷDR</td>
<td>delayed rectifier potassium current</td>
</tr>
<tr>
<td>I ML</td>
<td>intermediolateral column of the spinal cord</td>
</tr>
<tr>
<td>Iₜ</td>
<td>T-type calcium current</td>
</tr>
<tr>
<td>LJP</td>
<td>liquid junction potential</td>
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<tr>
<td>LTS</td>
<td>low threshold spike</td>
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<tr>
<td>NA</td>
<td>nucleus ambiguus</td>
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<tr>
<td>NTS</td>
<td>nucleus of the solitary tract</td>
</tr>
<tr>
<td>OT</td>
<td>oxytocin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>PVN-DVC</td>
<td>paraventricular nucleus neurons projecting to the dorsal vagal complex</td>
</tr>
<tr>
<td>PVN-NTS</td>
<td>paraventricular nucleus neurons projecting to the nucleus of the solitary tract</td>
</tr>
<tr>
<td>PVN-RVLM</td>
<td>paraventricular nucleus neurons projecting to the rostral ventrolateral medulla</td>
</tr>
<tr>
<td>RVLM</td>
<td>rostral ventrolateral medulla</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SFO</td>
<td>subfornical organ</td>
</tr>
<tr>
<td>SHR</td>
<td>spontaneously hypertensive rat</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethyl ammonium</td>
</tr>
<tr>
<td>TOR</td>
<td>transient outward rectification</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>VLM</td>
<td>ventrolateral medulla</td>
</tr>
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</table>
$V_m$ membrane potential
$VP$ vasopressin
Appendix B
Methodological Considerations

Retrograde injections

To identify PVN-RVLM neurons, rhodamine-labeled fluorescent latex microspheres were injected in the RVLM. This retrograde tracer results in highly restricted and well-defined injection sites [102], and is commonly used to trace CNS pathways, including PVN projections to brainstem nuclei [26,28,118,119,209]. While this tracer is not taken up by fibers in passage [102,103,109], we cannot rule out potential labeling of severed axons in the area of the injection, which could include PVN projections to the spinal cord [132]. Labeling of severed axons in our studies seems however unlikely, because injections that were misplaced, either rostrally, caudally or laterally to the RVLM, in areas containing PVN descending axons running towards the RVLM and/or the spinal cord [132], failed to retrogradely label neurons in the PVN.

Voltage-clamp recordings

Voltage- and space-clamp problems are often associated with voltage-clamp recordings in brain slices. We believe however that these potential errors were minimized in this study by the use of rigorous selection criteria for inclusion of neurons (see Methods), along with the fact that PVN neurons are known to be relatively electrotonically compact [133,185]. The good quality of our voltage-clamp experiments is supported by several lines of evidence, including: A) stable and relatively low series
resistance throughout the recordings (~10 MΩ, compared to neuronal input resistance of ~1000 MΩ), B) small voltage errors (~1-3 mV) associated with uncompensated series resistance, and C) lack of dependence of I_A rates of activation and inactivation on varying conditioning steps [133]. Although voltage errors cannot be completely eliminated when recording intact neurons in a slice preparation, we believe the results reported here to be relatively accurate. This is also supported by previous studies from hypothalamic neurons in which similar I_A voltage and kinetic properties were reported, including studies from acutely dissociated [36,87] or intact neurons in a brain slice preparation [133].

**Pharmacological considerations**

The voltage-dependent properties of I_A are known to overlap with other voltage-dependent conductances, in particular the T-type Ca^{2+} current, previously reported to be present in parvocellular and identified preautonomic PVN neurons [133,185]. Thus, in order to isolate I_A from the low-threshold T-type and other voltage-dependent Ca^{2+} current, voltage-clamp recordings were obtained in the presence of nominal 0mM extracellular Ca^{2+}, the Ca^{2+} chelator EGTA and the broad spectrum Ca^{2+} channel blocker Cd^{2+}. A drawback of this approach however, is that previous studies in other neuronal populations reported that extracellular divalent cations, including Ca^{2+} and Cd^{2+}, could affect the voltage-dependence of I_A activation and inactivation [51,87,179,203]. Thus, it is possible that the reported voltage-dependent activation and inactivation values of I_A do not reflect the physiological condition. This becomes important when comparing data obtained from voltage-clamp and current-clamp experiments in this study, since the latter were obtained in the presence of 2 mM Ca^{2+} and in the absence of Cd^{2+}.  

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$I_T$ has previously been described in non-identified parvocellular PVN neurons [133] to activate at a threshold of -59 mV. However, we observed the threshold of activation of $I_T$ to be -54 mV in PVN-RVLM neurons. This difference may be due to population differences between PVN-RVLM neurons and unidentified parvocellular neurons. Observed differences may also be due in part to variations in extracellular calcium concentrations as it has been described that increases in extracellular calcium concentrations can shift the gating of T-type channels in a positive direction [70]. While we used an extracellular calcium concentration of 5 mM, the study by Luther & Tasker utilized 10 mM suggesting that it is more likely that the differences observed in threshold are due to population differences as opposed to differences in extracellular calcium.

Early studies determined that T-type channels were more sensitive to inhibition by low concentrations of nickel than HVA [68,77]. However, the HVA R-type calcium channels have also been shown to be relatively nickel sensitive [160,210]. We, therefore, attempted to specifically inhibit $I_T$ by using the selective T-type channel blocker NNC 55-0396 (10-50 μM, Sigma Aldrich) [92,123] via bath application. Unfortunately, no effect was observed (data not shown). Therefore, a low concentration of nickel (100 μM) was used in order to reduce the possible impact of inhibition on HVA. Interestingly, nickel did not have an effect under sham conditions, only in hypertensive PVN-RVLM neurons, in which we have demonstrated an increase in $I_T$ activity. However, we cannot rule out the possibility of nickel partially inhibiting HVA and contributing to the enhanced spontaneous firing activity observed during hypertension.
V. REFERENCES


