CALL FOR PAPERS | Central Control of Fluid and Electrolyte Homeostasis

Salt-induced sympathoexcitation involves vasopressin V₁ₐ receptor activation in the paraventricular nucleus of the hypothalamus

Natalia Ribeiro,* Helena do Nascimento Panizza,* Karoline Martins dos Santos, Hildebrando C. Ferreira-Neto, and Vagner Roberto Antunes

Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

Submitted 7 July 2015; accepted in final form 31 August 2015

Salt-induced sympathoexcitation involves vasopressin V₁ₐ receptor activation in the paraventricular nucleus of the hypothalamus. Am J Physiol Regul Integr Comp Physiol 309: R1369–R1379, 2015. First published September 9, 2015; doi:10.1152/ajpregu.00312.2015.—A high-salt diet can lead to hydromineral imbalance and increases in plasma sodium and osmolality. It is recognized as one of the major contributing factors for cardiovascular diseases such as hypertension. The paraventricular nucleus (PVN) plays a pivotal role in osmotically driven sympathoexcitation and high blood pressure, the precise mechanisms of which are not fully understood. Recent evidence indicates that AVP released from magnocellular neurons might be involved in this process. Using a combination of in vivo and in situ studies, we wanted to investigate whether AVP, acting on PVN neurons, can change mean arterial pressure (MAP) and sympathetic nerve activity (SNA) in euhydrated male rats. Furthermore, we wanted to determine whether V₁ₐ receptors on PVN neurons would be involved in salt-induced sympathoexcitation and hypertension. In rats, 4 days of salt loading (NaCl 2%) elicited a significant increase in plasma osmolality (39 ± 7 mosmol/kgH₂O), an increase in MAP (26 ± 2 mmHg, P < 0.001), and sympathoexcitation compared with euhydrated rats. Microinjection of AVP into the PVN of conscious euhydrated animals (100 nl, 3 μM) elicited a pressor response (14 ± 2 mmHg) and a significant increase in lumbar SNA (100 nl, 1 mM) (19 ± 5%). Pretreatment with a V₁ₐ receptor antagonist, microinjected bilaterally into the PVN of salt-loaded animals, elicited a decrease in lumbar SNA (−14 ± 5%) and MAP (−19 ± 5 mmHg), when compared with the euhydrated group. Our findings show that AVP plays an important role in modulating the salt-induced sympathoexcitation and high blood pressure, via V₁ₐ receptors, within the PVN of male rats. As such, V₁ₐ receptors in the PVN might contribute to neurogenic hypertension in individuals consuming a high-salt diet.

A high dietary intake of salt is one of the major contributing factors to cardiovascular diseases, such as hypertension (11, 12, 32). High-salt diets can lead to a hydromineral imbalance and increases in plasma sodium concentrations and osmolality. This, in turn, can activate central hypothalamic nuclei that elevate sympathetic outflow and arterial blood pressure (BP), leading to a disorder referred to as salt-sensitive neurogenic hypertension (33).

Osmotic perturbations are primarily sensed by osmosensing neurons located in brain regions that lack a blood-brain barrier termed circumventricular organs (CVOs). Under systemic hyperosmotic conditions, the CVOs are activated and send excitatory inputs to the paraventricular nucleus of the hypothalamus (PVN), an important integrative center involved in the autonomic and neuroendocrine functions to maintain body fluids and cardiovascular homeostasis (14, 19, 28). The PVN lies adjacent to the third ventricle in the anterior hypothalamus, and it is composed of magnocellular neurons (MCNs) and parvocellular neurons (PCN). The PCN send projections to autonomic nuclei, such as the rostral ventrolateral medulla (RVLM) and intermediolateral cell column located in the brain stem and spinal cord, respectively (7, 24, 30). Both of these cardiovascular centers are involved in the control of sympathetic function and the maintenance of arterial BP. The MCNs are responsible for the synthesis of AVP and oxytocin, peptide hormones that are released not only into the bloodstream via axonal projections to the neurohypophysis but also locally within the PVN via dendritic exocytosis release (3, 13, 23).

Although it is well established that hyperosmolality can increase sympathetic outflow resulting in elevations in BP (1, 6, 10, 26, 35) and that PVN plays a pivotal role in this response (1, 26, 31, 35), the mechanism by which hyperosmolality activates PVN neurons to increase sympathetic nerve activity (SNA) is not fully understood. In this regard, recent evidence indicates that AVP might act as one of the key neurotransmitters/neuromodulators involved in controlling sympathetic outflow in situations of increased plasma osmolality (29).

An increase in plasma osmolality is an important stimulus that triggers the central release of AVP at the level of hypothalamic supraoptic nucleus (SON) and PVN (13, 16). Son et al. (29) demonstrated that dendritically released AVP from a single stimulated MCN can directly evoke an increase in the discharge of neighboring presympathetic PVN to RVLM projecting neurons—an effect largely blocked by local application of a V₁ₐ receptor antagonist (29). This interaction between neurosecretory and presympathetic neurons suggests the potential involvement of AVP in hyperosmotically induced sympathoexcitation and hypertension under conditions of high salt intake. Given the results of these recent findings, the aim of this study was to investigate whether AVP, acting on PVN neurons, is involved in salt-induced sympathoexcitation. We postulated that 1) AVP can activate PVN neurons to increase BP and SNA and 2) the increased sympathetic activation and hypertension observed in salt-loaded male rats could be related to an endogenous AVP action at the PVN level.

* N. Ribeiro and H. do Nascimento Panizza contributed equally to this work.

Address for reprint requests and other correspondence: V. R. Antunes, Dept. of Physiology and Biophysics, Institute of Biomedical Sciences, Univ. of São Paulo, Av. Prof. Lineu Prestes, 1524, 05508-900, São Paulo (SP), Brazil (e-mail: antunes@icb.usp.br).

http://www.ajpregu.org 0363-6119/15 Copyright © 2015 the American Physiological Society
Here, we demonstrate that AVP activation of V_{1A} receptors within the PVN contributes to the sympathoexcitation and hypertension observed following chronic salt loading.

**METHODS**

**Ethical approval.** All experimental procedures were performed in accordance with the Ethical Principles in Animal Experimentation and were approved by the Ethical Committee for Animal Research of the Institute of Biomedical Sciences, University of São Paulo (ICB/USP) (protocol no. 111-108/2011). In vivo implanted rats were killed by an overdose of pentobarbital sodium (>60 mg/kg body wt) given intravenously.

**Animals.** Male Wistar rats 10–12 wk of age (290–350 g) were used for the in vivo studies, and 3–4-wk-old rats (50–80 g) for in situ studies. All animals were obtained from the colony bred at the ICB/USP, and kept at a constant temperature of 22–24°C and a relative humidity of 50–60% under a controlled light-dark cycle (12:12 h; lights on 6:00 AM) with normal rat chow and drinking water ad libitum.

**Intracerebral microinjection and blood pressure recording in conscious rats.** Four days before the experiment, rats were anesthetized with a mixture of ketamine chloride (100 mg/kg ip) and xylasine chloride (20 mg/kg ip) and placed in a stereotaxic head frame (David Kopf Instruments, Tujunga, CA) with the incisor bar set at −3.3 mm below the interaural point. A local vasoconstrictor anesthetic (3% lidocaine chloral hydrate with norepinephrine bitartrate, 1:50,000) was injected subcutaneously into the scalp region to avoid bleeding after the surgical incision. The skin overlaying the skull was reflected back, and bilateral stainless-steel guide canulas (0.6 mm OD, 24 gauge, 15 mm of length) were placed through a 2-mm burr hole drilled over the sagittal midline suture overlaying the PVN (AP = −1.2 mm from bregma, ML = ±0.3 mm from sagittal venous sinus, DV = −4.8 mm from skull surface), according to coordinates derived from a rat brain atlas (21). For microinjection into the lateral ventricle (LV), unilateral stainless-steel 23-gauge guide canulas (10 mm of length) were implanted immediately above the LV at the following coordinates: AP: −1 mm from bregma, ML: −1.6 mm lateral to midline, and DV: −3.5 mm below the skull surface. Canulas were fixed to the skull with dental acrylic resin (Clássico Artigos Odontológicos, Campo Limpo Paulista, São Paulo, Brazil), anchored by two small stainless-steel watch screws and occluded by a tight-fitting mandrel to avoid obstruction. After the surgical procedures, all animals received a prophylactic, broad-spectrum antibiotic (penicillin and streptomycin, 1,200,000 UI/ml; Fort Dodge, Campinas, São Paulo, Brazil) and a nonsteroidal anti-inflammatory drug, ketoprofen (Biofen 1% - Biofarmá Química e Farmacêutica LTDA, Jaboticabal, São Paulo, Brazil) subcutaneously. Three days after guide canulas were implanted and 1 day prior to experimentation, the rats were reanesthetized with a mixture of ketamine chloride (100 mg/kg ip) and xylasine chloride (20 mg/kg ip), and the femoral artery was catheterized (PE-10 connected to PE-50; Clay Adams, Parsippany, NJ) for measurement of pulsatile arterial pressure (PAP), mean arterial pressure (MAP), and heart rate (HR). The femoral vein was also catheterized for systemic drug delivery. The catheters were tunneled and exteriorized in the back of the neck so that MAP and HR could be monitored under conscious freely moving conditions. The cardiovascular parameters were monitored 24 h after the catheterization by connecting the arterial catheter, previously heparinized, to a pressure transducer (model CDX III, Cobe Labs, Lakewood, CO) connected to an amplifier (ML224 Quad Bridge Amp, ADInstruments, New South Wales, Australia), and this was connected to a digital data acquisition system (PowerLab, AD-Instruments). The sampling rate for the hemodynamic measurements was 1 kHz. PAP, MAP, and HR were analyzed off-line at the end of experiments. After the surgery, animals were individually housed. Intracerebral microinjections were performed in conscious freely moving rats using a 1-μl syringe (Hamilton, Reno, NV) connected by a polyethylene tube PE-10 to an injection needle (33 gauge, Small Parts, Miami Lakes, FL) 3.5 mm or 1.0 mm longer than the guide canula for the PVN or LV, respectively. The needle was carefully inserted into the guide canula, and the volume injected was always 100 nl for PVN, or 1 μl for LV.

In situ studies. Nerve recordings were performed in a decorticate, anesthesia-free, arterially perfused in situ preparation of rat (DAPR), as previously described (1). Briefly, rats were deeply anesthetized with halothane (5%) until loss of the paw withdrawal reflex. The stomach, intestines, and spleen were ligated and removed via midline laparotomy. The sternum was split and the rib cage was retracted to allow access to the mediastinum. The pericardium was removed, and the left phrenic nerve was isolated. The animal was submerged in cooled Ringer solution (see composition below), and the cerebral hemispheres were exposed by removal of the parietal bones. The cerebral cortices, hippocampus, and thalamic area were removed by gentle aspiration. The removal of these structures abrogates the need for further anesthetic use in this preparation. The preoptic area and its adjacent septal nuclei and hypothalamic areas remained totally intact.

The preparation was skinned and transferred to the recording chamber. A double-lumen perfusion canula was inserted into the ascending aorta via the left ventricle. The preparation was perfused at a flow rate of 28 ± 2 ml/min using a roller pump (Watson Marlow 505S, UK), with Ringer solution containing an oncotic agent (polyethylene glycol 20,000, 1.5%; Sigma, St. Louis, MO), gassed with carbogen (95% O_{2} and 5% CO_{2}), warmed to 32°C and filtered using a nylon screen (pore size: 25 μm). After respiration-related movements commenced, a neuromuscular blocker (vecuronium bromide, 4 mg/ml, Vecuron, Cristina, São Paulo, Brazil) was added to the perfusion solution to mechanically stabilize the preparation. The second lumen of the canula was used to monitor aortic perfusion pressure.

**PVN microinjection and nerve recordings in the DAPR.** The phrenic nerve and the lumbar sympathetic chain (L2 and L3) were visualized and dissected using a binocular microscope. Phrenic nerve activity (PNA) and lumbar sympathetic nerve activity (LSNA) were recorded from their distal ends using a glass suction bipolar electrode held in a three-dimensional micromanipulator. Rhythmic ramping PNA gave a continuous physiological index of preparation stability and viability. Preparations that did not show ramping PNA were deemed unreliable and not included in the study. Signals were AC-amplified (NL104, Neurolog, UK) and band-pass filtered (100 Hz-3 kHz) and displayed on a computer using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Sympathetic nerve activity exhibited marked respiratory modulation and was attenuated by an increase in perfusion pressure (arterial baroreceptor stimulation). For the intracerebral microinjections, the head of the DAPR was fixed by ear bars and a nasal clamp mounted on the perfusion chamber. The head of each preparation was fixed to an exact set of coordinates to allow accurate and consistent placement of micropettes (borosilicate glass capillaries, 1B150F-4; 1.5/0.8 OD/ID in mm; World Precision Instruments, Sarasota, FL). Microinjections into the PVN were performed on the basis of stereotaxic coordinates relative to the superior colliculus, i.e., 2.5–2.7 mm rostral, 0.3–0.5 mm lateral to midline and 3.2–3.4 mm below the brain surface (1). The volume microinjected either unilaterally and/or bilaterally (100 nl each side) was determined by viewing the movement of the meniscus through a binocular microscope fitted with a precalibrated reticule in the eyepiece.

Osmotic stimuli. The same salt loading protocol was performed for both in vivo and in situ studies. Animals were placed in individual cages and randomly assigned to one of two cohorts: 1) euhydrated group, maintained on standard chow diet and given tap water; and 2) salt-loaded group, maintained on standard chow diet and given 2% NaCl solution in place of the tap water for 4 days prior to the microinjection into the PVN and nerve recording. For the in situ studies, euhydrated animals were perfused with isosmotic Ringer solution containing 120 mM NaCl, which resulted in solution of 290

AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00312.2015 • www.ajpregu.org

Downloaded from http://ajpregu.physiology.org/ by 10.220.33.6 on August 4, 2017
mosmol/kgH₂O, while in the salt-loaded animals, the Ringer solution osmolality was prepared by adjusting the final concentration of NaCl (135 mM) to match the plasma osmolality, about 320 mosmol/kgH₂O, of the animal subjected to 4 days of salt loading. The osmolality was measured by a vapor pressure osmometer (Vapro model 5600; Wescor Logan, UT).

Chemicals and solutions. The composition of the Ringer solution was (in mM): 120 NaCl (290 mosmol/kgH₂O) and 135 NaCl (320 mosmol/kgH₂O), 24 NaHCO₃, 5 KCl, 2.5 CaCl₂, 1.25 MgSO₄, 1.25 KH₂PO₄, 10 dextrose. Molar concentrations of other chemicals and drugs used in the experiments were [Arg⁸]-vasopressin acetate salt (AVP; vasopressinergic receptor agonist, 3 mM); prazosin (α₁-adrenoceptor antagonist, 1 mg·kg⁻¹·ml⁻¹); hexamethonium chloride (nicotinic receptors antagonist at autonomic ganglia level, 5 mM); Evan’s Blue dye (2% wt/vol). The drug solutions were freshly dissolved in sterile saline (NaCl; 154 mM), and sodium bicarbonate was added to ensure a final pH of 7.4. All salts and drugs were purchased from Sigma-Aldrich unless otherwise stated.

Histological analysis. At the end of all experiments, Evan’s Blue dye (2% wt/vol) was microinjected (100 nl) into the PVN to mark the injection sites. The brain was removed and fixed in 4% paraformaldehyde in 0.1 M PBS and 20% sucrose. Coronal sections (40 μm thick) were cut using a cryostat (CM1900; Leica, Switzerland) and thaw-mounted on gelatin-subbed glass microscope slides. Brain sections were visualized under light microscopy, and the injection sites were mapped according to the rat brain atlas of Paxinos and Watson (21). Only data in which the microinjections were confirmed to be within the PVN were analyzed.

Data analyses. The statistical analyses were performed by using GraphPad Prism 4.02 (GraphPad Software, San Diego, CA). For the in vivo data, after each microinjection, MAP was measured at the point of maximum deviation from baseline values before injection. All data passed normality and equal variance tests and were analyzed by one-way or two-way ANOVA for repeated measures followed by Bonferroni’s post hoc test for multiple comparisons. In situ data were acquired using biopotential AC amplifiers and filters (Neurolog, Digitimer, Hertfordshire, UK) and collected using a CED 1401 A-D interface [Cambridge Electronic Design (CED), Cambridge, UK] and a computer running Spike 2 software (CED) with custom-written scripts for data acquisition and on-line and off-line analyses. LNSA was expressed as a percentage of the baseline values. The noise for LNSA was assessed by application of hexamethonium at the end of each experiment. To analyze the duration of increased LNSA evoked by AVP microinjected into the PVN, a cursor was positioned at the beginning of the response, corresponding to the time of the injection of VP into the PVN, and a second cursor was positioned at the time when LNSA returned to baseline values. Noise was subtracted from the data prior to analysis. A one-way or two-way ANOVA for repeated measures followed by Bonferroni’s post hoc test was used depending upon the experimental protocol used. The results presented are expressed as means ± SE, and n represents the number of animals used in each group. The significance level was set at P ≤ 0.05.

Fig. 1. AVP microinjected into paraventricular nucleus of the hypothalamus (PVN) of conscious rats elicits an increase in the blood pressure. A: traces of the pulsatile arterial pressure (PAP) and mean arterial pressure (MAP) recordings from one representative euvhydrated rat that received a unilateral microinjection (arrow) of AVP (arrow) or saline (vehicle control, arrow) into the PVN. B: maximal changes in MAP (mmHg) elicited by microinjection of AVP (3 μM) or saline (vehicle control) into the PVN, outside of the PVN or into the lateral ventricle (LV) and intravenously (i.v.). C: maximal changes in MAP (mmHg) elicited by microinjection of AVP (3 μM) into the PVN before and after pretreatment with prazosin (PRZ; 1 mg/kg/ml iv; P < 0.01). Results are shown as means ± SE, *P ≤ 0.05 compared with the AVP-PVN misplaced. AVP-LV, saline-PVN, and AVP (i.v.) injection groups; One-way repeated-measures ANOVA with Bonferroni’s post hoc test. The numbers inside the parentheses indicate the number of animals used for each experimental protocol.
RESULTS

Exogenously applied vasopressin into the PVN increases blood pressure and sympathetic nerve activity in euhydrated animals. To determine whether activation of vasopressin receptors in the hypothalamus elicits changes in the MAP and sympathetic outflow, we performed microinjections of AVP into the PVN of euhydrated conscious rats and in the DAPR. Figure 1A shows traces of the pulsatile and MAP from one representative conscious animal unilaterally microinjected with 100 nl of exogenous AVP into the PVN (3 μM concentration). The microinjection of AVP into the PVN evoked a significant increase in MAP, a plateau, in average, 90 s after injection (ΔMAP: +14 ± 2 mmHg; Fig. 1B) before returning to baseline. The maximal increase in MAP (2 ± 2 mmHg) was completely abolished 10 min after intravenous pretreatment with prazosin, an α1 adrenoceptor antagonist (ΔMAP: −2 ± 2 mmHg; Fig. 1C).

In a separate group of rats, we performed a set of in situ DAPR experiments to determine the effects of AVP microinjections into the PVN on LSNA of euhydrated animals. Figure 2A shows representative traces of a gradual increase in LSNA elicited by microinjection of AVP (1 mM–100 nl each side) into the PVN. The maximal increase in LSNA was reached 15 min after the microinjection (19 ± 5%). Microinjection of saline (vehicle control) did not significantly alter LSNA (1 ± 2%) when assessed over the same time course (Fig. 2B), with no significant changes in the perfusion pressure at any time evaluated (data not shown). To test whether the sympathoexcitation was specific to the actions of AVP in the PVN, we also analyzed data from animals where the injection pipette was placed outside of the PVN (misplaced). In such cases, microinjections of AVP failed to elicit any significant change in the LSNA (2 ± 3%). Analysis of the area under the curve confirmed that there was a significant increase in the LSNA produced by microinjection of AVP into the PVN compared with the saline and misplaced groups (Fig. 2C).

Salt-induced hypertension relies upon sympathetic nerve activation. To validate our salt loading (2% NaCl in the drinking water) protocol, we measured plasma osmolality,
MAP, and LSNA in the salt-loaded rats and compared these to euhydrated animals (given tap water).

At in vivo studies the Fig. 3A shows representative traces of blood pressure levels in conscious euhydrated and salt-loaded rats before (basal) and after intravenous injection of the α₁ adrenoceptor antagonist, prazosin. The basal MAP of salt-loaded rats was significantly higher (137 ± 3 mmHg) than in euhydrated rats (110 ± 1 mmHg; Fig. 3B). Following prazosin treatment, the reduction in the MAP of salt-loaded rats was greater (ΔMAP: −40 ± 4 mmHg) than in euhydrated rats (ΔMAP: −15 ± 3 mmHg), which is indicative of greater sympathetic activation in the salt-loaded rats (Fig. 3C).

Similarly, in situ studies in the DAPR have shown that the basal LSNA of euhydrated and salt-loaded rats was significantly different. Rats that were salt-loaded showed higher LSNA (1.93 ± 0.21 μV) when compared with euhydrated animals (0.98 ± 0.15 μV), as can be seen in Fig. 4A. Likewise, the reduction in the LSNA after ganglionic blockade with hexamethonium was significantly greater in salt-loaded rats (−39 ± 1%) compared with euhydrated rats (−26 ± 3%; Fig. 4B). These findings are consistent with the higher sympathetic tone observed in conscious salt-loaded rats contributing to the salt-induced hypertension (Fig. 3C). Plasma osmolality was significantly higher in salt-loaded group (333 ± 3 mosmol/kgH₂O) when compared with euhydrated animals (295 ± 6 mosmol/kgH₂O; Fig. 4C).

Antagonism of vasopressin V₁a receptors within the PVN decreases the salt-induced sympathoexcitation and hypertension. To elucidate whether endogenous action of AVP on V₁a receptors within the PVN contributes to hypertension and the sympathetic hyperactivity in salt-loaded rats, we performed bilateral microinjection of V₁a receptor antagonist (V₁a.ant) into the PVN of salt-loaded DAPR model rats and conscious rats, respectively. Bilateral microinjection of V₁a.ant (1 μM) into the PVN of salt-loaded DAPR model animals elicited a decrease in the LSNA with a significant and maximal reduction observed at 15 min after injection (−14 ± 5%; Fig. 5, A and B) when compared with euhydrated rats over the same time course (10 ± 3%).

In a separate group of in vivo studies in conscious salt-loaded rats, V₁a.ant (1 μM) microinjected bilaterally into the PVN elicited a significant reduction in MAP at 10 min (ΔMAP: −19 ± 5 mmHg) when compared with the control group in which microinjections were placed outside of the PVN (misplaced) over the same time course (Fig. 6B). Microinjections of saline (vehicle control) into the PVN produce no significant changes in the MAP of 4 days salt-loaded rats (Fig. 6, A and B).

PVN microinjection sites. The placement of each microinjection was verified at the conclusion of each experiment by injecting Evan’s blue dye (100 nl) to mark the injection site. As demonstrated in the schematic diagrams (Fig. 7A) and representative photomicrographs of typical injection sites (Fig. 7B), all of the microinjections reported here were made within the borders of the PVN as defined by the rat brain atlas of Paxinos and Watson (21). Microinjections located outside of the PVN had no significant effects on LSNA, thus demonstrating that the effects of AVP were specific to the PVN.

![Graph showing MAP and PAP comparisons between euhydrated and salt-loaded conditions](image)

**Fig. 3.** The elevated blood pressure in salt-loaded animals relies upon sympathetic overactivation. A: traces of basal levels of the PAP and MAP from one representative euhydrated and salt-loaded conscious animal after receiving an injection of prazosin (PRZ; 1 mg·kg⁻¹·ml⁻¹), an α₁ adrenoceptor antagonist intravenously, B: scatter graph of mean (solid lines) basal levels of MAP (mmHg) of the euhydrated (○; n = 5) and salt-loaded rats (●; n = 5), C: delta (Δ) changes in MAP (mmHg) of euhydrated (open bars; n = 5) and salt-loaded (solid bars; NaCl 2%, n = 5) rats after intravenous injection of prazosin (PRZ; 1 mg·kg⁻¹·ml⁻¹ iv). The data were analyzed using a Student’s t-test. A probability value of P ≤ 0.05 was considered to be significant for comparison between euhydrated and salt-loaded animals.
only V1a receptors have been well characterized in the rat which AVP is a ligand (namely, V1a, V1b, and V2 receptors), While there are three different G protein-coupled receptors are mainly located in the parvocellular region of the PVN. and blood pressure control. Centrally projecting AVP neurons priors, osmoregulation, hydromineral balance, thermoregulation, and multiple physiological functions within the central nervous system, such as learning and memory processes, sexual behaviors, osmoregulation, hydromineral balance, thermoregulation, and blood pressure control. Centrally projecting AVP neurons are mainly located in the parvocellular region of the PVN. While there are three different G protein-coupled receptors for which AVP is a ligand (namely, V1a, V1b, and V2 receptors), only V1a receptors have been well characterized in the rat brain. It has been suggested that V1a receptors are the predominant receptors responsible for the central actions of AVP (2).

Here, we demonstrated that AVP can act within the PVN, likely on V1a receptors expressed on parvocellular neurons, to increase SNA and BP. These data are supported by the study of Son et al. (29), who demonstrated dense V1a receptor immunoreactivity on presympathetic RVLM-projecting PVN neurons. From a functional standpoint, stimulation of vasopressin-expressing PVN neurons resulted in membrane depolarization and increased firing rate of PVN-RVLM neurons (29).

DISCUSSION

The major findings of the study are 1) AVP applied to the PVN evoked significant increases in the blood pressure and SNA of euhydrated rats; and 2) antagonism of V1a receptors within the PVN reduced the higher SNA and blood pressure in salt-loaded rats. Taken together, these data provide evidence of a significant involvement of vasopressin V1a receptors within the PVN in the control of sympathetic outflow and blood pressure changes of rats under salt-loaded conditions.

Vasopressin is a neurohypophysial hormone that regulates multiple physiological functions within the central nervous system, such as learning and memory processes, sexual behaviors, osmoregulation, hydromineral balance, thermoregulation, and blood pressure control. Centrally projecting AVP neurons are mainly located in the parvocellular region of the PVN. While there are three different G protein-coupled receptors for which AVP is a ligand (namely, V1a, V1b, and V2 receptors), only V1a receptors have been well characterized in the rat brain. It has been suggested that V1a receptors are the predominant receptors responsible for the central actions of AVP (2).

Here, we demonstrated that AVP can act within the PVN, likely on V1a receptors expressed on parvocellular neurons, to increase SNA and BP. These data are supported by the study of Son et al. (29), who demonstrated dense V1a receptor immunoreactivity on presympathetic RVLM-projecting PVN neurons. From a functional standpoint, stimulation of vasopressin-expressing PVN neurons resulted in membrane depolarization and increased firing rate of PVN-RVLM neurons (29).

Using an in situ approach of DAPR preparation, we show that the microinjection of AVP into the PVN leads to an increase in LSNA. This preparation is advantageous, as it eliminates the need for anesthetic use, which has been shown to have significant depressive effects on the neural control of autonomic function. We have validated and used this preparation in the past to study central control of hyperosmolality-induced sympathoexcitatory responses, in which the integrity of connections between circumventricular organs, including subfornical organ and PVN were preserved (1). Another advantage of the preparation is the removal of all circulating blood-borne substances, such as ANG II and other hormones, which can affect sympathetic nerve discharge and obfuscate the data. To determine whether the sympathoexcitatory drive mediated by AVP is translated to blood pressure changes, we extended our investigation to include intact, conscious, in vivo rats experiments. In the conscious freely moving rat, AVP microinjection into the PVN also led to an increase in the arterial blood pressure, which is sympathetically mediated as prior intravenous infusion of α1 adrenoceptor antagonist, prazosin, abolished the salt-induced hypertension.

The temporal profile of responses elicited by microinjection of AVP into the PVN on sympathetic outflow and blood pressure in both in vivo and in situ studies warrants discussion. It can be argued that these gradual and prolonged responses might be related to the half-life of AVP in the brain, which is ~20 min (18). This considerable half-life suggests that AVP could interact with neighboring synapses to facilitate long-

![Image](http://ajpregu.physiology.org/)

Fig. 4. LSNA and plasma osmolality are higher in studies of the decorticate, anesthetic-free, arterially perfused in situ preparation of salt-loaded animals. A: representative traces of changes in raw and integrated (∫) LSNA signals of two groups of animals: one that had free access to water (euhydrated) and the other to 2% NaCl (salt-loaded) before and after application of hexamethonium (HX; 5 mM, arrow) in the perfusate. B: percentage of reduction in the magnitude of the LSNA (%) of euhydrated (open bars; n = 6) and salt-loaded animals (solid bars; n = 6) after ganglionic blockade with HX. C: plasma osmolality levels of euhydrated (open bars; n = 6) and salt-loaded animals (solid bars; n = 7). The data were analyzed using a Student’s t-test. A probability value of P ≤ 0.05 was considered to be significant for comparison between euhydrated and salt-loaded animals.
lasting responses by modulating the firing pattern(s) of neighboring groups of neurons (17). Moreover, these gradual increases in the SNA and arterial pressure caused by microinjection of AVP into the PVN could also be related to the spread of the injectate, the concentration of which could also vary from the center of the injection’s site to the more peripheral areas of the PVN. It is unlikely that AVP has cardiovascular effects in nuclei that are immediately adjacent to the PVN, since we did not observe any changes in arterial pressure or SNA in animals with misplaced injections. Additionally, we noted that the onset of changes in the blood pressure was more rapid than changes to the sympathetic response observed in in situ experiments; this discrepancy could be due to the different concentrations of AVP or related to the different methodologies used. In the in situ studies, we used a reduced preparation, and although the neural circuit around the PVN is fully intact, the reestablishment of basal blood pressure in this preparation might be mediated faster than the sympathetic response. Furthermore, in in vivo studies of conscious rats, AVP was microinjected into the PVN at a lower concentration (3 μM) compared with in situ studies (1 mM), but enough to elicit a significant rise in BP of conscious rats. In the in situ studies, we used two different doses of AVP, and only the higher dose produced an increase in the SNA. We believe that the lower dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions. Despite the fact that a higher dose of AVP was without effect because of the relatively high flow rate of the brain perfusion, which is typical of the DAPR preparation. This may have resulted in rapid washout and/or a decrease in AVP receptor binding. It also explains the delayed onset of AVP’s physiological actions.

Fig. 5. Antagonism of $V_{1a}$ receptors in the PVN reduces the sympathoexcitation elicited by high-salt intake in DAPR studies. A: traces of changes in raw and integrated (f) LSNA signals from one representative animal that received bilateral microinjections of a $V_{1a}$ receptor antagonist ($V_{1a}$ant, arrows) into the PVN of salt-loaded animals. B: percentage change (basal as 100%, before injections) in the magnitude of the LSNA (%) and the duration of response (min) of euhydrated ($n = 4$) and salt-loaded ($n = 5$) animals that received a bilateral microinjection of $V_{1a}$ receptor antagonist ($V_{1a}$ant; 1 μM) or saline (vehicle control; $n = 7$) into the PVN. Results are presented as means ± SE. *$P < 0.05$ is related to the basal level (before microinjection). #$P < 0.05$ is related over the same time course of comparison between salt-loaded animals and euhydrated. Two-way repeated-measures ANOVA with Bonferroni’s post hoc test.
that AVP concentration in the extracellular fluid of the supraoptic nucleus is 100 to 1,000 times higher than that found in blood, suggesting that higher AVP concentrations may be required for modulating neuronal activity in the SON (20) and possibly also within the PVN.

It is well established in the literature that chronic increases in plasma osmolality can trigger a sustained sympathoexcitation, and generate structural and functional changes in certain brain nuclei, including those involved in the synthesis and release of AVP (4, 8, 22, 33). Thus, given that AVP can increase sympathetic outflow through its action in the PVN, we tested the hypothesis that endogenous AVP action at the level of PVN neurons might provide a functional physiological relevance in the control of SNA and blood pressure under conditions in which systemic osmolality was increased by high salt intake. To this end, rats were subjected to 4 days of high salt intake. We then blocked V1a receptors specifically within the PVN, while simultaneously recording SNA (in in situ studies) and BP (in vivo studies). The antagonism of V1a receptors within the PVN decreases SNA and blood pressure, which were significantly elevated after 4 days of high salt intake. Our findings are in accord with those reported by Son et al. (29), who demonstrated in in vivo studies on anesthetized rats that bilateral microinjections of V1a receptor antagonist into the PVN attenuated the increase in RSNA caused by acute intracarotid infusion of hypertonic saline. Taken together, these findings demonstrate that AVP contributes to the sympathoexcitation and high blood pressure induced by either acute or chronic osmotic challenges, which involves activation of via V1a receptors within the PVN. Although we cannot discount it entirely, given the dose, the small injection volume, and the fact that the PVN has an intact blood-brain barrier, it is highly

Fig. 6. V1a receptor blocker in the PVN of conscious rats reduces the high-salt-induced hypertension. A: traces of changes in the PAP and MAP of a representative salt-loaded animal that received bilateral microinjections of a V1a receptor antagonist (V1a-ant, arrows) into the PVN of salt-loaded animals or saline (vehicle control, arrows). B: MAP (mmHg) changes and the duration of response (min) of salt-loaded (SL) animals that received a bilateral microinjection (arrow) of vasopressin V1a receptor antagonist (V1a-ant, 1 μM; n = 3) or saline (vehicle control; n = 3) into the PVN compared with the control group where microinjections were outside of the PVN (misplaced; n = 3). Data are presented as means ± SE. *P ≤ 0.05 and #P ≤ 0.05 denote a significant difference to the basal level (before AVP microinjection) and over the same time course compared with saline and misplaced groups, respectively. Two-way repeated-measures ANOVA with Bonferroni’s post hoc test.
unlikely that the V1a receptor antagonist leaked into the circulation to block V1a receptors located in the periphery (27).

We propose that the salt-induced sympathoexcitation and hypertension might result from an imbalance in the pattern of activity of PVN neurons in response to an increase of plasma osmolality, since several studies support the idea that AVP may influence neuronal interactions in a complex manner. For example, it has been shown that the response of MCNs of the supraoptic nucleus, evoked by hyperosmotic stimulus, leads to coactivation of excitatory and inhibitory inputs (15), which could involve the participation of AVP. Considering its potential to mediate both excitatory and inhibitory effects, it is reasonable to speculate that a salt loading condition might cause a loss of balance between the excitatory/inhibitory mechanisms within the PVN, which, in turn, could trigger downstream effects, such as increasing SNA and arterial blood pressure.

In addition, it has been shown elsewhere that neuropeptides, such as AVP, can be released from dendrites and exert their function as autocrine or paracrine signals to modulate the neuron’s own activity, or even act at distant neuronal targets to evoke long-lasting changes (17). For example, Son et al. (29) demonstrated that activation of magnocellular vasopressinergic neurons within the PVN triggers the release of AVP, which diffused a short distance to modulate presympathetic PVN-RVLM-projecting neurons. These findings support the hypothesis of dendritic AVP release from neurosecretory MCNs. Bearing this in mind, we propose that osmosensitive neurons in the forebrain detects osmotic changes (20) in the plasma evoked by high salt intake, and through their axonal projec-

Fig. 7. Confirmation of microinjections sites. A: schematic diagram of coronal sections of the hypothalamus at the level of the PVN modified from the rat brain atlas of Paxinos and Watson (21) [Modified with permission from Elsevier, The Rat Brain in Stereotaxic Coordinates. San Diego, CA: Elsevier Academic, 5th ed., 2005], showing the center of microinjections of AVP (star) and V1aant into the PVN of salt-loaded (open cross) and euhydrated animals (△) performed in the in situ studies. Asterisks (*) represent microinjection sites made outside of the PVN. B: representative photomicrographs of coronal sections of the hypothalamus showing bilateral (in situ studies) and unilateral (conscious rats) microinjections into the PVN. Arrows indicate the position of the micropipette tip. Scale bar = 500 μm. PaDC, paraventricular hypothalamic nucleus, dorsal cap; PaLM, paraventricular hypothalamic nucleus, lateral magnocellular part; PaMP, paraventricular hypothalamic nucleus, medial parvicellular part; PaV, paraventricular hypothalamic nucleus, ventral part; Pe, periventricular hypothalamic nucleus; Arc, arcuate hypothalamic nucleus; 3V, third ventricle.
tions, influence the neurosecretory MCNs in the PVN to trigger the endogenous dendritic release of AVP, which, in turn, stimulates the presynaptic neuronal population to drive sympathetic overactivation and hypertension. This assertion is merely speculative, since we have not measured the release of AVP within the PVN during the chronic salt-loading condition.

Methodological considerations. Here, we clearly demonstrate that a high salt intake during 4 days can increase plasma osmolality, blood pressure levels (in vivo conscious rats studies), and SNA (in situ DAPR studies) compared with euhydrated animals. However, a methodological consideration has to be given to the protocol of osmotic stimulus used in this study. Taking the results of our in situ (DAPR) experiments with sympathetic nerve recordings, rats were perfused with a hypertonic Ringer solution (~320 mosmol/kgH2O); this osmolality was matched to the plasma osmolality of animal subjected to 4 days of salt intake. To be certain that the changes in the LSNA were due to the chronic hyperosmotic stimulus of 4 days exposure to high salt intake, and not simply to the acute effects of increased perfusate osmolality, we performed pilot experiments in which we started perfusion with isotonic Ringer perfusate (290 mosmol/kgH2O). Interestingly, when salt-loaded rats were exposed to isotonic perfusate from the beginning of perfusion, the LSNA level exhibited a small reduction. Then, we decided not to perform the experiments with isotonic Ringer perfusate, as doing so could compromise the data interpretation. On the other hand, we have also tested starting perfusion of DAPR-euhydrated rats with hypertonic Ringer solution (~320 mosmol/kgH2O), to verify whether the LSNA would be affected at this osmotic condition, and we have noticed that euhydrated DAPR rats exposed to hypertonic perfusate had no significant change in the basal LSNA. Collectively, it seems that 4 days of high salt intake (2% NaCl in solution) in place of drinking water is unlikely to be an acute hyperosmotic stimulus, but an interesting model to investigate neural homeostatic regulation of blood pressure under osmotic challenges.

One possible caveat of the present study is that while we demonstrated that the V1a receptor antagonist applied into the PVN has attenuated the salt-induced sympathoexcitation and hypertension, we did not show specificity of its action in terms of whether blockade of oxytocin receptors was affected as a consequence of nonspecific drug action. However, as we have previously shown (1) that V1aR antagonist (Manning compound) applied intrathecally was able to reduce about 70% the sympathoexcitiation elicited by its agonist [V1 agonist (Phe2,Ile3,Orn8-AVP)], we are confident that the antagonist efficacy was primarily blocking V1a receptors.

Perspectives and Significance

According to Toney and Stocker (33), modern diets, containing high amounts of sodium, are contributing to chronic changes in plasma osmolality. As a consequence, there is a dramatic increase in the prevalence of salt-sensitive diseases, such as hypertension and heart failure. Furthermore, there is growing evidence of an association between cardiovascular diseases that lead to salt retention and increased sympathetic activity (5, 9, 34). Thus, the sustained increase in plasma osmolality can induce functional and/or structural changes in specific autonomic brain nuclei involved in the control of BP, and here, we have shown an important physiological role for AVP, via V1a receptors, within the PVN in modulating the salt-induced sympathoexcitation and hypertension. On the other hand, in humans with salt-sensitive hypertension, the plasma osmolality tends to be maintained at the normal level, which could account for a lesser role of central action of AVP within the PVN contributing to osmotically driven sympathoexcitation and high blood pressure. Salt-sensitive hypertension in humans is an important issue under debate, and we still need well-controlled studies to understand the precise pathophysiological mechanisms of persistent osmosensory activation of SNA that could potentially contribute to a high blood pressure in a high-salt diet intake condition.

Collectively, our present findings shed additional light on the complex mechanisms underlying the generation, central integration, and maintenance of neurogenic hypertension under high-salt diet conditions in animal models.

ACKNOWLEDGMENTS

We thank Dr. Song T. Yao for helpful suggestions and language editing.

GRANTS

This study was supported by São Paulo Research Foundation (FAPESP) Grants 10/17997-0 and 13/06206-0 and the National Council for Scientific and Technological Development (CNPq). H. do Nascimento Panizza was the recipient of a CNPq fellowship. N. Ribeiro H. C. Ferreira-Neto, and K. M. dos Santos were recipients of FAPESP fellowships nos. 09/13047-0, 10/05037-1, and 13/05037-1, respectively.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author’s contributions: Ribeiro N and Ferreira-Neto HC performed the in situ experiments, collection and analysis of data. Antunes VR is responsible for the manuscript. Panizza HN and Martins dos Santos K performed in vivo experiments, collection and analysis of data. Antunes VR is responsible for the conception and design of experiments, writing, and editing the manuscript.

REFERENCES


