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VASOPRESSIN AND V1BR GENE EXPRESSION IS INCREASED IN HYPOTHALAMIC PVN OF BORDERLINE HYPERTENSIVE RATS

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Keyword:	Vasopressin, vasopressin receptors, borderline hypertension, stress, hypothalamic paraventricular nucleus
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**VASOPRESSIN AND V1BR GENE EXPRESSION IS INCREASED IN
HYPOTHALAMIC PVN OF BORDERLINE HYPERTENSIVE RATS**

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ABSTRACT

Vasopressin (VP) is a neurohypophyseal peptide best known for its role in maintaining osmotic and cardiovascular homeostasis. The main sources of VP are the supraoptic and paraventricular (PVN) nuclei of the hypothalamus, which co-express the vasopressin V1a and V1b receptors (V1aR and V1bR). Here we investigate the level of expression of VP and VP receptors in the PVN of borderline hypertensive rats (BHRs), a key integrative nucleus in neuroendocrine cardiovascular control. Experiments were performed in male BHRs and Wistar rats (WR) equipped with radiotelemetry device for continuous hemodynamic recordings, under baseline conditions and after saline load without or with stress. The autonomic control of the circulation was evaluated by spectral analysis of blood pressure (BP) and heart rate (HR) variability and baro-receptor reflex sensitivity (BRS) using the sequence method. Plasma VP was determined by radioimmunoassay, and VP, V1aR and V1bR gene expression by RT-qPCR. Under baseline conditions, BHRs had higher BP, lower HR and enhanced BRS in respect to WRs. BP and HR variability was unchanged. In the PVN, overexpression of VP and V1bRs genes were found and plasma VP was increased. Saline load downregulated V1bR mRNA without affecting VP mRNA expression, plasma VP and the BP. Adding stress increased BP, BP and HR low frequency sympathetic spectral markers, decreased plasma VP without altering the level of expression of VP and VP receptors in PVN. It follows that overexpression of VP and V1bR in the PVN is a characteristic trait of BHR, and that sympathetic hyperactivity underlies stress-induced hypertension.

KEY WORDS: Vasopressin, vasopressin receptors, borderline hypertension, stress, hypothalamic paraventricular nucleus

1 INTRODUCTION

2 Vasopressin (VP) is a neurohypophyseal peptide hormone engaged in an abundance of
3 physiological and behavioural regulatory processes, but it is best known for its importance in
4 maintaining osmotic and cardiovascular homeostasis. The main sources of VP in the brain are
5 the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus ^{1,2}. It is well
6 established that PVN is a key integrative site for neuroendocrine and autonomic responses
7 relevant for cardiovascular regulation. Large magnocellular neurons (MCNs) of the PVN
8 synthesize VP, which is axonally transported to the neurohypophysis for systemic secretion ^{1,2}.
9 In the periphery, VP fosters water reabsorption in the kidneys and vasoconstricts blood vessels
10 to increase peripheral resistance supporting circulation ³. Smaller parvocellular neurons of the
11 PVN comprise a neuroendocrine portion which synthesizes VP and releases it in to the
12 hypophyseal portal circulation, and a pre-autonomic neuronal subgroup responsible for the
13 sympathetic nervous system activation that contributes to hypertension ⁴. These latter neurons
14 modify sympathetic outflow by virtue of projections to the brain stem autonomic nuclei in the
15 rostral part of the medulla (RVLM vasomotor neurons) and the intermediolateral column
16 of the spinal cord (IML), enabling them to modulate cardiovascular and renal homeostasis ⁵.
17 VP is also released from somata and dendrites of MCNs where it performs auto-regulation via
18 specific co-localised vasopressin receptors (V1aR and V1bR) ^{6,7}. Osmotic challenges have
19 been shown to modify the expression of V1aRs, modulate VP release and sympathetic outflow
20 in normotensive rat strains ⁸⁻¹⁰. V1bRs in the PVN were also found to be implicated in the
21 blood pressure (BP) increase caused by VP microinfusion into the PVN ¹¹. However, the
22 possible roles of VP, V1aRs and V1bRs in the PVN in the pathogenesis of borderline
23 hypertension has not been elucidated.

1   We hypothesized that changes in the gene expression of V1aR and V1bR receptors in
2   PVN may modulate VP gene expression and release and thus contribute to the genesis of
3   borderline hypertension in rats (BHR) and their susceptibility to develop overt hypertension
4   when exposed to repeated environmental stressors ¹². Therefore, we evaluated the gene
5   expression of VP, V1aR and V1bR in the PVN of BHRs as well as plasma VP and
6   cardiovascular autonomic control under baseline physiological condition and when submitted
7   to a 24-weeks long salt load with and without repeated stress.

For Review Only

1 METHODS

2 All experimental procedures in this study conform to the guidelines from Directive
3 2010/63/EU of the European Parliament on the protection of animals used for scientific
4 purposes, as well as UK Animal Act 1986. The experimental protocol was approved by the
5 School of Medicine University of Belgrade Ethics review board (# 323-07-04083/2016-05/7),
6 and all studies involving animals comply with the ARRIVE guidelines for reporting
7 experiments involving animals^{13,14}.

8 *Animals*

10 Two strains of rats, Wistar rats (WR) and BHRs, were included in the study. BHR were
11 produced in a local animal facility as an F1 generated by crossbreeding spontaneously
12 hypertensive rat (SHR) females and normotensive WR males. All animals were matched
13 regarding their age (12 weeks old) and weight (280-330 g), and were housed in a controlled
14 environment (12 h/12 h light-dark cycle, temperature $21\pm 2^{\circ}\text{C}$ and humidity $65\pm 9\%$) with
15 access to standard food pellets (0.2% sodium content; Veterinarski zavod, Subotica, Republic
16 of Serbia) and tap water or 0.9% saline solution *ad libitum*. The number of rats enrolled in
17 experimentation was determined in 'Power Sample Size Calculation' software
18 (<http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize>) for power of 90%
19 and type I error probability of 0.05.

21 *Implantation of radiotelemetry probe in abdominal aorta*

22 Animals were anesthetized using a combination of ketamine ($100\text{ mg}\cdot\text{kg}^{-1}$, I.M.) and
23 xylazine ($10\text{ mg}\cdot\text{kg}^{-1}$, I.M.). The depth of anesthesia was verified by the lack of corneal and
24 pedal reflexes, at the beginning and throughout the surgery. Rat body temperature was

maintained by a heating pad (Harvard Apparatus, Holliston, MA, USA). Following medial abdominal 3 cm-long incision and intestine retraction, a catheter tip of radiotelemetry device (TA11-PA C40; DSI, Transoma Medical, St Paul, MN, USA) was inserted into aorta, fixed with 3M Vetbond™ and tissue cellulose patch (DSI, Transoma Medical). Perioperatively, rats were treated with gentamicin (25 mg•kg⁻¹, i.m.) and carprofen (5 mg•kg⁻¹ daily, s.c.) to prevent secondary bacterial infections and to reduce pain, respectively. Postoperatively, animals were housed individually (30 × 30 × 30 cm Plexiglas cage) in a separate surrounding under controlled laboratory conditions. They were monitored every day until full recovery.

Experiment design

Fourteen days following surgery rats were fully recovered and randomly allocated in four groups subjected to different protocols. In group 1, Wistar rats drinking tap water were kept under baseline physiological conditions for 24 weeks. In group 2, BHRs drinking tap water were kept under baseline conditions for 24 weeks. In group 3, BHRs were drinking 0.9% saline solution for 24 weeks, while in group 4, BHRs were drinking 0.9% saline solution and were exposed to repeated heterotypic stressors to minimize conditioning, also for 24 weeks. Heterotypic stress was produced by applying alternating stress batteries. Battery 1 lasted 4 weeks: crowding stress (rats were housed in a reduced living space: 70 cm²/100g body weight) plus shaking stress (200 cycles/min for 30 min), for 6 days per week. Battery 2 lasted 6 weeks: isolation in opaque Plexiglas cages (300 cm²/100g body weight) plus inclination at 40° for 60 minutes followed by 2-min-long air jet directed to the top of rats' head, for 6 days per week. Battery 1 was repeated for 3 times, and battery 2 was repeated for 2 times, in total for 24 weeks. On each seventh day (day without exposure to stress), SBP, DBP, HR, BRS and spectral parameters were monitored and shown after completion of each stress battery.

Cardiovascular signal processing and analysis

Arterial BP was digitalized at 1000 Hz in Dataquest A.R.T. 4.0 software, (DSI, Transoma Medical). Systolic (SBP) and diastolic BP (DBP) and pulse interval (PI) or its inverse heart rate (HR) were derived from the arterial pulse pressure wave as maximum, minimum and $dP/dt_{\max}$ inter-distance, respectively.

Evaluation of the spontaneous baroreceptor reflex by the sequences method

The method is explained in detail elsewhere¹⁵. A stream of, at least four, consecutively increasing or decreasing SBP values and PI values delayed by three, four or five beats with respect to SBP, were considered as baro-receptor reflex sequence¹⁶. The following parameters were calculated as follows:

- *Baroreceptor reflex sensitivity* (BRS, $\text{ms} \cdot \text{mmHg}^{-1}$) as linear regression coefficient averaged over all identified sequences ($\text{PI} = \text{BRS} \cdot \text{SBP} + \text{const.}$, where fitting of the curve is done in a least square method);
- *Baroreceptor effectiveness index* (BEI) as ratio of the number of SBP-PI sequences versus number of SBP ramps
- *Operating range* (OR) as a rectangle surface covering 95% of SBP-PI sequences.
- *Set Point* (SP) as a median of all SBP-PI sequences.

Spectral analysis of BP and HR

Spectral analysis of SBP, DBP and HR based on fast Fourier transformation, was performed after linear de-trending, nine-point Hanning window filtering and resampling at 20Hz¹⁷. Spectra were obtained on 30 overlapping 2048 points time series of SBP, DBP and HR. The BP (mmHg^2) and HR (bpm^2) spectral powers were calculated for the whole spectrum (0.0195–3Hz) and in the three frequency ranges: very low frequency (VLF, 0.0195–0.195 Hz),

low frequency (LF, 0.195–0.8Hz) and high frequency (HF, 0.8–3Hz). The LF SBP and LF DBP and LF/HF HR are recognized autonomic markers in both clinical^{18,19} and experimental practices²⁰

Blood and tissue collection for evaluation of plasma VP and mRNA expression of VP, V1aR and V1bR in the rat PVN

At the end of the last recording session rats were euthanized using guillotine (Harvard Apparatus, Holliston, Massachusetts, USA) for fast decapitation. The blood was collected from the trunk in chilled plastic tubes with heparin and the brains were removed and snap frozen with powdered dry ice. Plasma was obtained after centrifugation (20 min, 1600 g, 4°C) and stored at -80 °C for up to six months before the hormone extraction and lyophilisation. Lyophilised plasma was stored at -20 °C for six months until the radioimmunoassay.

The specific antibody for VP radioimmunoassay was obtained from Peninsula (T4561, San Carlos, CA, USA). The VP extraction and measurements were performed by using the specific radioimmunoassay techniques that were previously described by Husain *et al*²¹. 1973. The VP measurement was performed in duplicate. The radioimmunoassay sensitivity range was from 0.4 to 32.0 pg/ml and intra- and inter-assay coefficients of variation were 7.9–9.75%, respectively.

By using a cryostat (Leica Microsystems CM1900, Leica Microsystems, Nussloch GmbH, Nussloch, Germany), 60 µm caudal-rostral brain slices were taken and stained with Toluidine blue (1% in 70% ethanol, Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) for mapping and verification of the hypothalamus²². Both left and right PVN unstained tissue were punched taking 1 mm in diameter, using a 15-G Sample Corer (Fine Science Tools Inc., Foster City, CA, USA, cat.no 18035-01), and stored in RNase-free tubes at -80°C.

1 *RNA extraction*

2 Tissue samples were homogenized in 1 mL TRIzol reagent (Invitrogen, Thermo Fisher
3 Scientific, Waltham, MA USA) by vortexing for 30 s. Samples were centrifuged at 12000 rpm
4 for 12 minutes at 4°C. Supernatants were aspirated by pipette into new tubes, while the pellet
5 of cellular debris was discarded. Extraction was performed with 200 µL chloroform (Sigma-
6 Aldrich Co. Ltd.), added to each sample followed by vortexing. After 2-3 minutes of standing
7 at room temperature, samples were centrifuged for 15 min, at 12 000 rpm, at 4°C. Aqueous
8 upper phase, containing the RNA were decanted into fresh tubes; without disturbing the
9 interphase or lower organic phase in which both the DNA and proteins were located. Total
10 RNA from aqueous phase was then precipitated with 1 volume (~350 µL) of ethanol (EtOH
11 100% v/v), and purified using the RNeasy Mini Kit (Qiagen, Qiagen Ltd., Manchester, UK)
12 according to manufacturer's protocol.

14 *cDNA synthesis*

15 cDNA synthesis was performed using QuantiTect Reverse Transcription Kit (Qiagen,
16 Qiagen Ltd., Manchester, UK) using 100 ng of input RNA. cDNA samples were then diluted
17 to a concentration of 2 ng·µL⁻¹ for use in real time qPCR.

19 *Real time quantitative polymerase chain reaction (qPCR) and data analysis*

20 Primers for the housekeeping gene RPL19 (Ribosomal protein L19: 5'-
21 GCGTCTGCAGCCATGAGTA-3' and 5'-TGGCATTGGCGATTTCGTTG-3') were used to
22 allow normalization of receptor expression between samples. Other primers for rat genes used
23 in this study were VP (5'-TGCCTGCTACTTCCAGAACTGC-3' and 5'-
24 AGGGGAGACACTGTCTCAGCTC-3') synthesized by Eurofins MWG Synthesis GmbH,
25 Ebersberg, Germany, V1aR (Rn Avpr1a 1 SG QuantiTect Primer Assay, QT00402990,NM

053019) obtained from Qiagen (Qiagen, Qiagen Ltd., Manchester, UK), V1bR (5'-TGCCACATTCCTGGAGTACCT-3', 5'-AGGACGGTTAACCAAGTAG TGAGATG-3') provided by Invitrogen. All primers were designed using GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). Analysis was performed using an Applied Biosystems ViiA7 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and processed using ViiA 7 Software v1.1 (Applied Biosystems, Foster City, CA, USA). The Δ cycle threshold (ΔC_t = sample gene C_t – housekeeper gene C_t) was calculated for each sample and then exponentiation was applied to each sample (Power 2).

Drugs

Ketamine (Ketamidor), xylazine (Xylased®) and carprofen (Rimadyl®) were purchased from Marlofarma (RS). Gentamicin injections were obtained from Hemofarm (Vršac, RS).

Statistics

Results are presented as mean value $\pm$ standard error of the mean. BP, HR, spectral and baroreceptor reflex parameters statistical differences between WR and BHR strain were estimated using Student's T-test (two-sample T-test) in SPSS Statistics v.20 software (IBM Corporation, NY, USA). qPCR data was analysed by One way ANOVA followed by Tukey post hoc, while data collected during the follow-up period were assessed by ANOVA for repeated measures within experimental groups in SPSS Statistics v.20 software. Mixed Effect Regression model was used to compare data between different experimental groups overtime in R-3.5.0 software (The R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was considered at $p < 0.05$.

1 RESULTS

2 *Plasma VP and expression of the VP, V1aR and V1bR genes in the PVN of WR and BHR under* 3 *baseline conditions and following saline loading with or without stress*

4 Plasma VP was increased in BHRs under baseline physiological conditions in respect
5 to WRs. (Figure 1). BHRs exhibited a three-to-four-fold increase of VP and V1bR mRNA
6 expression levels in the PVN as compared to WRs while V1aR mRNA levels did not change
7 (Figure 1).

8 Exposure of BHRs to saline load did not affect plasma VP concentrations nor VP and
9 V1aR mRNA levels, but reduced V1bR mRNA levels with respect to BHR controls kept under
10 baseline conditions (Figure 1). Exposure of salt loaded BHRs to heterotypic stressors decreased
11 plasma VP concentration compared to BHR controls and salt loaded BHRs. In stressed rats VP,
12 V1aR and V1bR mRNA levels did not change and were comparable to BHR controls (Figure
13 1).

15 *BP, HR, their short-term variability and the baroreceptor reflex in BHRs and WRs under* 16 *baseline conditions*

17 Under baseline conditions, BHRs exhibited higher SBP, DBP and lower HR values
18 compared to WRs (Table 1). Spectral analysis of BP and HR short-term variability revealed no
19 differences between the experimental groups (Figure 2). BRS and OR of BHRs were increased
20 with respect to WRs under baseline physiological conditions and the baro-receptor reflex was
21 re-set toward higher SBP values (Table 1).

BP, HR, their short-term variability and BRS in saline loaded BHRs with or without exposure to stress

Saline load alone did not affect SBP and DBP, but it decreased HR in respect to baseline values. In these rats BRS did not change overtime in respect to baseline values and in respect to BHR controls (Figure 3). Saline loaded BHRs exposed to stress showed a statistically significant increase of SBP over time with respect to baseline values and BHRs controls. DBP increase did not reach statistical significance. In these rats, BRS also increased whilst HR decreased over time in respect to baseline conditions (Figure 3).

In saline loaded BHRs autonomic spectral markers indicate a reduction of sympathetic influences on the blood vessels and the heart: LF-SBP and LF-DBP decreased over time with respect to baseline conditions, and LF-HR and LF/HF-HR were lower compared to BHR controls. In contrast, autonomic spectral markers LF-SBP and LF-DBP and LF/HF-HR were enhanced in saline loaded and stressed BHRs with respect to BHR controls (Figure 4).

1 DISSCUSION

2   For the first time to our knowledge, we have described strain specific differences in VP
3 and its receptors mRNA within the PVN of BHRs compared with normotensive controls. Under
4 baseline conditions, BHRs exhibit elevated mean values of SBP, DBP, reduced HR and
5 increased BRS, operating range and baroreceptor reflex resetting with respect to control WR.
6 In BHRs qPCR analysis of the PVN revealed increased V1bR and VP gene expression and VP
7 plasma concentration was increased. Saline load did not affect BP, BRS, plasma VP and VP
8 gene expression in BHRs. However, V1bR gene expression decreased. When BHRs were
9 exposed to saline load plus stress they developed overt hypertension and had decreased plasma
10 VP. In these rats spectral markers reflecting sympathetic hyperactivity to blood vessels (LF-
11 BP) and the heart (LF/HF HR) were enhanced. Neither VP nor VP receptor gene expression
12 in the PVN were changed in these rats.

13   The BHR is the first filial offspring of crosses between SHR and normotensive Wistar
14 rats and thus possesses genetic information from both the normotensive and the hypertensive
15 parent. The aetiology of borderline hypertension is not well understood and is different from
16 genetic hypertension (SHR). Our present results suggest the involvement of VP in the
17 pathogenesis of borderline hypertension. It has been documented previously that VP both
18 centrally and peripherally contributes, but does not play a key role in the pathogenesis of
19 genetic hypertension²³⁻²⁸. It has also been suggested that increased expression of VP mRNA
20 in the PVN and SON is a constitutive trait of SHR, found in the pre-hypertensive stage in the
21 SHR²⁸. VP released from the magnocellular division of the PVN into the systemic circulation
22 targets V2Rs in the collecting ducts of the kidneys to conserve water²⁹, and V1aRs in blood
23 vessels to increase peripheral resistance. These effects of VP are important for support of the
24 circulation and are lifesaving under hypovolemic and hypotensive conditions³⁰⁻³². At a molar

level, VP has been shown to be the most potent vasoconstrictor³³. However, when applied systemically to normovolemic and normotensive rats, VP only moderately increases BP because it increases the BRS and more effectively decreases HR, which then more efficiently opposes increased peripheral resistance³⁴. The effect of VP on the BRS was found to be mediated via the area postrema, a structure lacking a blood brain barrier, which is abundantly connected to the nucleus of the solitary tract (NTS), where baro-receptor reflex afferents terminate³⁵. There is pharmacological evidence that both types of VP receptors, V1a and V2, are involved in BRS modulation in the rat area postrema³⁶⁻³⁸. Thus, in present experiments, increased expression of the VP mRNA in the PVN of BHR rats may result in increased synthesis and release of VP into the blood. This may increase peripheral resistance and at the same time sensitize the baro-receptor reflex to reduce HR more effectively and keep BP in the borderline range. It is of interest to emphasize here that the baro-receptor reflex is one of the key controllers of VP release³⁹. However, in BHRs exhibiting chronically increased BP the baro-receptor reflex fails to suppress VP release into the circulation regardless increased sensitivity. This could be due to the resetting of the baro-receptor reflex to higher BP values and no changes in BEI (Table 1). The concomitant increase of BRS and of baro-receptor reflex operating range in borderline hypertension may lead to its exhaustion, when exposed to environmental stressors.

Our experiments suggest that vasopressin V1bR could be involved in the autocrine regulation of VP release in BHRs under baseline physiological conditions. V1bRs are abundantly expressed in the PVN and are co-localized with V1aR in VP secreting neurons, in both magnocellular and parvocellular divisions^{6,40}. Gouzènes *et al*⁶ provided evidence *in vivo* that VP released somato-dendritically by exocytosis, regularizes the firing pattern of MCNs to best fit the axonal release of VP to physiological demand. Vesicles containing VP prepared for somatodendritic release are positive for both V1aR and V1bR receptors. A number of reports

mention V1aR in autocrine regulation of VP release ⁴¹. In rats, Hurbin *et al* ⁴¹ have shown that dehydration up-regulates the V1aR mRNA in MCNs and V1aR receptor content in exocytotic granules. They also noted a down regulation of V1aR mRNAs in MCNs, and V1aR receptor content in exocytotic granules during water over-load. Son and colleagues ⁸ further demonstrated that V1aRs located on neurons in the parvocellular division of PVN are stimulated by VP released by MCNs in the magnocellular division to provide an integrated humoral and neurogenic response to osmotic challenge. Ribeiro and colleagues ⁹ observed that V1aRs in the PVN are involved in the mediation of sympatho-excitation during salt load in normotensive rats. Lozić and coworkers ¹⁰ investigated the role of V1aRs in the PVN during stress and showed that they reduce BRS under baseline physiological conditions, and increase the neurocardiogenic stress response. Our present results do not indicate a change in V1aR expression in the PVN of BHRs under baseline conditions, when exposed to saline load and when exposed to saline load plus stress. Rather, our results point to increased expression of V1bR as a possible mechanism involved in autocrine positive feed-back control of VP release in BHR.

In the parvocellular division of the PVN, VP is expressed in a number of neurons that project to the brainstem vasomotor centre in the RVLM and to pre-ganglionic sympathetic neurons located in the IML column of the spinal cord, where V1aRs are found ⁵. In clinical and experimental practice, sympathetic outflow to the blood vessels and the heart can be assessed indirectly by autonomic spectral markers ²⁰. In the present experiments no changes in LF-BP and LF/HF HR spectral markers were observed under baseline conditions in BHRs (Figure 2), suggesting that increased expression of VP and V1bR in the PVN occurs in the magnocellular division and does not alter baseline neurogenic cardiovascular control in BHRs.

The concept that hypertension derives from an interaction between genetic and environmental factors was first proposed by Folkow ⁴². Of the environmental factors, dietary

sodium intake and environmental (psychosocial) stress are considered important. DiBona and Rios ⁴³ have shown that intravenous isotonic saline infusion exaggerates natriuresis in SHR due to increased renal sympathetic nerve activity (RSNA) withdrawal. In SHR and Dahl rats Koepke and colleagues ^{44,45} have further shown that increased dietary sodium intake augments efferent RSNA in response to environmental stress leading to greater renal vasoconstriction and profound antidiuretic and antinatriuretic response. They have also shown that BHR rats exposed to high sodium intake have a response similar to SHR rats, whilst BHRs exposed to low sodium intake (up to 1% w/v) does not and respond like normotensive controls ⁴⁶. This is in line with our findings that BHRs drinking saline (corresponding to low sodium intake) exhibited sympathetic withdrawal, as documented indirectly by LF-BP spectral decrease, allowing effective natriuresis, as in normotensive rats. A decrease in V1bR in the PVN of salt loaded BHRs also suggests removal of positive feed-back autocrine control on VP synthesis/release. Altogether, these data exclude the possibility that hydromineral imbalance occurs in BHR exposed to saline load contributing to BP dysregulation.

In present experiments, BHRs drinking isotonic saline and exposed to heterotypic stressors for 24 weeks showed higher BP values with respect to baseline conditions and BHR controls throughout the observation period. The lack of statistical significance in DBP increase could be due to improved BP control by baro-receptor reflex since BRS increased over time, though partial habituation cannot be excluded. Compared to BHR controls, SBP, LF-SBP, LF-DBP and LF/HF-HR constantly increased to reach highest values by the end of the stress protocol. An increase of LF-BP and LF/HF HR in present experiments indicates increased sympathetic outflow respectively to the blood vessels ^{47,48} and the heart ⁴⁸⁻⁵¹ suggesting that this is the mechanism underlying stress-induced systolic hypertension. The possibility that autonomic spectral markers reflect increased sensitivity of peripheral vasculature and cardiac

tissue to catecholamines is unlikely since saline load below 1% NaCl has been shown not to modulate cardiovascular tissue reactivity to catecholamines in the BHR ⁵².

Our results do not support a role of peripheral VP in stress-induced hypertension in BHRs. Stress did not affect the level of expression of V1bR and VP in the PVN in respect to baseline conditions. Moreover, in BHRs exposed to saline load and stress, there was a decrease in plasma VP. This is not an unexpected finding since stressors provoking strong emotional reactions have been reported to inhibit systemic VP secretion ⁵³. In our experiments, BHRs were exposed to air-jet stress that induces startle and a panic reaction. We also changed the housing of BHRs and introduced another emotional, novelty paradigm. The present experiments show that the combination of these two paradigms reduces VP secretion without affecting its mRNA steady-state abundance. Nonetheless we cannot exclude the possibility that stress-induced hypertension also suppressed VP release. It is of note to mention that concomitant saline load with stress blunts the HPA axis responsiveness to stress ⁵⁴.

The present study has a limitation that needs to be addressed. The main drawback is the lack of data on V1aR and V1bR receptor protein levels in the PVN of both WR and BHRs under different experimental conditions. V1aR and V1bR are G protein coupled receptors (GPCRs) and as such they are important regulators of cellular function. As GPCR mRNA level and protein do not necessarily correlate, detection at protein level is important. GPCRs can be identified either using specific radioligands or antibodies. However, suitable radioligands for V1aR and V1bR are missing, while the selectivity of antibodies is doubtful due to great structural homology (up to 85 %) between V1aR, V1bR and oxytocin receptors ⁵⁵, all found in the PVN of rats. The lack of antibody selectivity to vasopressin and oxytocin receptors is not an exception, but rather a rule for GPCR antibodies ⁵⁶. And a number of reports discourage the use of antibodies for GPCR protein detection ⁵⁷⁻⁶². At present state of the art, it seems that the expression of mRNA is

1 the only reliable approach for GPCRs assessment. Thus, we have used this technology to
2 quantify changes in the expression of the V1aR and V1bR receptors at the level of steady-
3 state mRNA abundance (see Figure 1).

4 V1aR and V1bR cell surface density is dynamic and changes under different
5 physiological conditions due to either changes in mRNA expression or receptor
6 internalization involving β -arrestin or clathrin-coated vesicles, marking the end of
7 peptide action ⁶³. Internalized receptors have different fates. They can undergo
8 degradation and reduces protein level or recycle to the cell surface without affecting the
9 protein level. V1aR have been shown to recycle rapidly, and V1bR have been reported to
10 be preponderantly located in the cytoplasm even unstimulated ⁶⁴, and none are being
11 degraded following internalization. Therefore, we believe that it is unlikely that the
12 steady-state abundance of the V1aR and V1bR is not related to mRNA level in this study.

13 In conclusion, the present results show, for the first time, that over-expression of VP
14 and V1bR genes in the PVN is a characteristic trait of the borderline hypertension in the rat.
15 Overexpression of VP and of V1bRs in the PVN does not seem to modulate neurogenic control
16 of the circulation in BHRs under baseline conditions, but is associated with increased plasma
17 VP, that may act at the same time to increases peripheral resistance and to increase BRS
18 preserving BP at borderline level. Exposure of BHRs to prolonged isotonic saline load and
19 stress increased sympathetic outflow to blood vessels and the heart inducing overt
20 hypertension. At the same time plasma VP decreased and the expression of VP, V1bR and
21 V1aR genes in the PVN was unaffected. This suggests that sympathetic hyperactivity and not
22 peripherally released VP underlies stress-induced hypertension in BHRs.

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8   **AUTORS CONTRIBUTIONS**

9   All authors contributed to design, performance, analysis, reporting and writing of the
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11   **CONFLICT OF INTEREST**

12   Authors declare no conflict of interest

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Figure 1. Plasma VP and VP, V1aR and V1bR in the PVN of WR and BHR under baseline conditions and following saline loading with or without stress. Note the increase of plasma VP, VP and V1bR gene expression in the PVN of BHRs under baseline conditions with respect to WRs. Saline decreased V1bR gene expression. Adding stress reduced plasma VP without affecting VP, V1aR and v1bR gene expression. In this and the following figures WR-Wistar rats; BHR-borderline hypertensive rats; VP-vasopressin; V1aR-vasopressin receptor type 1a; V1bR-vasopressin receptor type 1b. Values are mean of at least six rats $\pm$ s.e.m.. * $p < 0.05$; ** $p < 0.01$ vs. WR baseline; † $p < 0.05$; †† $p < 0.01$ vs. BHR baseline; ‡‡ $p < 0.01$ vs. BHR saline (One-Way ANOVA followed by *post hoc* Tukey).

Figure 2. Autonomic spectral markers in BHRs and WRs under baseline conditions. In this and the following figures LF SBP-low frequency systolic blood pressure spectral component; LF DBP-low frequency diastolic blood pressure spectral component; LF HR-low frequency heart rate spectral component; LF/HF HR-low frequency to high frequency heart rate ratio. Values are mean of at least six experiments $\pm$ s.e.m. (Student's T-test).

Figure 3. BP, HR and BRS in BHRs exposed to saline load without or with stress. Mark the increase in SBP and BRS in BHRs exposed to saline solution plus heterotypic stress. HR was decreased in BHRs drinking saline with or without exposure to stress. Values are mean of at least seven rats $\pm$ s.e.m.. * $p < 0.05$, *** $p < 0.001$ (ANOVA for repeated measures) vs. baseline; † $p < 0.05$ vs. control BHR group (Hierarchical linear regression).

1 **Figure 4. Autonomic spectral markers in BHRs exposed to saline load without or with**
2 **stress.** In saline loaded BHRs without exposure to stress LF SBP and LF DBP decreased over
3 time. LF HR and LF/HF HR were lower compared to BHR controls. Note that LF SBP, LF
4 DBP and LF/HF HR are higher in saline loaded plus stressed BHRs compared to BHR controls.
5 Values are mean of at least seven rats $\pm$ s.e.m.. * $p < 0.05$ (ANOVA for repeated measures) vs.
6 baseline; † $p < 0.05$; †† $p < 0.01$ vs. control BHR group (Hierarchical linear regression).

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Table 1. BP, HR and baroreceptor reflex in BHRs and WRs under baseline conditions

	WR	BHR
SBP (mmHg)	115 ± 3	134 ± 3 **
DBP (mmHg)	82 ± 2	99 ± 2 **
HR (bpm)	321 ± 12	269 ± 6 ***
BRS (mmHg·ms)	1.1 ± 0.1	1.6 ± 0.2*
BEI	0.6 ± 0.03	0.63 ± 0.02
OR (ms·mmHg)	114.6 ± 36.5	205 ± 2.0 *
SP (mmHg)	105.1± 3.2	123.6±3.2**

Values are mean of experiments ± s.e.m.. SBP-systolic blood pressure; DBP-diastolic blood pressure; HR-heart rate; BRS-baro-receptor reflex sensitivity. BEI-baro-receptor effectiveness index; OR-operating range; SP-set point. **p<0.01, *** p<0.001 vs. WR.

Figure 1. Plasma VP and VP, V1aR and V1bR in the PVN of WR and BHR under baseline conditions and following saline loading with or without stress. Note the increase of plasma VP, VP and V1bR gene expression in the PVN of BHRs under baseline conditions with respect to WRs. Saline decreased V1bR gene expression. Adding stress reduced plasma VP without affecting VP, V1aR and v1bR gene expression. In this and the following figures WR-Wistar rats; BHR-borderline hypertensive rats; VP-vasopressin; V1aR-vasopressin receptor type 1a; V1bR-vasopressin receptor type 1b. Values are mean of at least six rats $\pm$ s.e.m.. * $p < 0.05$; ** $p < 0.01$ vs. WR baseline; † $p < 0.05$; †† $p < 0.01$ vs. BHR baseline; ††† $p < 0.01$ vs. BHR saline (One-Way ANOVA followed by post hoc Tukey).

184x147mm (300 x 300 DPI)

Figure 2. Autonomic spectral markers in BHRs and WRs under baseline conditions. In this and the following figures LF SBP-low frequency systolic blood pressure spectral component; LF DBP-low frequency diastolic blood pressure spectral component; LF HR-low frequency heart rate spectral component; LF/HF HR-low frequency to high frequency heart rate ratio. Values are mean of at least six experiments $\pm$ s.e.m. (Student's T-test).

189x146mm (300 x 300 DPI)

Figure 3. BP, HR and BRS in BHRs exposed to saline load without or with stress. Mark the increase in SBP and BRS in BHRs exposed to saline solution plus heterotypic stress. HR was decreased in BHRs drinking saline with or without exposure to stress. Values are mean of at least seven rats $\pm$ s.e.m.. * $p < 0.05$, *** $p < 0.001$ (ANOVA for repeated measures) vs. baseline; † $p < 0.05$ vs. control BHR group (Hierarchical linear regression).

271x183mm (300 x 300 DPI)

Figure 4. Autonomic spectral markers in BHRs exposed to saline load without or with stress. In saline loaded BHRs without exposure to stress LF SBP and LF DBP decreased over time. LF HR and LF/HF HR were lower compared to BHR controls. Note that LF SBP, LF DBP and LF/HF HR are higher in saline loaded plus stressed BHRs compared to BHR controls. Values are mean of at least seven rats $\pm$ s.e.m.. *p<0.05 (ANOVA for repeated measures) vs. baseline; †p<0.05; ††p<0.01 vs. control BHR group (Hierarchical linear regression).

308x195mm (300 x 300 DPI)