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OVER-EXPRESSION OF V1A RECEPTORS IN PVN MODULATES AUTONOMIC CARDIOVASCULAR CONTROL

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Abstract

The hypothalamic paraventricular nucleus (PVN) is a key integrative site for the neuroendocrine control of the circulation and of the stress response. It is also a major source of the neuropeptide hormone vasopressin (VP), and co-expresses V1a receptors (V1aR). We thus sought to investigate the role of V1aR in PVN in cardiovascular control in response to stress. Experiments were performed in male Wistar rats equipped with radiotelemetric device. The right PVN was transfected with adenoviral vectors (Ads) engineered to over-express V1aR along with an enhanced green fluorescent protein (eGFP) tag. Control groups were PVN transfected with Ads expressing eGFP alone, or wild-type rats (Wt). Rats were recorded with and without selective blockade of V1aR (V1aRX) in PVN under both baseline and stressed conditions. Blood pressure (BP), heart rate (HR), their short-term variabilities, and baroreflex sensitivity (BRS) were evaluated using spectral analysis and the sequence method, respectively. Under baseline physiological conditions,V1aR rats exhibited reduced BRS and a marked increase of BP and HR variability during exposure to stress. These effects were all prevented by V1aRX pretreatment. In Wt rats, V1aRX did not modify cardiovascular parameters under baseline conditions, and prevented BP variability increase by stress. However, V1aRX pretreatment did not modify baroreflex desensitization by stress in either rat strain. It follows that increased expression of V1aR in PVN influences autonomic cardiovascular regulation and demarcates vulnerability to stress. We thus suggest a possible role of hypothalamic V1aR in cardiovascular pathology.

Keywords

Vasopressin, V1a receptor, paraventricular nucleus, adenoviral vector, baroreflex, blood pressure variability, heart rate variability

Abbreviations

BRS, baroreflex sensitivity; VLF, very low frequency short-term variability; LF, low frequency short-term variability; HF, high frequency short-term variability; VP, vasopressin; OT, oxytocin; OTR, oxytocin receptor; V1aR, vasopressin V1a receptor; V1bR, vasopressin V1b receptor; V2R, vasopressin V2 receptor; V1aRX, vasopressin V1a receptor antagonist, PVN, paraventricular nucleus; NTS, nucleus of the solitary tract; RVLM; rostroventrolateral medulla; IML, intermediolateral column of the spinal cord.

1. Introduction

It is well established that vasopressin (**VP**) is an important neuropeptide in cardiovascular homeostasis, both as a hormone and as a neurotransmitter / modulator (Japundžić-Žigon, 2013). VP is mainly synthesized in the paraventricular (**PVN**) and supraoptic (**SON**) nuclei of the hypothalamus. Neurosecretory neurons of SON and of the magnocellular part of the PVN project to neurohypophysis wherefrom VP is released in the systemic circulation, to act as a hormone in hydromineral homeostasis and blood pressure control (Swanson and Sawchenko, 1983; Burbach *et al*., 2001). Axons of VP containing neurons of the parvocellular part of the PVN project to eminentia mediana (Rivier and Vale, 1983; Herman and Cullinan, 1997), the limbic system and amygdala, and to the brainstem and spinal cord where VP acts as a neurotransmitter / modulator influencing, respectively, ACTH release, emotions and autonomic functions (Sawchenko and Swanson, 1982; Geerling *et al*., 2010). Although VP containing neurons in PVN that participate in neuroendocrine and autonomic cardiovascular control are anatomically segregated, they act in concert in response to physiological challenges requiring a multimodal homeostatic response. This neuronal coordination, at least for hyperosmotic challenges, has been shown to involve intranuclear, somato-dendritic release of VP and activation of V1a receptors on somata and dendrites of pre-sympathetic neurons of the PVN (Stern, 2001; Son et al., 2013; Stern, 2015).

Emotional stress is another challenge that demands complex behavioral reaction requiring coordinated adjustments of neuroendocrine and autonomic responses. We have previously reported that central V1a receptors are important in the modulation of the cardiovascular response to stress (Milutinović *et al*., 2006; Stojičić*et al*., 2008; Milutinović-Smiljanić *et al*., 2013). Here we investigate the role of PVN V1a receptors in autonomic adjustment of the cardiovascular system to emotional stress. We hypothesized that, by increasing the number of V1aR in PVN or by selectively blocking their activity, we could alter autonomic cardiovascular control and, even more importantly, influence the stress response. The findings show for the first time that over-expression of V1aR in PVN demarcates a rat phenotype vulnerable to emotional stress.

2. Methods

All experimental procedures in this study conformed to European Communities Council Directive of November 24, 1986 (86/609/EEC) and comply with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). The experimental protocol was approved by the Faculty of Medicine University of Belgrade Ethics review board.

2.1. Animals

Twelve week old male Wistar rats (280-320g) bred at the local animal facility were used in the experiments. Rats were housed individually in a controlled environment (12h/12h light dark-cycle, temperature 21±2°C and humidity 60± 5%) with access to standard pelleted chows (0.2 % w/v sodium content, Veterinarski zavod, Subotica) and tap water *ad libitum*. The number of rats in each protocol was calculated statistically taking into account intra-group variability, using the 'Power Sample Size Calculation' software freely available at: http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize for power of 90% and type I error probability of 0.05. At the end of the experiment, the rats were euthanized using injection of three anesthetics (0.1ml, i.p. of T61® solution).

2.2. Surgery

Under combined ketamine (100 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.) anesthesia rats underwent two surgical procedures at ten days interval. A 3 cm-long medial abdominal incision was made and the intestine retracted to expose the abdominal aorta. The tip of the catheter of the radiotelemetric probe (TA11-PA C40, DSI, St. Paul, MN, USA) was inserted into the abdominal aorta using a 21G needle. The inserted catheter was fixed with 3M Vetbond™ and tissue cellulose patch (DSI, St. Paul, MN, USA). The transmitter was attached to the anterior abdominal wall and the wound was closed by suture. In order to prevent infection, neomycin and bacitracin were sprayed topically, and the rats received gentamicin parenterally (25 mg/kg i.m.) three days before, and again on the day of surgery. Pain was reduced by carprofen (5 mg/kg/day, s.c.) on the day of surgery and for the next two days. Each rat was housed in a Plexiglas cage (30 cm x 30 cm x 30 cm) and left to recover fully for 10 days.

The second surgery was performed in rats under the same combined anesthesia and postoperative care protocol. Rats head was mounted in stereotaxic frame and the skin was incised 3 mm to expose the scull. A hole was opened with dental drill to position 23G guide above right PVN (AP = 1.8 mm caudal from bregma, LAT = 0.4 mm from midline, 6.5 mm beneath the scull; Paxinos and Watson, 2005) and fixed with dental cement. On the day of experimentation 7.5 mm-long 30G needle was used for microinfusion of drugs into the PVN. At the end of experiment, rats' brain was removed and dissected at microinfusion site. The 5 μm sections were then dried and stained with cresyl violet acetate (0.1% w/v) and cover-slipped with DPX mountant (VWR International Ltd, Lutterworth, UK).

2.3. Adenoviral vector production

The cDNA clone of the rat V1aR in pcD2 was generously provided by Dr. Stephen Lolait, University of Bristol (Morel *et al*., 1992). The V1aR sequences were amplified from pcD2 using Phusion High-Fidelity DNA polymerase (New England Biolabs Ltd, Hitchin, UK) and primers (5'-GCCTCGAGGGCTCTGTACGGACA-3') and (5'-CTGGATCCAAAAGTCCCTCCCAAGAGTC-3'). The PCR product was digested with XhoI and BamHI and ligated into compatible restriction sites of adenoviral vector pacAd5.CMV.IRES.GFP (Cambridge Bioscience Ltd., Cambridge, UK). Adenoviral vector pacAd5.CMV.GFP was used as a control (eGFP). The adenoviruses were generated by co-transfection of viral shuttle and backbone (pacAd5 9.2-100) vectors in HEK293T cells by calcium phosphate method in accordance with manufacturer's guidelines (Cambridge Bioscience Ltd., Cambridge, UK). Adenoviruses were purified by two rounds of CsCl ultracentrifugation and desalted using Slide-A-Lyzer dialysis cassettes (Pierce). The purified viruses were aliquoted and stored at -80^{o}C. The virus titers were determined in triplicate by standard plaque assay.

2.4. Transfection

Ten days after fitting the telemetry device, injection of Ads into the right PVN of rats was performed under combined ketamine xylazine anesthesia. The head of the rat was mounted in the stereotaxic frame and the skin was incised 3 mm to expose the scull. The stereotaxic coordinates of PVN (AP = 1.8 mm caudal from bregma, LAT = 0.4 mm from midline) were derived from the rat brain atlas (Paxinos and Watson, 2005). A glass micropipette was slowly positioned at 7.6 mm beneath the skull for infusion of virus (titer $4 \cdot 10^{10}$ pfu/ml) in 50 nl, pressure injected in one minute. In sham rats, a glass micropipette was slowly positioned at 7.6 mm beneath the skull. After removal of micropipette a chronic guide cannula was positioned as described in the section 2.2 surgery. Rats were left to recover for seven days. This time is necessary for maximal expression of transfected gene (Lonergan T *et al*., 2005).

2.5. Tissue preparation & collection

Hypothalamic PVN were identified using Toluidine blue (Sigma Aldrich; Sigma Aldrich Company LTD, Poole, Dorset, UK; 0.1% in 70% EtOH) staining, in conjunction with

a brain map (Paxinos and Watson, 2005) for reference. Following identification, 60 µm caudal-rostral slices were taken from PVN using a cryostat (Leica Microsystems CM1900, Leica Microsystems Nussloch GmbH, Nussloch, Germany) maintained between -18ºC and -20ºC. Bilateral tissue punches of Left and Right PVN were obtained using a micro-punch (1mm diameter; Item Number. 18035-01, 15G, Fine Science Tools (USA) Inc., Foster City, CA, USA) and stored in RNase-free microcentrifuge tube on dry ice, or at -80ºC, until extraction.

2.6. RNA extraction

To each sample tube, 1ml TRIzolLysis Reagent (Life Technologies, Paisley, UK; Cat No. 15596-018) was added and samples were mixed by vortexing for 10 s. Samples were then allowed to stand at room temperature for 5min prior to centrifugation (10300 rpm, 4ºC) for 10min, in order to pellet any cellular debris. The resulting supernatant was collected, and added into a new microcentrifuge tube containing200 µl of chloroform with amylenes as stabilizer ≥ 99% (v/v) (Sigma Aldrich). Samples were then mixed by vortex for 20 s and then allowed to stand for 5 min at room temperature. To separate organic and aqueous phases, samples were centrifuged for 15min (11200 rpm, 4ºC), and the aqueous phase (350 µl from approx. total 500 µl) was collected and added into a new microcentrifuge tube containing1volume (350 µl) of 70% (v/v) EtOH, in order to precipitate total RNA. Further purification was performed via the use of the RNeasy Mini Kit according to manufacturer's protocol (Qiagen; Qiagen LTD., Manchester, UK; Cat No. 74104). Purified RNA was then quantitatified using an Implen Gene flow Nanophotometer, and then stored at - 20ºC until cDNA synthesis.

2.7. cDNA synthesis

Using a QuantiTect Reverse Transcription Kit (Qiagen, Cat No. 205313), 100 ng RNA was reverse transcribed to produce cDNA, which was then diluted to a concentration of 2ng/µl for use in qPCR.

2.8. RT-qPCR& Expression Analysis

Primers for the housekeeping gene Rpl19 (Ribosomal protein L19 - Fwd: GCGTCTGCAGCCATGAGTA, Rev: TGGCATTGGCGATTTCGTTG) & eGFP (enhanced Green fluorescent protein – Fwd: ATCATGGCCGACAAGCAGAAGAAC Rev: GTACAGCTCGTCCATGCCGAGAGT) were obtained online from Eurofins MWG Operon

(Eurofins MWG Synthesis GmbH., Ebersberg, Germany; http://www.eurofinsgenomics.eu/), with primer for V1aR (Rn_Avpr1a_1_SG QuantiTect Primer Assay, QT00402990, NM_053019) obtained from Qiagen. Expression via qPCR was analyzed for all genes on a 96 well PCR plate (MicroAmp Fast 96-Well Reaction Plate (0.1 mL), Ref; 4346907, Applied Biosystems, Foster City, CA, USA), with each well containing; 2 ng of cDNA (1µl per well) along with 11 µl of Mastermix (Sybr Green (FastStart Universal Sybr Green Master (with ROX), Roche Diagnostics - Ref. 04913914001), Forward & Reverse Primers and RNase-free water). All samples were run in duplicate. Following sample & Mastermix addition, the plate was covered with a clear adhesive seal (Micro Amp Optical Adhesive Film, Ref; 210404056, Applied Biosystems) and centrifuged for 30s to ensure proper mixing of reagents and to remove air bubbles. RT-qPCR analysis was performed using an Applied Biosystems ViiA^{TM} Real Time PCR System for High Resolution Melt experiments in conjunction with the ViiA^{TM} 7 Software v1.2. All qRT-PCR reactions were followed by dissociation curve analysis. Relative quantification of gene expression was performed using the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

All procedures were carried out in an RNase-free environment and all solutions made up using RNase-free water/reagents.

2.9. Tissue preparation and immunohistochemistry

At the end of experiments anesthetized rats were perfused transcardially with 150 ml of 0.1 M phosphate-buffered saline (PBS pH 7.4) followed by 300 ml of 4% (w/v) paraformaldehyde (PFA) in 0.1 M PBS. The brains were removed, post-fixed overnight in 4% paraformaldehyde followed by three-day-incubation in gradually increasing sucrose solutions (10-30% (w/v)) and frozen over liquid nitrogen. Free-floating coronal sections (35µm) of the forebrain were collected in 24-well tissue culture plates and washed in PBS (3x10min). Sections were then incubated in animal-free blocking solution (Vector Laboratories Ltd., Peterbourough, UK) for 30 minutes, washed in PBS (3 x 10minutes) and incubated in primary antibodies diluted in 0.1M PBS and 0.3% (v/v) Triton X-100 (Sigma-Aldrich Co. Ltd., Poole, Dorset, UK). Primary antibodies used were goat polyclonal anti-V1aR antibody (1:50, Santa-Cruz Biotechnology, Heidelberg, Germany catalogue number: sc-18096), mouse monoclonal anti-neuron nuclear antigen (NeuN) antibody (1:100) and mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (1:100) (both from Chemicon International, Temecula, CA, USA). Sections were incubated in primary antibodies for 48 hours at 4ºC and rinsed three times

in PBS (10 minutes each). After incubation in biotinylated anti-mouse IgG raised in horse (1:500, Vector Laboratories Ltd, Peterborough, UK, catalogue number: BA 2001) for one hour, sections were transferred in wells containing secondary donkey anti-goat CF594 antibody (1:500, Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) and streptavidin conjugated Alexa Fluor 647 (1:500, Invitrogen Ltd, Paisley UK) diluted in PBS containing 0.3% (v/v) Triton X-100 for one hour. Following further rinses (3 x 10 min), sections were mounted onto slides in 0.5% gelatin, air-dried and cover slipped with antifade mounting medium for fluorescence (Vectashield^{TM}, Vector Laboratories Ltd., Peterborough, UK). Images were observed using Zeiss Axioskop 20 fluorescent microscope. ImageJ software, freely available athttps://imagej.nih.gov/ij/download.html was used to observe colocalization of V1aR, eGFP, NeuN or GFAP.

2.10. Vasopressin dose response and V1aR antagonist dose determination

Experiments were performed to determine the selective dose of V1aX microinjected in PVN of conscious, freely moving rats (n=6). Following vehicle application (200 nL/min 0.9% w/v NaCl), increasing doses of VP (30 ng, 100 ng and 300 ng) in a volume of 200 nL were microinfused in PVN for 1 minute, at 2 hours interval. Arterial pulse pressure was recorded for 60 minutes after drug administration. Five days later, V1aRX and VP were co-administered in the PVN of rats to test their blocking efficacy.

2.11. Experimental design

All experiments started around 10 a.m. in quiet surrounding under controlled environmental conditions, in rats housed individually in Plexiglas cages (30 cm x 30 cm x 30 cm). Cardiovascular parameters were recorded for 20 minutes under baseline conditions and 10 minutes during exposure of rats to stress as well as during recovery. Stress was induced by directing air-jet (compressed in a bottle under 1 bar) to the top of rats' head avoiding the snout. Wild type rats were microinfused with 200 nL/min of pyrogen-free saline in the PVN (Wt group, n=6) or with 300 ng/200 nL of V1aR antagonist ($V1aRX_{Wt}$ group n=6); eGFP rats were not micoinfused in PVN (n=6) and rats over-expressing V1aR in PVN received either 200 nL/min saline (V1aR group, n=6) or 300 ng/200 nL of V1aR antagonist ($V1aRX_{V1aR}$ group, n=6).

2.12. Cardiovascular signal processing and analysis

Arterial blood pressure was digitalized at 1000 Hz in Dataquest A.R.T. 4.0 software, (DSI, St. Paul, MN, USA). Systolic BP (**SBP**), diastolic BP (**DBP**), mean BP (**MBP**) and pulse interval (**PI**) or its inverse, heart rate (**HR**), were derived from the arterial pulse pressure as maximum, minimum, integral of the arterial pulse pressure wave and inter-beat interval of the arterial pulse pressure wave, respectively. For each registration period mean value of SBP, MBP, DBP, HR and PI was calculated, and again averaged for the whole experimental group (values shown in tables and graphs).

2.13. Evaluation of the spontaneous baroreflex by the method of sequences.

The method is explained in details elsewhere (Bajić *et al*. 2010). Briefly, a spontaneous baroreflex sequence is a stream of consecutively increasing/decreasing SBP samples, followed by a stream of increasing/decreasing PI interval samples delayed by 3, 4 or 5 beats in respect to SBP. A threshold for sequence length was set to four beats (Lončar-Turukalo *et al*., 2011). The sensitivity of baroreflex [**BRS**, ms/mmHg] was assessed as a linear regression coefficient averaged over all identified sequences (PI=BRS·SBP+const, where fitting of the curve is done in a least square sense).

2.14. Spectral analysis of BP and HR

Before spectral analysis was performed, SBP, DBP and HR signals were re-sampled at 20 Hz and subjected to nine-point Hanning window filter and linear trend removal (Milutinović *et al,*. 2006; Stojičić *et al*., 2008). Spectra were obtained using a fast Fourier transform algorithm on 30 overlapping 2048 point time series involving in 410-s registration period of SBP, DBP and HR. The power spectrum of BP ($mmHg^2$) and HR (bpm^2) for 30 FFT segments was calculated for the whole spectrum (total volume, **TV**: 0.019-3 Hz) and in three frequency ranges: very low frequency (**VLF**: 0.019-0.2 Hz), low frequency (**LF**: 0.2-0.8 Hz) and high frequency (**HF**: 0.8-3 Hz) range. The LF oscillation of SBP and DBP spectrum (LF-SBP and LF-DBP) and the LF/HF ratio of the HR spectrum are recognized markers of sympathetic activity directed to blood vessels and the sympatho-vagal balance to the heart, respectively (Japundzic-Žigon, 1998).

2.15. Drugs

Vasopressin ([Arg8]-vasopressin acetate) was purchased from Sigma-Aldrich (Unichem Belgrade, RS) and dissolved in pyrogen-free saline. Selective vasopressin V1a receptor antagonist $d(CH_2)_5[Tyr(Me)^2,Dab^5]AVP$ was kindly donated by professor Maurice

Manning from the University of Toledo, Ohio, USA, and was dissolved in pyrogen-free saline (Manning et al., 2012). Ketamine (Ketamidor®), xylazine (Xylased®), carprofen (Rimadyl®) and combination of embutramide plus mebezonium plus tetracaine (T61®) injections were purchased from Marlo Farma (Belgrade, RS). Gentamicin injections (Gentamicin®) and bacitracin plus neomycin spray (Bivacyn®) were purchased from Hemofarm (Vršac, RS).

2.16. Statistics

Cardiovascular parameters are presented as mean ± standard error of the mean. Multiple comparisons between experimental groups were performed by ANOVA for repeated measures followed by post *hoc* Bonferroni test using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was considered at $p<0.05$.

3. Results

3.1. Verification of microinjection sites and Ads expression

The position of the micropipette and of the guide cannula in the PVN at the end of each experiment was verified histologically (Figure 1). The extent of virus spread was carefully examined and quantified. We observed that the dorsoventral, mediolateral and rostrocaudal extent of the injection covers a sphere of approximately 400-500 μm in diameter. The efficacy of Ad transduction and expression was verified by quantification of V1aR mRNA expression (Figure 2) and eGFP fluorescence (Figure 3B, 4B) at the site of transfection. We found that V1aRs are over-expressed in both neurons (Figure 3) and astrocytes (Figure 4).

3.2. Vasopressin dose response and V1aR antagonist dose determination

VP microinfused in PVN in a dose of 30 ng/200nL/min did not affect SBP, DBP, MBP and HR, while VP in doses of 100 ng / 200 nL/min and 300 ng / 200 nL/min induced statistically significant increases in SBP, DBP, MBP, and statistically significant decreases in HR that lasted up to 20 minutes (Table 1). Both the hypertensive and bradycardic responses induced by 100 ng of VP were prevented by co-administration of 300 ng/200nL/min V1aRX (Table 1).

3.3. Cardiovascular parameters in rats over-expressing V1aR in the PVN

Under baseline conditions, mean values of SBP, MBP, DBP, HR did not differ between sham injected Wt, eGFP and V1aR rats (Table 2). BRS was decreased in rats over-expressing V1aR in respect to sham injected Wt and eGFP control rats. Spectral analysis of BP and HR under basal physiological conditions revealed that SBP and DBP short-term variability (Figure

5), and HR short-term variability (Figure 6), were comparable between V1aR over-expressing rats, sham injected Wt rats and e-GFP rats.

Exposure of rats to stress increased mean values of SBP, MBP, DBP, HR and decreased sBRS in sham injected Wt and eGFP transfected rats (table 2). Rats over-expressing V1aR in PVN exposed to stress exhibited similar increases in SBP, DBP and MBP compared to controls, and sBRS was also decreased (Table 2). Total BP variability increased in all experimental groups of rats exposed to stress due to an increase in variability in the LF and HF spectral bands. The increase of HF-SBP and HF-DBP variability was more pronounced in rats over-expressing V1aR (Figure 5). In these rats, stress also induced a marked increase in LF-HR and HF-HR variability as well as in the LF/HF HR ratio (Figure 6).

3.5. Effect of V1aRX microinjected in the PVN on cardiovascular parameters of Wild type rats

Under baseline physiological condition, microinfusion of V1aRX into the PVN of conscious Wt rats had no effect on mean levels of SBP, DBP, MBP, HR (Table 3, their variabilities (Figure 7 and Figure 8),and on BRS (Table 3).

V1aRX pre-treatment of Wt rats did not modify the stress-induced increases in SBP, DBP, MBP, HR and baroreflex desensitization (table 3). Nonetheless, in these rats, LF-SBP and LF-DBP variability did not increase during stress, and stress also failed to enhance HF-SBP and HF-DBP variability (Figure 7). V1aRX pre-treatment did not modify HR variability in Wt rats exposed to stress (Figure 8).

3.6. Effect of V1aRX microinjected in the PVN on cardiovascular parameters of rats over-expressing V1aR

Under baseline physiological conditions, microinjection of V1aRX to rats over-expressing V1aR in PVN did not affect basal values of SBP, DBP, HR and their variabilities (Figure 7 and Figure 8). However, V1aRX restored the decrease in BRS observed under baseline conditions in rats over-expressing V1aR, but did not modify baroreflex desensitization by stress (Table 3).

Moreover, V1aRX pretreatment reduced the increases in LF-SBP, HF-SBP, LF-DBP, HF-DBP variabilities seen with stress (Figure 7), and prevented the increase in LF-HR variability and LF/HF HR ratio (Figure 8) observed in stressed rats over-expressing V1aR in the PVN.

4. Discussion

The present study shows, for the first time, that rats over-expressing V1aR under baseline physiological conditions exhibit reduced BRS and, when exposed to stress, they respond with a marked increase in both BP and HR variability. Our results also show that, under baseline physiological conditions, V1aRs in PVN do not modify cardiovascular short-term variability and BRS of Wt rats, but rather appear to mediate a stress-induced increase in BP variability. These findings suggest that a rat phenotype of increased expression of V1aR in the PVN demonstrate cardiovascular vulnerability compared to wild type controls.

Increased cardiovascular variability is a recognized risk factor for cardiovascular diseases (Lombardi, 2002; Narkiewicz and Grassi, 2008; Parati *et al*., 2012) and its complications such as stroke (Lattanzi *et al*. 2015a). Furthermore, Lattanzi and colleagues reported in aseries of clinical studies (Lattanzi *et al*., 2014a; 2014b, 2015b) that patients suffering from Alzheimer disease exhibit greater BP variability compared to age-matched controls, and that greater SBP variability in this patient population predicted faster cognitive decline, supporting the concept that vascular disease aggravates cognitive impairment. Therefore, elucidating the underpinning central mechanism/s could help identify new therapeutic target/s. Our results point to the importance of brain V1aR, particularly the level of expression/density of V1aR in the PVN, in relation to BP variability. In wild type rats, V1aR in the PVN mediates an increase of BP variability following stress, and this was augmented in rats over-expressing V1aRs in PVN. Moreover, in rats over-expressing V1aR in PVN, stress also increased HR variability. Accumulated evidence indicates that BP short-term variability arises from the coordinated activity of numerous homeostatic mechanisms (Japundžić-Žigon, 1998). The low frequency oscillations in the spectra of BP are the result of sympathetic nervous system and the baroreceptor reflex activity, whilst very low frequency oscillations are generated by vasoactive mechanisms, mainly the renin-angiotensin system, and are opposed by baroreflex activation (Grichois *et al*., 1992). Conditions with increased sympathetic activity, such as hemorrhage and stress, are characterized by enhanced LF-BP variability that can be prevented by sympatholytic drugs (Ponchon and Elghozi, 1997; Kanbar *et al*., 2007). Thus has been proposed that LF-BP may be a marker of sympathetic activity directed to blood vessels. A number of neurons from the parvocellular division of the PVN project to the rostral part of the ventrolateral medulla (RVLM) where sympathetic outflow to the vasculature and, in part, to the heart originates (Hallbeck *et al*., 2001; Geerling *et al*., 2010). It can be assumed that neurons projecting from PVN to RVLM modulate peripheral vascular resistance and the magnitude of LF-BP oscillation during stress. Morphological studies indicate an abundance of

V1a receptors in the PVN (Ostrovski *et al*., 1994; Hernando *et al*., 2001). Using a dual immunocytochemical labeling procedure, it has been shown that those receptors are located on somata and dendrites of magnocellular neurons in PVN (Berlove and Piekut, 1990; Hurbin *et al*., 1998) and that during osmotic challenge, intranuclear release of VP increases or inhibits the firing of magnocellular neurons to optimize the systemic release of VP (Neumann *et al*., 1993; Gouzène *et al*.,1998; Ludwig *et al*., 1994; Ota *et al*., 1994; Ludwig and Leng, 2006). In the present study, we did not apply an osmotic or a hypovolemic challenge to induce intranuclear and systemic release of VP from neurons located in the magnocellular part of the PVN. Moreover, the increase in BP variability observed in rats over-expressing V1aR in this study cannot be attributed to any peripheral effect of VP since peripherally released VP exerts the opposite effect and buffers BP variabilityby enhancing BRS (Japundzic-Zigon, 2001). The present finding, that selective vasopressin V1a receptor antagonist microinjected in PVN of wild type rats and rats over-expressing V1a receptors respectively, prevented or reduced the increase in LF BP variability by stress, suggests that V1a receptors in the PVN participate in the genesis of stress-induced increases in LF-BP variability. Moreover, we observed that in rats over-expressing V1aR in PVN there was a marked increase in cardiac LF-HR variability and also in the LF/HF-HR ratio, suggesting a shift in the autonomic control of the heart towards the sympathicus. This effect was antagonized by vasopressin V1aR antagonist microinjected in PVN, confirming that ectopic V1aRs are functional and increase sympathetic drive to blood vessels and to the heart during stress. Increased sympathetic responsiveness to stress may trigger cardiovascular and cerebral events. Altogether, our findings show that the V1aR over-expressing rat phenotype is more susceptible to stress than wild type controls.

An interesting finding of this study is that the V1aR mediates an increase in HF-BP oscillations in wild type rats. This increase was more pronounced in rats over-expressing V1aRs in the PVN, and could be prevented by V1aR antagonist pre-treatment. The HF-BP oscillation is created by negative intra-thoracic pressure during breathing (Japundzic *et al*., 1990). It may increase as a result of thoracic vessels unloading either by blood loss (Ponchon and Elghozi, 1997) or by blood redistribution due to vasodilatation (Japundzic *et al*., 1990). Since the rats in present experiments were well hydrated and eupnoic (as judged by the position of the respiratory HF peak at 1.6 Hz - 1.8 Hz in both HR and BP spectra), we believe that the increase of HF-BP can be attributed to stress-induced changes in respiratory pattern. Deeper inspiration imposed by stress may have induced greater distension of thoracic vessels and unloading that could increase the HF-BP oscillations (Elghozi *et al*., 1991). It is well established that PVN neurons project to brainstem areas that affect breathing pattern, primarily

the pre-Bötzinger complex in the medulla (PreBötC; Koizumi *et al*., 2013). The PreBötC generates and transmits the rhythmic activity producing inspiration (Smith *et al*., 1991; Rekling and Feldman, 1998). Microinjections of vasopressin into the PreBötC have been reported to stimulate respiration, an effect that involvesV1a receptors (Kc *et al*., 2002). Similar effects on respiration were obtained with microinjections of the excitatory transmitter L-glutamate into the PVN (Yeh *et al*., 1997). Also intracerebroventricular injection of VP has been shown to increase HF-BP variability (Milutinović *et al*., 2006). In our study, the effect of V1aR over-expression in PVN on HF-BP magnitude could reflect an increase in the alveolar surface for blood oxygenation to support the active coping strategy observed in present experiments. In rats over-expressing V1aR in PVN, stress also increased cardiac HF-HR oscillations. HF-HR depicts respiratory sinus arrhythmia (RSA), a natural phenomenon that characterizes healthy young hearts. HF-HR can be abolished by atropine or vagotomy, (Akselrod *et al*., 1985; Japundzic *et al*., 1990) and its diminution has been found to be a bad prognostic sign in heart disease (Huikuri and Stein, 2013). This discovery is intriguing and implies that V1aR over-expressed in PVN gives rise to a new trait in the transfected rats that improves RSA. The finding that vasopressin enhances vagal activity to the heart is not new and has been described in hemorrhagic shock (Peuler *et al*., 1990). However, the site of action of VP has yet to be determined, but it might involve the PVN, since microinjcetions of L-glutamate into caudal PVN produce bradycardia (Darlington *et al*., 1989).

Another important finding is that only in rats over-expressing V1aR in the PVN we observed modulation of baroreflex sensitivity under baseline but not stressed conditions. The effects of V1aR were suppressed by microinjection of the V1aR antagonist into the PVN, again showing that ectopic V1aRs are functional. This also confirms the principle that the receptor response to the ligand depends upon receptor density, which may affect both the quantity and the quality of the response (Kenakin, 1997). It is well known that VP modulates BRS both peripherally and centrally. Peripherally, VP has been shown to increase BRS via the area postrema, whislt centrally VP suppress the BRS via the NTS (Unger *et al*., 1986; Brattström *et al*., 1990; Dufloth *et al*., 1997). Morphological studies indicate that parvocellular neurons containing VP have direct projections to NTS and that the NTS expresses theV1aR (Ostrovski *et al*., 1994). In our experiments, selective blockade of V1aR in PVN did not prevent baroreflex desensitization by stress. This is not surprising since emotional stress is not associated with intranuclear or peripheral release of VP. Stress was found to increase intranuclear release of oxytocin (OT) (Callahan *et al*., 1989; Callahan *et al*., 1992; Nishioka *et al*., 1998), and a role for the oxytocin receptor (OTR) in the stress response was further supported by

pharmacological and genetic modulation of OTR in the PVN (Lozić *et al*., 2014). Also, we have shown that during exposure of rats to air-jet stress, selective blockade of central VP receptors do not affect the response of the hypothalamo-pituitary axis as judged by the concentration of blood corticosterone (Stojičić *et al*., 2008), suggesting that VP released into the portal circulation during air-jet stress does not modulate ACTH release into the blood stream. Nonetheless, physiological stimuli know to increase VP release into the blood stream, such as hyperosmotic challenge, have been reported to increase intranuclear release of VP (Son *et al*., 2013) and that its role is to coordinate the optimal firing rate of the whole population of magnocellular neurons (Gouzènes *et al.*, 1998), and also to activate pre-sympathetic neurons in the parvocellular division of PVN. This inter-neural cross-talk has been shown to involve V1aRs in PVN (Son *et al*., 2013), supporting the present findings.

It is important to emphasize that the ectopic V1aR could be localized on any neuron in the PVN. The PVN synthesizes over 30 different neurotransmitters (Pyner, 2009), including GABA, NO and glutamate produced by neurons involved in tonic inhibition of pre-ganglionic sympathetic neurons under baseline physiological conditions, as well as in OT-, and dopamine-producing neurons that selectively modulate this tonic inhibitory signal (Pyner, 2009). Furthermore, we have shown that ectopic V1aRs are expressed on both neurons and astrocytes. Thus, there is a possibility that ectopic V1aR located on astrocytes could be involved (Doherty *et al*., 2011; Tasker *et al*., 2012) in the modulation of neuronal activity in the PVN.

4.1. Conclusion

The present findings show for the first time that over-expression of V1aRs in PVN of rats decreases BRS under baseline conditions, and induces a marked increase in BP and HR short-term variability during exposure to emotional stress. In contrast, V1aRs in PVN of Wt rats do not affect baseline BRS, but mediate BP variability increase induced by stress. These findings suggest that somato-dendritically released VP and the level of expression (i.e. density) of the V1aR in the PVN modulate autonomic cardiovascular control during baseline and stressful physiological conditions and demarcate vulnerability to stress. This implies a possible role of the level of expression of the V1aR in the PVN in cardiovascular pathology, especially hypertension and heart failure, whose poor prognosis is associated with baroreflex desensitization and augmentation of cardiovascular short-term variability.

Table 1. Effects of vasopressin and vasopressin co-administered with V1a receptor antagonist on blood pressure and heart rate

	SBP (mmHg)	MBP (mmHg)	DBP (mmHg)	HR (bpm)
0.9% NaCl (200 nL)	118 ± 2	92 ± 1	79 ± 3	375 ± 5
AVP (30 ng · 200 nL^{-1})	125 ± 5	98 ± 5*	85 ± 3	405 ± 33
AVP (100 ng · 200 nL^{-1})	169 ± 13***	134 ± 11***	117±13**	289 ± 16**
AVP (300 ng · 200 nL^{-1})	172 ± 10 ***	138 ± 9 ***	121 ± 9**	281 ± 8***
V1aRX (300 ng · 200 nL^{-1}) **plus AVP** (100 ng · 200 nL^{-1})	116 ± 2	96 ± 3	86 ± 4	360 ± 10

Values are mean of six experiments ± s.e.m.. In this and the following tables SBP stands for systolic blood pressure, MBP for mean blood pressure, DBP for diastolic blood pressure and HR for heart rate. V1aRX is a selective vasopressin V1a receptor antagonist. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs.* 0.9% NaCl.

Table 2. Cardiovascular parameters in rats over-expressing V1a receptors in PVN

		SBP (mmHg)	MBP (mmHg)	DBP (mmHg)	HR (bpm)	BRS (ms/mmHg)
Wt	Baseline	116 ± 3	98 ± 4	89 ± 3	350± 16	2.1 ± 0.1
	Stress	136 ± 2***	115 ± 5***	105 ± 3***	425± 22***	1.5 ± 0.2*
eGFP	Baseline	116 ± 5	97 ± 3	88 ± 2	338± 21	2.0 ± 0.1
	Stress	139 ± 3***	118 ± 3***	108 ± 3 ***	428 ± 15***	1.3 ± 0.4*
V1aR	Baseline	112 ± 2	94 ± 3	84 ± 3	323 ± 13	1 ± 0.1 ††‡
	Stress	135 ± 3 ***	116 ± 3 ***	103 ± 5 ***	389 ± 12 **†‡	1.5 ±0.5

Values are mean of six experiments ± s.e.m.. Wt: wild type rats; eGFP: enhanced green fluorescent protein transfected rats; V1aR: rats over-expressing V1a receptor in PVN. *p<0.05, **p<0.01, ***p<0.001 *vs*. baseline; †p<0.05 ††p<0.01 vs. Wt and ‡p<0.05 *vs*. eGFP.

Table 3. Cardiovascular parameters in Wild type rats and rats over-expressing V1a receptors in PVN, treated with selective V1aR antagonist

		SBP (mmHg)	MBP (mmHg)	DBP (mmHg)	HR (bpm)	BRS (ms/mmHg)
Wt	Baseline	116 ± 3	98 ± 4	89 ± 3	350 ± 16	2.1 ± 0.1
	Stress	136 ± 2***	115 ± 5***	105 ± 3***	425 ± 22**	1.5 ± 0.2*
V1aRX$_{Wt}$	Baseline	115± 4	94 ± 5	84 ± 4	365 ± 6	2.3 ± 0.2
	Stress	130 ± 5*	107 ± 6*†	96 ± 4*†	423 ± 18**	1.5 ± 0.4 *
V1aR	Baseline	112 ± 2	94 ± 3	84 ± 3	323 ± 13	1 ± 0.1††
	Stress	135 ± 3 ***	116 ± 3 ***	103 ± 5 ***	389 ± 12**	1.3 ± 0.5
V1aRX$_{V1aR}$	Baseline	119 ± 3	96 ± 2	85 ± 3	322 ± 7	1.8 ± 0.3$^{@}$
	Stress	143 ± 3**	120 ± 3**	109 ± 3**	448 ± 6$^{@**}$	1.1 ± 0.1*

Values are mean of six experiments ± s.e.m.. Wt: wild type sham injected rats; V1aRX$_{Wt}$: vasopressin V1a receptor antagonist treated Wt rats. V1aR: rats over-expressing V1a receptors; V1aRX$_{V1aR}$: rats over-expressing V1a receptors in PVN treated with vasopressin V1a receptor antagonist. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs*. baseline; $^{\dagger}p<0.05$ $^{\dagger\dagger}p<0.01$ *vs*. Wt and $^{@}p<0.05$, $^{@@}p<0.01$ *vs*. V1aR.

FIGURE 1 Verification of microinfusion site in PVN (-1.8 mm from Bregma). Representative picture. The arrow points to the mark made by chronic cannulation. Cresyl Violet, Magnification 4 x. Scale bar 500μm.

FIGURE 2 V1aR mRNA expression in the left and the right PVN of Ads transfected rats and wild type sham transfected rats. Note statistically significant increase of mRNA expression at the site of transfection with Ads.

FIGURE 3 Adenoviral vector transfections site in PVN neurons. Immunostaining to V1aR (A, A'), eGFP fluorescence (B, B'), neuron nuclear antigen - NeuN (C, C'), merged A and B and C (D, D'). A, B, C, D show transfected and A' B' C' D' non-transfected slices. Magnification 10 x. Scale bar indicates 100 μm.

FIGURE 4 Adenoviral vector transfections site in PVN astrocytes. Immunostaining to V1aR (A, A'), eGFP fluorescence (B, B'), glial fibrillary acidic protein - GFAP (C, C'), merged A and B and C (D, D'). A, B, C, D show transfected and A' B' C' D' non-transfected slices. Magnification 10 x. Scale bar indicates 100 μm.

FIGURE 5 Components of BP short-term variability in rats over-expressing V1aR in PVN

Under baseline physiological conditions, V1aR over-expressing rats exhibited similar SBP and DBP short-term variability to Wt and eGFP rats. Air-jet stress induced comparable increases in LF-SBP and LF-DBP variability in all rats. The stress-induced increases in HF-SBP and HF-DBP was accentuated in V1aR over-expressing rats. Empty bars indicate baseline values, black bars indicate stress values. Wt: wild-type rats; eGFP: rats transfected with enhanced green fluorescent protein in PVN; V1aR: rats over-expressing V1a receptors in PVN; LF-SBP: low frequency systolic blood pressure variability; HF-SBP: high frequency systolic blood pressure variability; LF-DBP: low frequency diastolic blood pressure variability; HF-DBP: high frequency diastolic blood pressure variability. Values are mean of 6 rats ± s.e.m.. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ *vs*. baseline; $^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.01$*vs*. eGFP; $^{\ddagger}p<0.05$; $^{\ddagger}p<0.05$;$^{\ddagger\ddagger}p<0.01$ *vs*. Wt

FIGURE 6 Components of HR short-term variability in rats over-expressing V1aR in PVN

Under baseline conditions, HR short-term variability was similar in V1aR rats, Wt and eGFP rats. Note however that air-jet stress induced marked increases in LF-HF, HF-HR and LF/HF-HR variability only in V1aR over-expressing rats. Empty bars indicate baseline values, black bars indicate stress values. Wt: wild-type rats; eGFP: rats transfected in PVN with enhanced green fluorescent protein; V1aR: rats over-expressing vasopressin V1a receptors in PVN; LF-HR: low frequency heart rate variability; HF-HR: high frequency heart rate. Values are mean of 6 rats ± s.e.m.. $^{*}p<0.05$ *vs*. baseline; $^{\dagger}p<0.05$ *vs*. eGFP rats; $^{\ddagger}p<0.05$ *vs.* Wt rats.

FIGURE 7 Effects of selective vasopressin V1a receptor antagonist microinjected in PVN on the components of BP short-term variability of Wild type rats and rats over-expressing V1a receptors in PVN.

Under baseline physiological conditions BP variability did not differ between V1aRX treated and non-treated rats. However, during exposure of Wt rats to stress, V1aRX prevented stress-induced LF-SBP, LF-DBP, HF-SBP and HF-DBP increases. In V1aR rats exposed to stress, the V1aRX reduced the LF-SBP, LF-DBP, HF-SBP and HF-DBP increases induced by stress. Empty bars indicate baseline values, black bars indicate stress values. Wt: wild-type rats; eGFP: rats transfected with enhanced green fluorescent protein; V1aR: rats over-expressing vasopressin V1aR in PVN and V1aRX: rats treated with selective vasopressin V1aR antagonist in PVN. LF-SBP: low frequency systolic blood pressure variability; HF-SBP: high frequency systolic blood pressure variability; LF-DBP: low frequency diastolic blood pressure variability; HF-DBP: high frequency diastolic blood pressure variability. Values are mean of 6 rats ± s.e.m.. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ *vs*. baseline conditions; $^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.05$ *vs*. Wt; $^{@}p<0.05$ $^{@@@}p<0.001$ *vs*. V1aR.

FIGURE 8 Effects of selective vasopressin V1a receptor antagonist microinjected in PVN on the components of HR short-term variability of Wild type rats and rats over-expressing V1a receptors in PVN.

Under baseline conditions, V1aRX did not affect HR short-term variability in Wt rats and V1aR over-expressing rats. However, in stressed V1aR rats, the V1aRX prevented the LF-HR increase, reduced the HF-HR increase, and abolished the LF/HF-HR increase. Empty bars indicate baseline values, black bars indicate stress values. Wt: wild-type rats; eGFP: rats transfected with enhanced green fluorescent protein; V1aR: rats over-expressing vasopressin

V1a receptors; V1aRX: rats treated with selective vasopressin V1aR antagonist in PVN. LF-HR: low frequency heart rate variability; HF-HR: high frequency heart rate variability. Values are mean of 6 rats ± s.e.m.. $^{*}p<0.05$ *vs*. baseline; $^{\dagger}p<0.05$ *vs*. Wt rats; $^{@}p<0.05$; $^{@@}p<0.01$ *vs*. V1aR rats.

5. References

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7. Author contributions

M.L., T.T., O.Š. performed animal studies, Ad vector transfections,cardiovascular hemodynamic studies, BRS and spectralanalysis of cardiovascular short-term variability. M.G. and M.L.constructed Ad vectors, A.M. performed qPCR analysis and M.L. and C.H.performed data processing and statistical analyses. N.J-Ž., D.M., J.P. designed the study and wrote the paper.

8. Conflicts of interest statement

The authors declare no conflicts of interest.

9. Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.