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RESEARCH PAPER

Overexpression of oxytocin receptors in the hypothalamic PVN increases baroreceptor reflex sensitivity and buffers BP variability in conscious rats

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BACKGROUND AND PURPOSE
The paraventricular nucleus (PVN) of the hypothalamus is an important integrative site for neuroendocrine control of the circulation. We investigated the role of oxytocin receptors (OT receptors) in PVN in cardiovascular homeostasis.

EXPERIMENTAL APPROACH
Experiments were performed in conscious male Wistar rats equipped with a radiotelemetric device. The PVN was unilaterally co-transfected with an adenoviral vector (Ad), engineered to overexpress OT receptors, and an enhanced green fluorescent protein (eGFP) tag. Control groups: PVN was transfected with an Ad expressing eGFP alone or untransfected, sham rats (Wt). Recordings were obtained without and with selective blockade of OT receptors (OTX), during both baseline and stressful conditions. Baroreceptor reflex sensitivity (BRS) and cardiovascular short-term variability were evaluated using the sequence method and spectral methodology respectively.

KEY RESULTS
Under baseline conditions, rats overexpressing OT receptors (OTR) exhibited enhanced BRS and reduced BP variability compared to control groups. Exposure to stress increased BP, BP variability and HR in all rats. In control groups, but not in OTR rats, BRS decreased during stress. Pretreatment of OTR rats with OTX reduced BRS and enhanced BP and HR variability under baseline and stressful conditions. Pretreatment of Wt rats with OTX, reduced BRS and increased BP variability under baseline and stressful conditions, but only increased HR variability during stress.

CONCLUSIONS AND IMPLICATIONS
OT receptors in PVN are involved in tonic neural control of BRS and cardiovascular short-term variability. The failure of this mechanism could critically contribute to the loss of autonomic control in cardiovascular disease.

Abbreviations

BRS, baroreceptor reflex sensitivity; DBP, diastolic arterial BP; DVN, dorsal nucleus of vagus; HR, heart rate; HF, high-frequency short-term variability; IML, intermediolateral column of the spinal cord; LF, low-frequency short-term variability; MBP, mean arterial BP; NAm, nucleus ambiguus; NTS, nucleus of the solitary tract; OT, oxytocin; OTX, selective non-peptide oxytocin receptor antagonist; PI, pulse interval; PVN, paraventricular nucleus; RVLM, rostroventrolateral medulla; SBP, systolic arterial BP; VLF, very low-frequency short-term variability

Introduction

In addition to its well established roles in reproduction and maternity, convincing evidence has accumulated in the past few decades to suggest that oxytocin (OT), a peptide hormone mainly synthesized in the hypothalamic paraventricular (PVN) and supraoptic nuclei, is also involved in the control of the circulation. Peripherally, an independent OT system has been discovered in the heart and the blood vessels, and is associated with heart development, heart renewal and natriuresis (Gutkowska and Jankowski, 2012; Japundzic-Zigon, 2013). OT has been shown to exert direct negative inotropic and chronotropic effects on the heart (Costa-e-Sousa *et al*., 2005), to produce weak vasoconstriction (Suzuki *et al*., 1992) and NO-dependent vasodilatation (Katusic *et al*., 1986).

In addition to its peripheral action, OT exerts endocrine and neuromodulator effects on the circulation (Haanwinckel *et al*., 1995; Randolph *et al*., 1998). OT neurons located in the parvocellular part of the PVN project to the brainstem vagal nuclear complex (nucleus of the solitary tract – NTS, nucleus ambiguus – NAm and dorsal nucleus of vagus – DVN), rostroventrolateral medulla (RVLM) and the intermediolateral column of the spinal cord (IML) where OT influences parasympathetic and sympathetic outflow to the heart and the blood vessels (Sawchenko and Swanson, 1982; Lang *et al*., 1983; Zerihun and Harris, 1983; Hosoya *et al*., 1995; Jansen *et al*., 1995; Hallbeck *et al*., 2001; Geerling *et al*., 2010). *In vivo* animal studies indicate that OT mediates the HR response to exercise (Martins *et al*., 2005) and HR adjustment to stress (Wsol *et al*., 2008). A reduction in OT mRNA in the PVN and OT receptor mRNA in the brainstem has been demonstrated in genetically hypertensive rats (Martins *et al*., 2005), while a decline in central OT was associated with increased cardiovascular reactivity to stress in rats surviving myocardial infarction (Wsol *et al*., 2009).

OT produces its effects by the stimulation of a specific OT receptor, well defined in terms of genes, protein structure and pharmacology (Rozen *et al*., 1995; Manning *et al*., 2012). OT receptors belong to the GPCR family coupled to PLC signalling pathways (see Alexander *et al*., 2013), and are widely distributed in the periphery and CNS (Freund-Mercier *et al*., 1987; Gimpl and Fahrenholz, 2001). The focus of the present work is the role of OT receptors found in the PVN, which have been reported to play an important role in autoregulation of magnocellular neuronal activity (Richard *et al*., 1997). We hypothesized that, by increasing the number of OT receptors in the PVN and by selectively blocking their activity, we can modulate PVN neuronal activity involved in autonomic cardiovascular control. To test this hypothesis, we used genetic tools, a microinjection of adenoviral vectors (Ads) carrying the tagged gene for the OT receptor into the PVN, to induce an overexpression of OT receptors, and pharmacological tools, microinjections of a selective OT receptor antagonist into the PVN of conscious transfected and non-transfected wild-type (Wt)rats, both during baseline and stressful conditions.

Methods

All experimental procedures in this study conformed to European Communities Council Directive of 24 November 1986 (86/609/EEC). The experimental protocol was approved by the School of Medicine University of Belgrade Ethics review board, and all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al*., 2010; McGrath *et al*., 2010).

Animals

Experiments were performed in 12-week-old male Wistar rats weighing 310–360 g bred at the local animal facility. Rats were housed individually in a controlled environment: 12 h/12 h light–dark cycle, temperature 21 ± 2°C and humidity 60 ± 5% with access to standard pelleted chows (0.2% w v^{-1} 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 available at: http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize for power of 90% and type I error probability of 0.05. At the end of the experiment, the rats were killed using a combination of three anaesthetics (0.1 mL, i.p. of T61® solution, Intervet, The Netherlands).

Surgery

Rats underwent two surgical procedures at 10 day intervals. The rats were anaesthetized with ketamine (100 mg·kg^{-1}, i.m.) and xylazine (10 mg·kg^{-1}, i.m.). The depth of anaesthesia was assessed by absence of pedal withrawal reflex and corneal reflex every 5 min, respiratory, chest wall movements, and when possible BP and HR monitoring. Body temperature was maintained by placing the animal on a heating pad (Harvard Apparatus, Holliston, MA, USA). A 3 cm long medial abdominal incision was made and the intestine was 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 aorta using a 21-G needle. The inserted catheter was fixed with 3M Vetbond™ and tissue cellulose patch (DSI). The transmitter was attached to the anterior abdominal wall and the wound was closed by suture. To prevent bacterial infection, neomycin and bacitracin were sprayed topically, and the rats were treated with gentamicin (25 mg·kg^{-1}, i.m.) 3 days before, and again on the day of surgery. To reduce pain, rats received carprofen (5 mg·kg^{-1} daily, s.c.) on the day of surgery and for the next 2 days. Each rat was housed in a Plexiglas cage (30 × 30 × 30 cm) and left to recover fully for 10 days.

The second surgery was performed in Wt rats under the same anaesthetics and postoperative care. The rat's head was mounted in a stereotaxic frame and the skin was incised 3 mm to expose the skull. A hole was opened with a dental drill to position a 23-G guide above the PVN (1.8 mm caudal from bregma, 0.4 mm lateral from midline, 6.5 mm beneath the skull; Paxinos and Watson, 2005) and fixed with dental cement. On the day of experimentation, a 7.5 mm long 30-G needle was used for micro-infusion of drugs into the PVN. At the end of experiment, the rat's brain was removed. After the centre of the micro-infusion site had been identified in the hypothalamus, the brains were fixed in 4% formalin for 48 h and paraffin-embedded. Five micrometre brain sections were stained with cresyl violet acetate (0.1% w v^{-1}) and coverslipped with DPX mountant (VWR International, Lutterworth, UK).

Adenoviral vector production

The cDNA clone of the rat OT receptor in pcD2 was generously provided by Dr Stephen Lolait, University of Bristol (Jeng *et al.*, 1996). The OT receptor was amplified from pcD2 using Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) and primers OT receptor_F (5′-GTCTCGAGCATGGAGGGCACGCCAGCA-3′) and OT receptor_R (5′-GCCGGATCCTCATGCTGAAGATGGCTGA-3′). The PCR product was digested with XhoI and BamHI and ligated into compatible restriction sites of adenoviral vector pacAd5.CMV.IRES.GFP (Cell Biolabs, San Diego, CA, USA). Adenoviral vector pacAd5.CMV.GFP was used as a control. The adenoviruses were generated by co-transfection of viral shuttle and backbone (pacAd5 9.2–100) vectors in HEK293T cells by a calcium phosphate method in accordance with the manufacturer's instructions (Cell Biolabs). Adenoviruses were purified by two rounds of CsCl ultracentrifugation and desalted using Slide-A-Lyzer dialysis cassettes (Thermoscientific Pierce, Loughborough, UK). The purified viruses were aliquoted and stored at –80°C. The virus titres were determined in triplicate by a standard plaque assay.

Transfection

Ten days after the telemetry device had been fitted, a unilateral injection of Ads into the PVN of rats was performed under combined ketamine–xylazine anaesthesia. The head of the rat was mounted in the stereotaxic frame and the skin was incised 3 mm to expose the skull. The stereotaxic coordinates of PVN (1.8 mm caudal from bregma, 0.4 mm lateral from midline) were derived from the rat brain atlas (Paxinos and Watson, 2005). A glass micropipette was slowly positioned 7.6 mm beneath the skull for infusion of virus (titre 4 $x10^{10}$ pfu·mL^{-1}) in 50 nL, pressure-injected in 1 min. In sham rats, a glass micropipette was slowly positioned 7.6 mm beneath the skull. After removal of the micropipette, the skin above the trepanation was sutured and sprayed with antibiotics (neomycin and bacitracin). Immediately after transfection, a guide cannula was positioned 6.5 mm beneath the scull in n = 6 rats, for micro-infusion of the OT receptor antagonist. In the postoperative period rats were treated with gentamicin (25 mg·kg^{-1} i.m.) one day before, on the day of surgery and 2 days after surgery and carprofen (5 mg·kg^{-1} daily, s.c.) on the day of surgery and 2 days after. Rats were left to recover for 7 days, the time needed for maximal expression of the transfected gene (Lonergan *et al.*, 2005).

Evaluation of OT receptor expression

Tissue preparation and collection. Following death, brains were carefully removed and snap-frozen with powdered dry ice. Using a cryostat (Leica Microsystems CM1900, Leica Microsystems, Nussloch GmbH, Nussloch, Germany), 60 µm caudal-rostral sections were taken and stained with Toluidine blue (1% in 70% ethanol, Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) in order to map the hypothalamus (Paxinos and Watson, 2005) and 1 mm diameter punches were obtained using a 15-G Sample Corer (Fine Science Tools Inc., Foster City, CA, USA, catalogue no 18035-01) from both the left and the right PVN unstained tissue and stored in RNase-free tubes at –80°C.

RNA extraction. Tissue samples were mechanically homogenized in 1 mL QIAzol Lysis reagent (Qiagen, Qiagen Ltd., Manchester, UK) and allowed to stand for 5 min at room temperature before centrifugation (10 min, 10 120× *g*, 4°C), which removed debris from the sample. Extraction with 200 µL chloroform (Sigma-Aldrich Co. Ltd.) was then performed (15 min, 11 920× *g*, 4°C). Total RNA was then precipitated with 350 µL (1 volume) ethanol (70% w v^{-1}) and purified using the RNeasy Mini Kit (Qiagen, Qiagen Ltd., Manchester, UK) according to manufacturer's protocol. RNA quality and yield were confirmed using GeneQuant II RNA/DNA Calculator (Pharmacia Biotech, Piscataway, NJ, USA).

cDNA synthesis. cDNA synthesis was performed using QuantiTect Reverse Transcription Kit (Qiagen, Qiagen Ltd., Manchester, UK) using 100 ng of input RNA. cDNA samples were then diluted to a concentration of 2 ng·μL^{-1} for use in real time qRT-PCR. Validated proprietary primers for the OT receptor were obtained (Rn_OT receptor_1_SGl, Quantitect Primer Assay, Qiagen, Qiagen Ltd.). Primers for the 60 s ribosomal protein L19 (Rpl19) were also obtained for use as a housekeeping gene to allow normalization of OT receptor expression between samples (Fwd: GCGTCTGCAGCCATGAGTA, Rev: TGGCATTGGCGATTTCGTTG; Eurofins MWG Synthesis GmbH, Ebersberg, Germany).

Real time qRT-PCR and data analysis. Analysis was performed using an Applied Biosystems 7500 Real Time PCR System and processed using Applied Biosystems 7500 SDS 1.2 software (Applied Biosystems, Foster City, CA, USA). The Δ cycle threshold (Δ*Ct* = sample gene *Ct* – housekeeper gene Ct) was calculated for each sample (left PVN and right PVN) and then exponentiation was applied to each sample (Power 2). Finally, left PVN was given a standard numerical value of 1, and differences in left PVN versus right PVN were calculated (Power 2 right PVN/Power 2 left PVN).

Tissue preparation and immunohistochemistry

At the end of the experiments rats were anaesthetized and transcardially perfused with 100 mL of 0.1 M PBS (pH 7.4) at room temperature followed by 300 mL of 4% (w v^{-1}) paraformaldehyde in 0.1 M PBS. The brains were removed, stored and cryoprotected in fixative containing 20% sucrose overnight at 4°C and subsequently frozen at –80°C. Coronal

sections (35 μm) of the entire rostro-caudal axis of the forebrain were sectioned on a cryostat. The free-floating sections were collected in 24-well tissue culture plates containing PBS before being processed for immunohistochemical detection of OT receptors.

For immunohistochemical detection of OT receptors, we used commercially available goat polyclonal anti-OT receptor antibody (1:100, Santa-Cruz, USA, catalogue no sc-8103). Free-floating rat hypothalamic sections were incubated for 30 min in a blocking solution comprising 10% normal horse serum (NHS; Sigma-Aldrich Co. Ltd.), and 0.3% (v v^{-1}) Triton X-100 (Sigma-Aldrich Co. Ltd.) in 0.1 M PBS followed by rinses (3 × 10 min) in PBS. Sections were then incubated in goat anti-OT receptor primary antibody (dilution 1:100) in PBS containing 1% (v v^{-1}) NHS and 0.3% (v v^{-1}) Triton X-100 overnight. After the primary antibody incubation, sections were rinsed in PBS (3 × 10 min) before a 1 h incubation in PBS containing donkey Alexa Fluor 594 anti-goat IgG (dilution 1:100, Abcam, UK), 10% (v v^{-1}) NHS and 0.3% (v v^{-1}) Triton X-100 at room temperature. Following rinses in PBS (3 × 10 min) sections were mounted onto glass microscope slides with 0.5% (w v^{-1}) gelatine and allowed to air dry for several minutes. Once dry, the slides were dehydrated in ethanol (75%, 85%, 96% v v^{-1}), cleared in Histoclear (Raymond A Lamb Ltd., East Sussex, UK), and coverslipped in DPX mountant (VWR).

Pilot experiments

Pilot experiments were performed to determine the selective dose of OT receptor antagonist. Six rats equipped with a radiotelemetric device and intrahypothalamic cannula were used. Following vehicle application (200 nL·min^{-1} 0.9% NaCl), increasing doses of OT (30, 100 and 300 ng) in a volume of 200 nL were micro-infused for 1 min into the PVN of conscious rats, at 2 h intervals. Cardiovascular parameters were recorded between OT administrations for 60 min. Five days later, a non-peptide OT receptor antagonist (OTX) and OT were co-administered to rats to test OTX blocking efficacy. Subsequently, cardiovascular parameters were recorded for 60 min.

Experimental design

Seven days post-transfection, rats were subjected to experimentation. All experiments started around 10:00 h in quiet surroundings under controlled environmental conditions, following a 60 min period of baseline recordings of rats housed individually in Plexiglas cages (30 × 30 × 30 cm). Cardiovascular parameters were recorded for 20 min under baseline conditions and 10 min during exposure of rats (n = 6) to stress and during the recovery period until the BP and HR had returned to normal. Stress was induced by directing an air-jet (bottle-compressed under $1x10^5$ Pa) to the top of the rat's head avoiding its nose. A separate group of Wt rats (n = 6) and transfected rats (n = 6) equipped with a radiotelemetric device and intrahypothalamic cannula for drug injection, were subjected to a micro-infusion of OTX (300 ng·200 nL^{-1}; n = 6) or saline (200 nL·min^{-1}; n = 6) and their effects were recorded under baseline conditions for 20 min and 10 min during exposure to stress.

Cardiovascular signal processing and analysis

Arterial BP was digitalized at 1000 Hz in Dataquest A.R.T. 4.0 software, (DSI). Systolic arterial BP (SBP), diastolic arterial BP (DBP), mean arterial BP (MBP) and pulse interval (PI) or its inverse, 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, the mean values of SBP, MBP, DBP, HR and PI were calculated, and again averaged for the whole experimental group (values shown in tables and graphs).

Evaluation of the spontaneous baroreceptor reflex by the method of sequences

The method used is explained in detail elsewhere (Bajić *et al.*, 2010). Briefly, a spontaneous baroreceptor reflex 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 with respect to SBP. A threshold for sequence length was set to four beats (Lončar-Turukalo *et al.*, 2011). The sensitivity of baroreceptor reflex sensitivity (BRS, ms·$mmHg^{-1}$) 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).

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 9-point Hanning window filter and linear trend removal (Milutinović *et al.*, 2006; Stojičić *et al.*, 2008). Spectra were obtained using a fast Fourier transform (FFT) 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 $(beats\ min^{-1})^2$ 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 low-frequency (LF) oscillation of SBP and DBP spectrum (LF-SBP and LF-DBP) and LF/HR-HR are markers of sympathetic activity directed to blood vessels and sympatho-vagal balance to the heart respectively (Japundzic-Zigon, 1998).

Drugs

Non-peptide and selective OT receptor antagonist, desGly-NH_2-$d(CH_2)_5$[D-Tyr^2,Thr^4]-OVT, was generously provided by Professor Maurice Manning University of Toledo OH USA. OT acetate was purchased from Sigma-Aldrich (UniChem, Belgrade, Serbia). Ketamine, xylazine, carprofen (Rimadyl®) and combination of embutramide plus mebezonium plus tetracaine (T61®) injections were purchased from Marlo Farma (Belgrade, Serbia). Gentamicin (Gentamicin®) injections and bacitracin plus neomycin spray (Bivacyn®) were purchased from Hemofarm (Vršac, Serbia).

Statistics

Cardiovascular parameters are presented as mean ± SEM. Multiple comparisons of haemodynamic parameters among

experimental groups were performed by ANOVA for repeated measures followed by *post hoc* Bonferroni's test using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). Wilcoxon's signed–rank test was used to evaluate mRNA expression data. Statistical significance was considered at $P < 0.05$.

Results

Verification of micro-infusion sites and Ad expression

The position of the guide cannula in the PVN was confirmed histologically and shown in Figure 1. Efficacy of Ad transfection in PVN at micro-infusion site is illustrated by enhanced green fluorescent protein (eGFP) fluorescence (Figure 2A, C), OT receptor immunostaining (Figure 2B, C) and increased mRNA of OT receptors in PVN at Ad virus infusion site (Figure 2D).

Pilot experiments

The dose–response with OT shown in Table 1 was performed in $n = 6$ conscious Wt rats. OT micro-infused into the PVN at a dose of 30 ng·200 nL^{-1} did not affect SBP, DBP, MBP and HR, while OT in doses of 100 ng·200 nL^{-1} and 300 ng·200 nL^{-1} induced statistically significant and comparable increases in SBP, DBP, MBP and HR, over the duration of 30 min. The hypertensive effect and tachycardia induced by 100 ng of OT was prevented by pretreatment of rats with OTX at a dose of 300 ng. OT infused alone at a dose of 100 ng increased SBP from 133 ± 2 mmHg in saline-treated rats to 152 ± 1 mmHg ($P < 0.001$) or to 135 ± 4 mmHg (non-significant, $P > 0.05$) in 300 ng OTX-pretreated rats. DBP changed from 81 ± 4 mmHg in saline-treated rats to 93 ± 5 mmHg in 100 ng OT-treated rats ($P < 0.01$) or to 73 ± 5 mmHg in OTX-pretreated rats (non-significant, $P > 0.05$); MBP changed from 98 ± 2 mmHg in saline-treated rats to 112 ± 2 mmHg in OT-treated rats ($P < 0.01$) or to 94 ± 2 mmHg in OTX-pretreated rats (non-significant, $P > 0.05$). HR increased from 363 ± 14 beats min^{-1} to 446 ± 18 bpm in 100 ng OT-treated rats ($P < 0.01$) or to 395 ± 31 beats min^{-1} in OTX-pretreated rats (non-significant, $P > 0.05$).

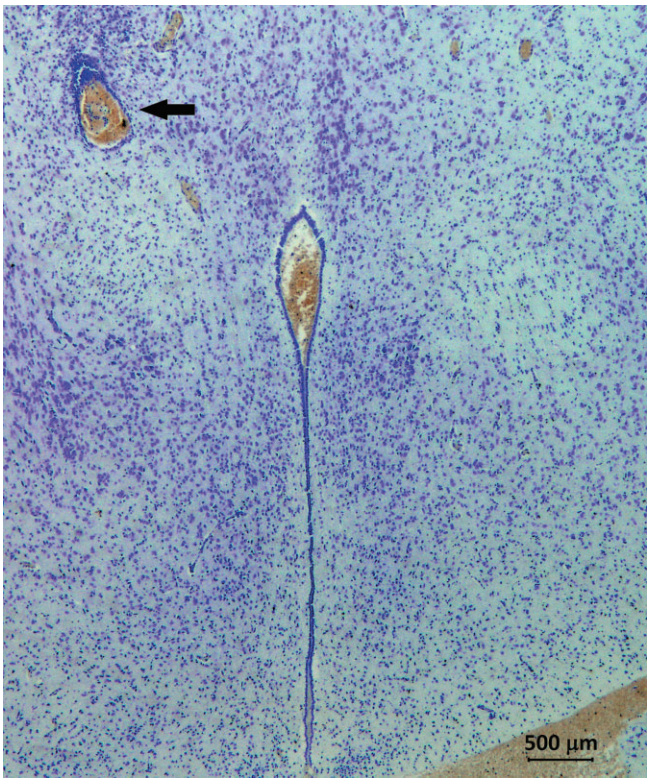

Figure 1

Micro-infusion site in PVN (–1.8 mm from Bregma). Representative picture. The arrow points to the mark made by chronic cannulation. Magnification 4×.

Cardiovascular parameters in rats overexpressing OT receptors in the PVN

Under baseline conditions and during exposure to stress, mean values of SBP, MBP, DBP, HR and BRS did not differ between sham-injected Wt and eGFP rats (Table 2). In contrast, rats overexpressing OT receptors in PVN (OTR group) exhibited increased values of SBP, MBP and enhanced BRS compared with eGFP and Wt controls. Exposure of rats to air-jet stress increased SBP, MBP, DBP and HR in all groups. In Wt and eGFP rats, a decrease in BRS occurred while in OTR rats BRS did not decrease (Table 2).

Spectral analysis of cardiovascular short-term variability revealed that under basal physiological conditions, BP short-term variability was comparable between Wt and eGFP rats (Figure 3). However, in OTR rats, a reduction in SBP and DBP total variability, due to the statistically significant decrease in VLF variability, was observed. Concomitantly, HF-SBP and HF-DBP variability increased.

When rats were exposed to air-jet stress, BP variability increased because of the increase in variability in all spectral bands. However, the increase in LF and HF variability in SBP and LF in DBP spectra was significantly smaller in OTR rats as compared with both eGFP and Wt groups (Figure 3).

Under baseline physiological conditions, HR variability in rats overexpressing OT receptors in PVN did not differ from eGFP or Wt controls (Figure 4). However, when rats were exposed to acute stressful conditions, OTR rats exhibited an increase in HR variability in all spectral bands without changes in LF to HF ratio (Figure 4) suggesting a concomitant sympathetic and vagal stimulation of the heart.

Effect of OTX on cardiovascular parameters in Wt rats and in rats overexpressing OT receptors in the PVN

In Wt rats, under baseline physiological conditions, a micro-infusion of OTX into the PVN significantly reduced BRS compared to non-treated rats, and had no effect on mean levels of SBP, DBP, MBP and HR (Table 2). In these rats, LF-SBP LF-DBP and HF-SBP spectral domains increased (Figure 5). HR variability was not affected significantly by OTX under baseline physiological conditions (Figure 6).

Wt rats pretreated with OTX and exposed to stress exhibited a similar increase in SBP to that of non-treated rats, but

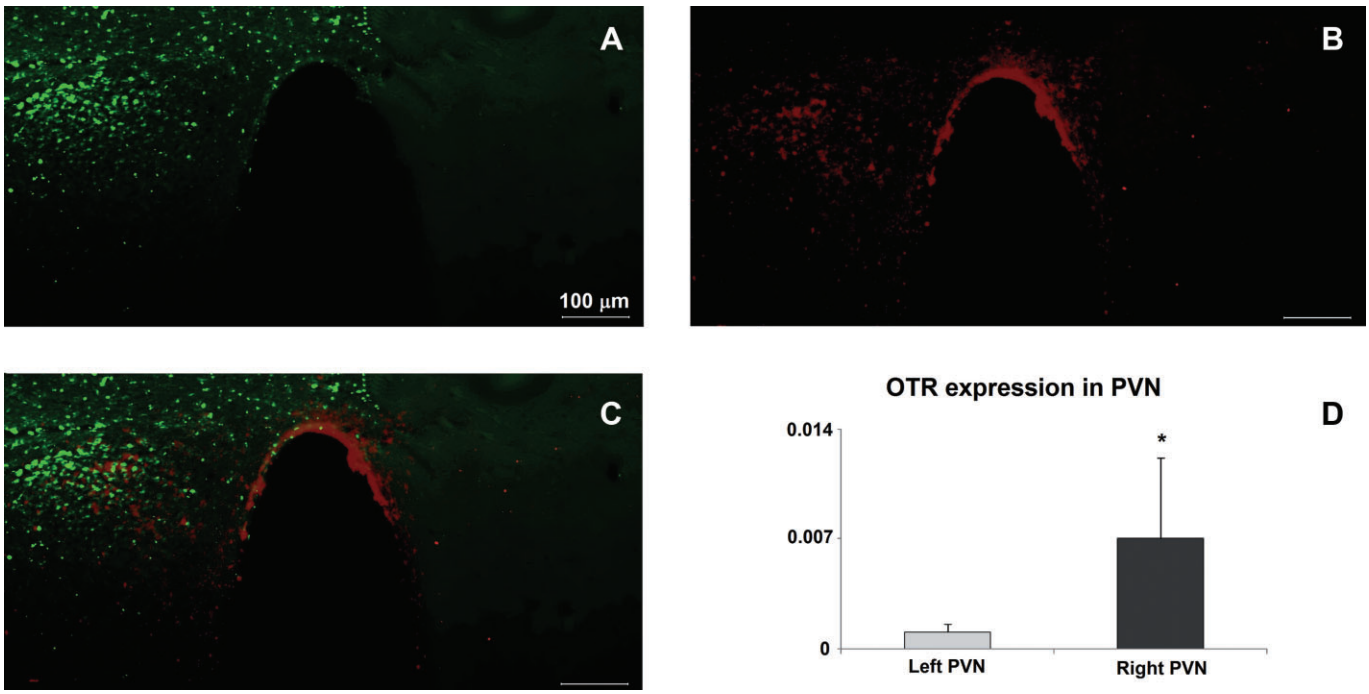

Figure 2

Adenoviral vector transfections site in PVN (–1.8 mm from Bregma). eGFP fluorescence (A), immunostaining of OT receptors (B) merged A and B (C), and relative mRNA expression of OT receptors in PVN (D). Magnification 10×.

Table 1

Effects of OT microinjected into the PVN on BP and HR of conscious Wt rats

	SBP (mmHg)	MBP (mmHg)	DBP (mmHg)	HR (beats min^{-1})
Saline (200 nL)	133 ± 2	98 ± 2	81 ± 4	363 ± 14
OT (30 ng·200 nL^{-1})	138 ± 1	99 ± 3	79 ± 3	348 ± 1
OT (100 ng·200 nL^{-1})	152 ± 1***	112 ± 2**	93 ± 5**	446 ± 18**
OT (300 ng·200 nL^{-1})	166 ± 11***	127 ± 3***	107 ± 6***	466 ± 16**

Values are mean of six rats ± SEM.
**P < 0.01; ***P < 0.001 versus saline.

the increases in MBP, DBP and HR were smaller and the BRS remained reduced (Table 3). In these rats, SBP and DBP variability (Figure 5) and HR variability increased in all spectral domains, as well as the ratio LF/HF-HR (Figure 6).

In rats overexpressing OT receptors, micro-infusion of OTX under baseline conditions reduced BRS in comparison with both non-treated OTR rats and Wt rats (Table 3). OTX had no effect on mean values of SBP, DBP, MBP and HR of OTR rats compared to non-treated OTR rats, but SBP and MBP were higher compared to Wt rats (Table 3). OTR rats pretreated with OTX and exposed to stress exhibited higher increase in MBP, DBP and HR than Wt rats pretreated with OTX and exposed to stress (Table 3). In OTX-treated OTR rats, both under baseline and stressful conditions, SBP, DBP (Figure 5) and HR variability (Figure 6) increased in all spectral domains. Also, in rats overexpressing OT receptors in PVN and pretreated with OTX, the increase in LF/HF-HR occurred both under baseline and stressful conditions (Figure 6) compared to non-treated OTR rats and Wt OTX-treated rats.

Discussion and conclusions

The findings of the present work suggest that OT receptors in PVN tonically modulate autonomic cardiovascular control both under baseline and stressful physiological conditions. In Wt rats, application of OTX under baseline conditions reduced BRS and unbuffered BP variability. When exposed to stress, BRS remained reduced and HR variability increased in these rats, especially in LF frequency domain, pointing to the domination of sympathetic control of the heart. In rats over-

Table 2

BP, HR and BRS in rats overexpressing OT receptors in the PVN

		SBP (mmHg)	MBP (mmHg)	DBP (mmHg)	HR (beats min^{-1})	BRS (ms·mmHg^{-1})
Wt	Baseline	117 ± 5	94 ± 4	82 ± 4	340 ± 12	2.3 ± 0.2
	Stress	139 ± 6***	120 ± 5***	110 ± 7***	450 ± 21***	1.3 ± 0.5*
eGFP	Baseline	118 ± 5	96 ± 4	85 ± 3	341 ± 17	2.0 ± 0.16
	Stress	138 ± 5***	122 ± 4***	108 ± 3***	448 ± 17***	1.3 ± 0.4*
OTR	Baseline	134 ± 3†,‡‡	106 ± 2††,‡‡	86 ± 2	351 ± 14	2.9 ± 0.3†,‡
	Stress	149 ± 5**,†	115 ± 2**,‡	96 ± 3**	430 ± 20**	3.4 ± 0.4††,‡‡

Values are mean of six rats ± SEM.
*P < 0.05; **P < 0.01; ***P < 0.001 versus baseline.
$^{\dagger}P$ < 0.05; $^{\dagger\dagger}P$ < 0.01 versus eGFP-transfected rats.
$^{\ddagger}P$ < 0.05; $^{\ddagger\ddagger}P$ < 0.01 versus Wt rats.

Table 3

Effects of selective OT receptor antagonist (OTX) microinfused into the PVN on BP, HR and BRS of wild-type rats and rats overexpressing OT receptors

		SBP (mmHg)	MBP (mmHg)	DBP (mmHg)	HR (beats min^{-1})	BRS (ms·mmHg^{-1})
Wt	Baseline	117 ± 5	94 ± 4	82 ± 4	340 ± 12	2.3 ± 0.2
	Stress	139 ± 6***	120 ± 5***	110 ± 7***	450 ± 21***	1.3 ± 0.5*
OTX_{Wt}	Baseline	123 ± 1	98 ± 2	85 ± 2	317 ± 16	1.0 ± 0.2‡
	Stress	145 ± 5***	106 ± 3**,‡‡	87 ± 2‡‡	394 ± 9‡,**	1.5 ± 0.2
OTR	Baseline	134 ± 3‡‡	106 ± 2‡‡	86 ± 2	351 ± 14	2.9 ± 0.3‡
	Stress	149 ± 5**	115 ± 2**,‡	96 ± 3**	430 ± 20**	3.4 ± 0.4‡‡
OTX_{OTR}	Baseline	136 ± 5‡	103 ± 3‡	87 ± 3	323 ± 12	1.2 ± 0.2$^{\ddagger,@@}$
	Stress	151 ± 6**	111 ± 5**,‡	94 ± 1**,‡	414 ± 5**,‡	1.7 ± 0.6$^{@@}$

Values are mean of six rats ± SEM.
*P < 0.05; **P < 0.01; ***P < 0.001 versus baseline.
$^{\ddagger}P$ < 0.05; $^{\ddagger\ddagger}P$ < 0.01 versus Wt rats.
$^{@@}P$ < 0.01 versus OTR rats.

expressing OT receptors, the increase in BRS was noted under baseline conditions and it remained increased during exposure to stress. These findings suggest that ectopic OT receptors in PVN are functional, and that their increased number may potentiate the physiological effects of naturally occurring ligands at physiological concentrations. Furthermore, the administration of OTX to OTR rats confirmed the functionality of ectopic OT receptors, that is OTX reduced BRS and debuffered cardiovascular short-term variability under baseline and stressful conditions, and these effects were clearly more pronounced in OTR rats than in Wt rats.

It is well established that OT receptors are normally expressed in the PVN (Van Leeuwen *et al.*, 1985; Freund-Mercier *et al.*, 1987; Tribollet *et al.*, 1988; Yoshimura *et al.*, 1993; Adan *et al.*, 1995). Electrophysiological studies have shown that OT receptors in the PVN have an important function as part of an endogenous autocontrol mechanism (Richard *et al.*, 1997). For instance, during suckling, somatodendritically released OT was found to stimulate OT receptors on magnocelluar neurons in the PVN to increase the basal firing rate and establish a periodic bursting activity pattern. The underlying mechanisms involve priming of OT neurons and release of calcium from inositol triphosphate-sensitive intracellular stores (Inenaga and Yamashita, 1986; Moos and Richard, 1989; Richard *et al.*, 1997; Ludwig and Leng, 2006). Here, we provide evidence that a change in the expression of OT receptors in the PVN in conscious rats can affect neurogenic control of the circulation. Anatomical and electrophysiological studies indicate that 40% of the spinally projecting PVN neurons contain mRNA for OT (Pyner, 2009) and that they project to the NTS, DVN, NAc, RVLM and IML column of the spinal cord (Sawchenko and Swanson, 1982; Lang *et al.*, 1983; Zerihun and Harris, 1983; Hosoya *et al.*, 1995; Jansen *et al.*, 1995; Hallbeck *et al.*, 2001; Geerling *et al.*, 2010) where vagal and sympathetic outflow to the heart and the blood vessels is set. Functional studies by Russ and Walker (1994) revealed that exogenously applied OT enhances the BRS, and Higa and associates (2002) also reported that micro-injections

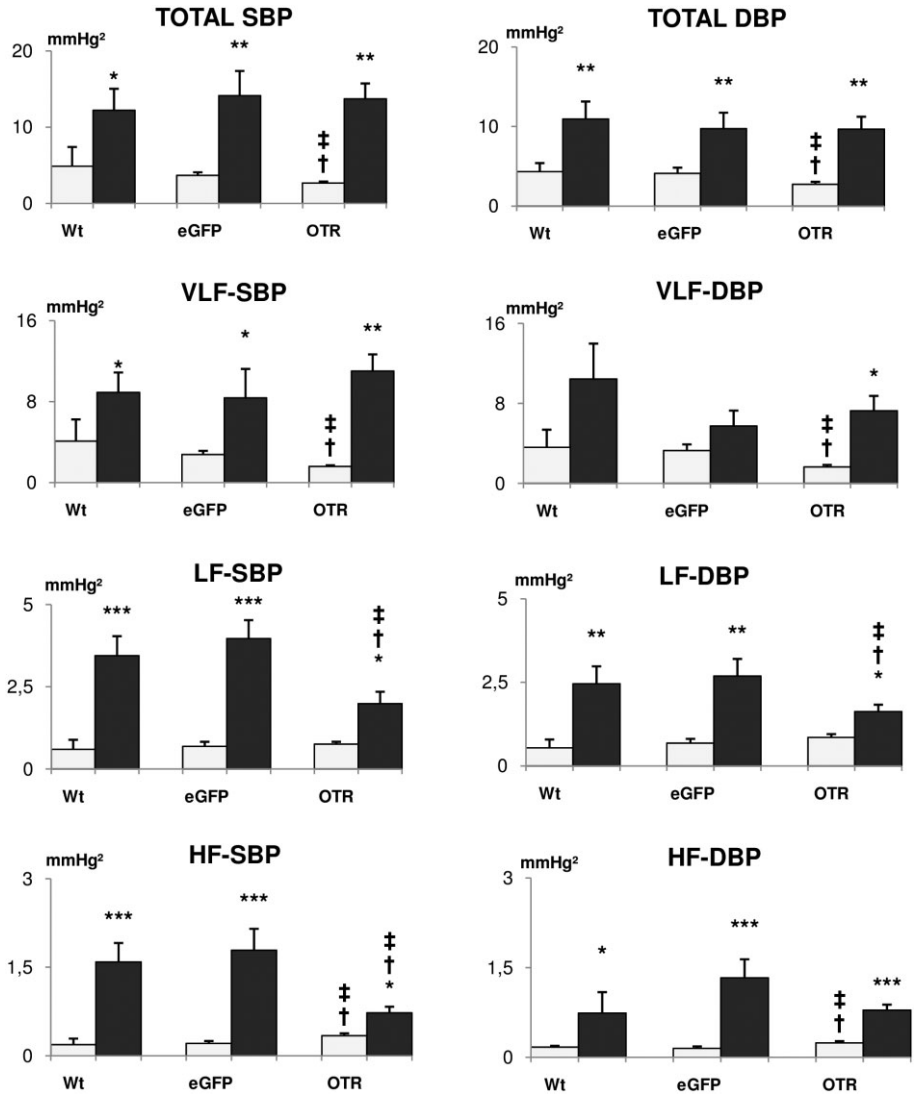

Figure 3

Components of BP short-term variability in rats overexpressing OT receptors in PVN. In OTR rats, under baseline physiological conditions a reduction in VLF-SBP and VLF-DBP variability and increase in HF-SBP and HF-DBP variability occurred. Note smaller increase in LF-SBP, LF-DBP and HF-SBP variability in OTR rats exposed to stress compared to controls. Open columns indicate baseline values, solid columns indicate stress values. TOTAL-DBP, total DBP variability; TOTAL-SBP, total SBP variability; VLF-DBP, very low-frequency DBP variability; VLF-SBP, very low-frequency SBP variability. Values are mean of six rats ± SEM. *$P < 0.05$; **$P < 0.01$;***$P < 0.001$ versus baseline; †$P < 0.05$ versus eGFP; ‡$P < 0.05$ versus Wt.

of OT into the NTS facilitate reflex bradycardia via the stimulation of OT receptors. Moreover in mice lacking the *OT* gene and OT peptide, Michelini and collaborators (2003) reported blunted baroreceptor reflex in response to pressure changes. In our experiments, overexpression of OT receptors in the PVN-enhanced BRS, and this effect on the baroreceptor reflex was also revealed by OTX administration to Wt rats. Although our experiments cannot indicate the identity of the transmitter, there is a possibility that OT receptors located on neurons in the parvocellular part of the PVN that project to cardiovascular centres in the medulla and the spinal cord could be stimulated by locally (somato-dendritically) released OT to enhance (autocontrol) axonal release OT in the vicinity of the NTS, where OT receptors have been shown to increase BRS. Alternatively, overexpression of ectopic OT receptors could have occurred on another neuron in the PVN, that is involved in the neural circuitry that alters autonomic cardiovascular control (direct ipsilateral projections to IML, RVLM and contralateral projections to both), or even on astroglial cells (Doherty *et al.*, 2011), to modulate total neuronal activity in the PVN (Tasker *et al.*, 2012). It is well established that NO, GABA and glutamate are crucial, while OT, vasopressin, dopamine, angiotensin II selectively modulate the tonic PVN signal for autonomic cardiovascular control (Pyner, 2009). Taken together, our results suggest that, in both transfected and Wt rats, OT receptors in the PVN activate downstream signalling pathways in neighbouring cells leading to neurotransmitter release in brainstem targets that increase BRS. They also suggest that the number of OT receptors expressed in the PVN may alter the level of tonic input from the PVN,

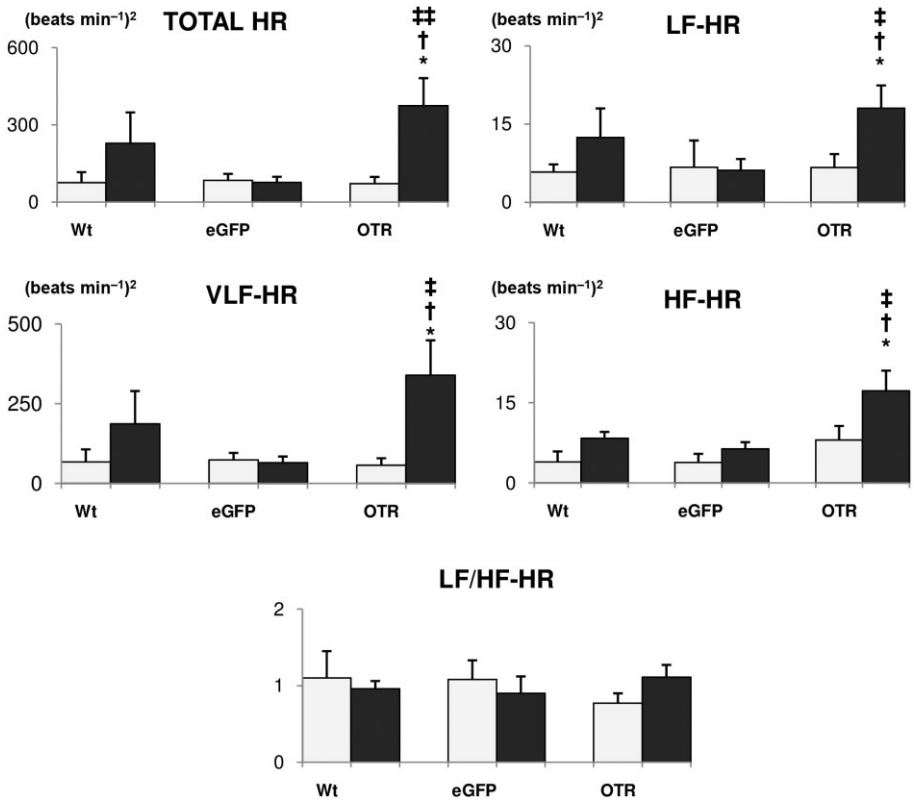

Figure 4

Components of HR short-term variability in rats overexpressing OT receptors in PVN. Note that HR variability in OTR rats was enhanced compared to control rats only during stressful conditions. Open columns indicate baseline values, solid columns indicate stress values. TOTAL-HR, total HR variability; VLF-HR, very low-frequency HR variability. Values are mean of six rats ± SEM. *$P < 0.05$ versus baseline; †$P < 0.05$ versus eGFP rats; ‡$P < 0.05$; ‡‡$P < 0.01$ versus Wt.

involved in the neurogenic cardiovascular control, without altering the amount of ligand released.

Our results further show that up-regulation of OT receptors in the PVN reduces BP variability in the VLF domain under basal physiological conditions. VLF-BP oscillations contribute most to overall BP short-term variability (Japundzic-Zigon, 1998), and dominate under basal conditions. They are created by multiple mechanisms acting in concert and opposition. Oscillation at ~0.1 Hz in rat originates from spontaneous myogenic activity of blood vessels (Stauss *et al.*, 2009), these can be enhanced by activation of the rennin-angiotensin system (Ponchon and Elghozi, 1996) and counteracted by the baroreceptor reflex, as suggested from results obtained in experiments where the baroreceptor reflex loop has been surgically or pharmacologically compromised (Japundzic *et al.*, 1990; Cerutti *et al.*, 1994). Therefore, in our experiments, the reduction of VLF variability could be related to the enhanced BRS. Experiments with OTX revealed that the overexpression of OT receptors buffers BP and HR variability in all spectral domains under baseline and stressful conditions and shifts the autonomic control of the heart to the vagus, while the buffering effect of OT receptors in the PVN of Wt rats on HR variability is confined to stress. In this context, it is important to mention that a better understanding of the central mechanisms that alter BRS, BP and HR variability, recognized markers of clinical outcome of cardiovascular diseases (Mancia *et al.*, 1994; Narkiewicz and Grassi, 2008), is of interest.

Our experiments indicate that the pharmacological effects of OT micro-infused into the PVN are hypertension and tachycardia. The failure of OTX in Wt to modulate BP and HR under baseline conditions indicates that OT has no tonic physiological influences on mean levels of BP and HR. Although in OTR rats, SBP and MBP were increased compared to non-transfected rats, this increase in SBP and MBP does not seem to be associated with an up-regulation of OT receptors in the PVN, as SBP and MBP remained increased after application of OTX. According to the literature, administration of OT exogenously can induce both hypotensive and hypertensive effects, depending on the route of administration. OT applied via the peripheral route was found to produce short-lasting hypertension followed by a longer-lasting hypotension (Petersson *et al.*, 1996). Centrally applied OT was reported to decrease BP (Petersson *et al.*, 1996; Petersson and Unväs-Moberg, 2007). It was further suggested that the central hypotensive effects of OT are mediated through axonal release in the locus coeruleus where OT increases the density and the affinity of α_2-adrenoceptors known to reduce sympathetic outflow (Petersson *et al.*, 2005). However, OT injected into the RVLM or NTS or DVN was found to increase

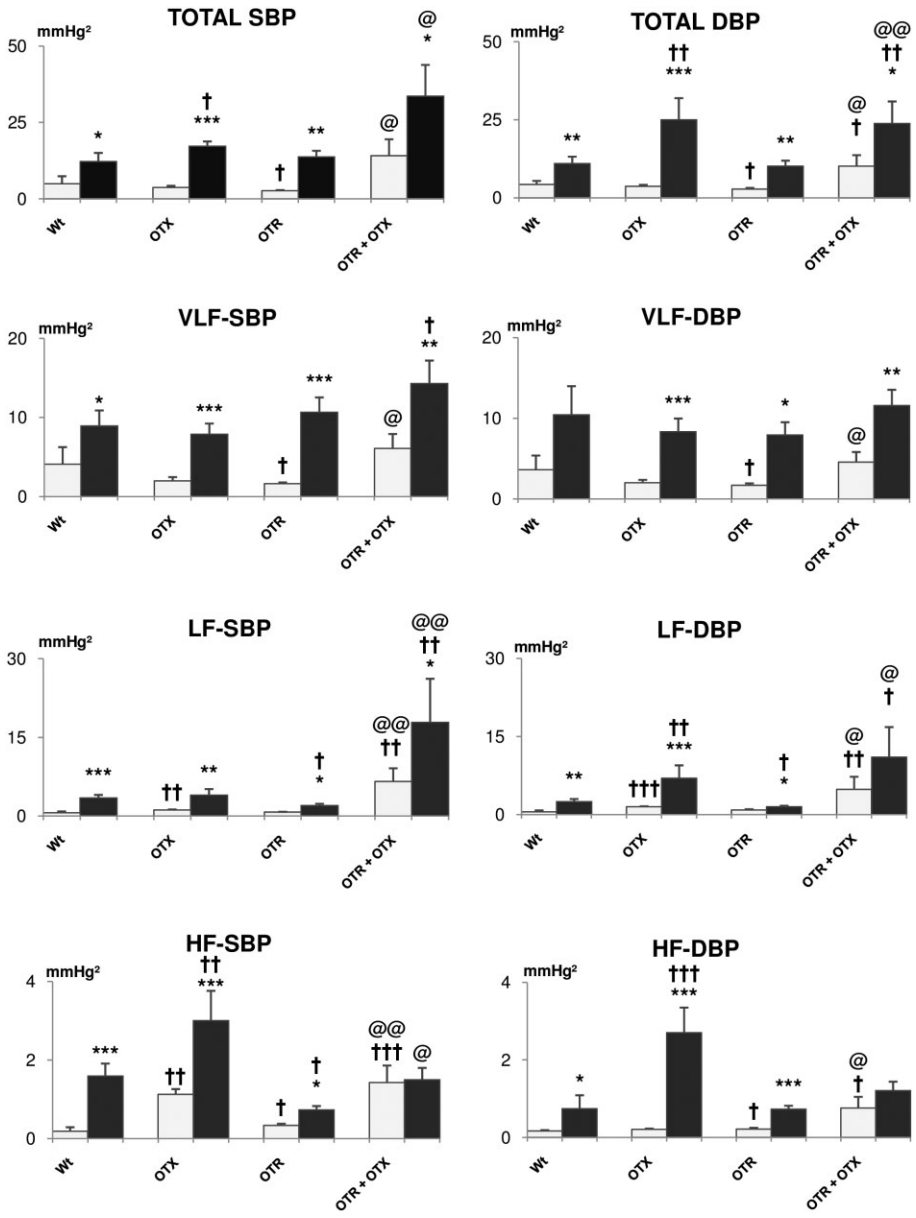

Figure 5

Effects of selective OT receptor antagonist (OTX) in PVN on the components of BP short-term variability of Wt rats and rats overexpressing OT receptors. Note that under baseline conditions OTX-treated Wt rats showed increased LF-SBP, LF-DBP and HF-SBP variabilities. During stress in these rats all the components of SBP and DBP variability increased. OTR rats treated with OTX exhibited increased SBP and DBP variability both under baseline and stressful conditions. Open columns indicate baseline values, solid columns indicate stress values. TOTAL-DBP, total DBP variability; TOTAL-SBP, total SBP variability. Values are mean of six rats ± SEM. *$P < 0.05$; **$P < 0.01$;***$P < 0.001$ versus baseline; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ versus Wt; $^{@}P < 0.05$; $^{@@}P < 0.01$ versus OTR rats.

BP (Mack *et al.*, 2002; Vela *et al.*, 2010. In OT-deficient mice, BP was found to be lower than in Wt mice, suggesting a tonic influence of endogenous OT on BP (Michelini *et al.*, 2003).

It is well recognized that the PVN is a major site for the integration of neuroendocrine and behavioural responsea to stress (Herman and Cullinan, 1997; Dampney and Horiuchi, 2003; Benarroch, 2005). Rats exposed to air-jet stress exhibit a startle reaction followed by freezing associated with increased BP and HR. Using microdialysis, Nishioka and co-workers (1998) found that stress increases the OT content of the PVN. It was also reported that specific PVN lesions, or micro-injection of antagonists i.c.v. or OT antisense oligonucleotides into the PVN (Callahan *et al.*, 1989; 1992) attenuated the HR response to stress. In our experiments, micro-infusions of OTX into the PVN of non-transfected rats prevented stress-induced DBP increase and reduced but did not prevent tachycardia. In transfected rats an up-regulation of OT receptors in the PVN did not modulate the HR response to stress, but evoked the increase in HR variability. The increase in HR variability is both sympathetically- and

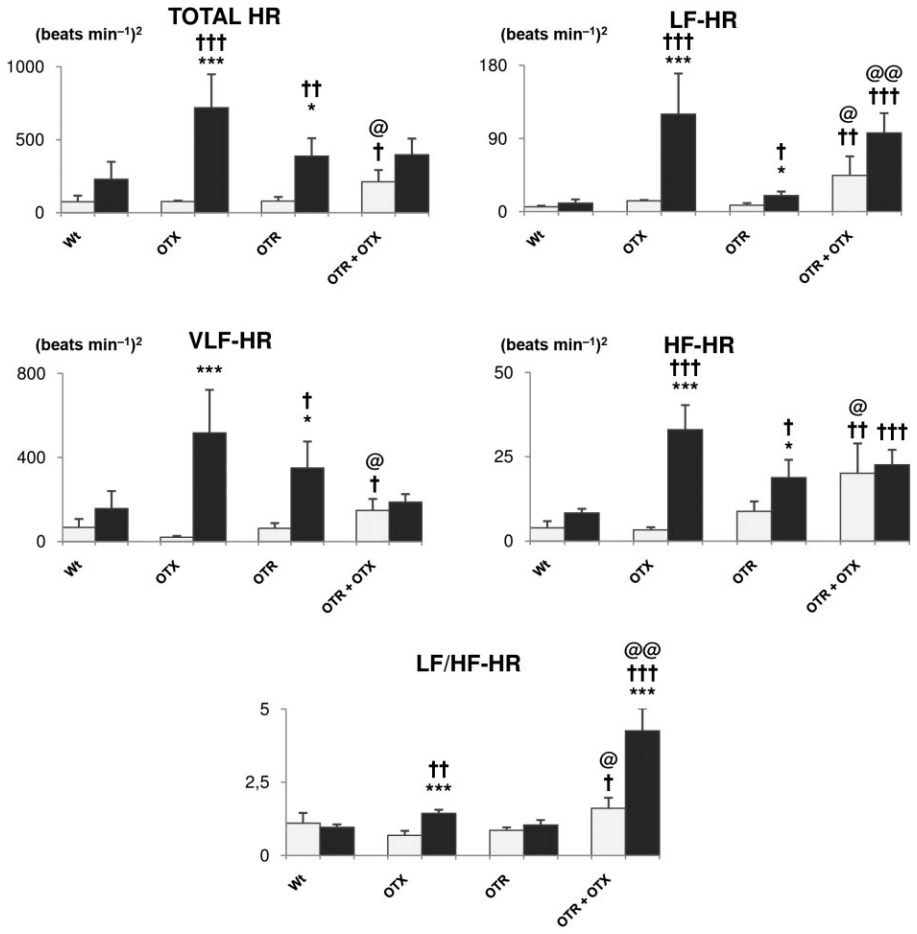

Figure 6

Effects of selective OT receptor antagonist (OTX) in PVN on the components of HR short-term variability of Wt rats and rats overexpressing OT receptors. Wt rats treated with OTX showed increased HR variability in all spectral domains only during stress as well as increased LF/HF-HR ratio. OTR rats treated with OTX exhibited increased HR variability during baseline and stressful conditions as well as increased LF/HF-HR ratio. Open columns indicate baseline values, solid columns indicate stress values. TOTAL-HR, total HR variability; VLF-HR, very low-frequency HR variability. Values are mean of six rats ± SEM. *$P < 0.05$; ***$P < 0.001$ versus baseline; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ versus Wt; $^{@}P < 0.05$; $^{@@}P < 0.01$ versus OTR rats.

vagally-mediated. Pretreatment of these rats with OTX, into the PVN, revealed that ectopic OT receptors buffer BP and HR variability and favour vagal control of the heart (according to changes in LF/HF–HR ratio). This effect on the heart was also unmasked in Wt rats pretreated with OTX but only during exposure to stress (Figure 5). The increase in the vagal influence on the heart during stress was reported to be useful in protecting the heart against sympathetic overstimulation, and involves cholinergic-induced NO synthesis in ventricles (Brack *et al.*, 2012). This protective effect of the vagus is life-saving during cardiac ischaemia, when sympathetic overstimulation triggers life-threatening arrhythmias and sudden death. This assumption is further supported by the work of Wsol and associates (2009) who reported reduced survival of rats after myocardial infarction because of the failure of brain OT to attenuate cardiovascular response to stress.

We also observed that overexpression of OT receptors in the PVN buffers the stress-induced BP variability response mediated by increased sympathetic outflow to blood vessels (LF) and stimulation of respiration (HF). OTX unmasked the buffering effect of OT receptors in PVN in both transfected and Wt rats. The buffering effect on BP variability could be mediated by magnocellular neurons expressing OT and projecting to the pre-Bötzinger region (Mack *et al.*, 2002; 2007). Another possibility is that stress-induced axonal release of OT in the amygdala activates a subpopulation of GABA interneurons that inhibit neurons in the medial amygdala projecting to the brainstem autonomic nuclei (Huber *et al.*, 2005; Viviani *et al.*, 2011; Knobloch *et al.*, 2012). This would attenuate the fear response, limit sympathetic activation and ease respiration, as reflected in HF and LF BP short-term variability. Also, we cannot rule out the possibility that other neurotransmitters synthesized in PVN neurons, especially vasopressin, could have affected BP variability during stress (Pyner, 2009). Our findings are in line with those from a number of animal studies that suggest OT activates an

anti-stress response (Windle *et al.*, 1997; 2004; Lee *et al.*, 2005; Grippo *et al.*, 2009). For instance OT is found to blunt restraint-induced hypothalamo–pituitary axes activation (Windle *et al.*, 1997; 2004), to decrease cardiovascular responding to isolation (Grippo *et al.*, 2009), to reduce anxiety-like behaviour (Windle *et al.*, 1997) and promote social interactions (Lee *et al.*, 2005). In OT knock-out mice, Bernatova and co-workers (2004) demonstrated an accentuated BP and corticosterone response during exposure to acute stress. In line with their findings, Wsol and colleagues (2008) reported that central application of an OT receptor antagonist enhanced the BP and HR increase to environmental stress. Clinical findings in humans also support a role for OT as an anti-stress hormone. Altemus and collaborators (2001) reported that lactating women have greater parasympathetic control of the heart, and Grewen and Light (2011) found that plasma OT in lactating women is correlated with lower cardiovascular reactivity to stress.

In conclusion, our results show for the first time that OT receptors in the PVN are involved in local (autocrine and/or paracrine) regulation of PVN neurons involved in tonic control of BRS and cardiovascular short-term variability. OT receptors in the PVN enhance the sensitivity of the baroreceptor reflex and buffer BP and HR short-term variability favouring vagal control of the heart. These effects are more pronounced in rats overexpressing OT receptors in the PVN than in Wt rats. Our findings open up new perspectives for elucidating the role of OT receptors in the PVN in cardiovascular disease and autonomic control of the circulation.

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Author contributions

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

Conflict of interest

The authors declare that there are no conflicts of interest.

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