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THE HYPOTHALAMIC OXYTOCIN RECEPTORS IN THE PARAVENTRICULAR NUCLEUS IN AUTONOMIC CARDIOVASCULAR CONTROL

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OKSITOCINSKI RECEPTORI U PARAVENTRIKULARNOM JEDRU HIPOTALAMUSA U AUTONOMNOJ KONTROLI KARDIOVASKULARNOG SISTEMA

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SUMMARY

The paraventricular nucleus (PVN) of the hypothalamus is an important integrative site of neuroendocrine control of the circulation. Herein I investigate the role of oxytocin receptors (OTRs) in the PVN in cardiovascular homeostasis. 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 over-express OTRs along with an enhanced green fluorescent protein (eGFP) tag. Control groups were PVN transfected with an Ad expressing eGFP alone or untransfected, sham rats (Wt). Rats were recorded without and with selective blockade of OTRs (OTX), both under baseline and stressful conditions. Baro-receptor reflex sensitivity (BRS) and cardiovascular short-term variability were evaluated using the sequence method and spectral methodology, respectively. Under baseline conditions OTR rats exhibited enhanced BRS and reduced blood pressure (BP) variability in comparison to eGFP and Wt rats. Exposure to stress increased BP, BP variability and heart rate (HR) in all rats. In eGFP and Wt rats, but not in OTR rats, BRS decreased during exposure to stress. Pre-treatment of OTR rats with OTX reduced BRS and enhanced BP and HR variability under baseline and stressful conditions. In Wt rats pre-treated with OTX, BRS was decreased and BP variability was increased under baseline and stress while HR variability was increased only during stress. OTRs in PVN are involved in tonic neural control of BRS and cardiovascular short-term variability. The failure of this mechanism could critically contribute to autonomic deregulation in cardiovascular disease.

Keywords: Oxytocin receptor, paraventricular nucleus of the hypothalamus, adenoviral vector, baro-receptor reflex, blood pressure variability, heart rate variability

Scientific field: Medicine

Scientific subfield: Molecular medicine, pharmacology

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REZIME

Paraventrikularno jedro hipotalamusa (PVN) predstavlja važan integrativni centar neuroendokrine kontrole cirkulacije. U radu je ispitivana uloga oksitocinskih receptora (OTR) u PVN-u u homeostazi kardiovaskularnog sistema.Eksperimenti su izvođeni na budnim, odraslim mužjacima Wistar soja pacova, kojima je ugrađen radiotelemetrijski uređaj radi praćenja i beleženja kardiovaskularnih parametara. Primenom tehnike *in vivo* genskog transfera, u PVN su jednostrano ubrizgani adenovirusni vektori, konstruisani tako da sadrže informaciju potrebnu za sintezu OTR i obeleživača-zelenog fluorescentnog proteina (OTR grupa).Jednoj grupi kontrolnih životinja je u PVN izvršen genski transfer adenovirusnih vektora koji dovode do ekspresije samo obeleživača (eGFP grupa), dok je druga grupa kontrolnih životinja ostala netransfecirana (Wt grupa). Snimanja kardiovaskularnih parametara su vršena u stanju mirovanja i tokom izlaganja stresu, i to na životinjama kojima u PVN nije ubrizgavan selektivni antagonist oksitocinskih receptora (OTX), ali i na životinjama čiji su paraventrikularni oksitocinski receptori blokirani primenom OTX. Senzitivnost baroreceptorskog refleksa (BRS) određivana je metodom sekvenci, dok je u proceni kratkoročnog varijabiliteta kardiovaskularnog sistema korišćena spektralna analiza. Tokom mirovanja, kod životinja u OTR grupi zabeleženo je povećanje BRS, kao i smanjenje varijabiliteta krvnog pritiska u poređenju sa eGFP i Wt grupom. Izlaganje stresu je u svim grupama dovelo do povećanja vrednosti krvnog pritiska, srčane frekvencije, kao i varijabiliteta krvnog pritiska . U eGFP i Wt grupi, ali ne i u OTR grupi pacova, izlaganje stresu je dovelo do smanjenja BRS. Primena OTX u OTR grupije

dovela do smanjenja BRS i povećanja varijabiliteta krvnog pritiska i srčane frekvencije u i u stanju mirovanja i u uslovima stresa. Selektivna blokada oksitocinskih receptora u PVN je u Wt grupi pacova dovela do smanjenja BRS i povećanja varijabiliteta krvnog pritiska pod bazalnim uslovima i tokom stresa, dok je do povećanja varijabiliteta srčane frekvencije došlo samo tokom izloženosti stresoru. OTR u PVN su uključeni u mehanizme toničke nervne kontrole barorefleksa i kratkotrajnog varijabiliteta kardiovaskularnog sistema, čiji poremećaj može ozbiljno doprineti autonomnoj deregulaciji u kardiovaskularnim oboljenjima.

Ključne reči: Oksitocinski receptor, paraventrikularno jedro hipotalamusa, adenovirusni vektor, baroreceptorski refleks, varijabilitet krvnog pritiska, varijabilitet srčane frekvencije

Naučna oblast: Medicina
Uža naučna oblast: Molekularna medicina, farmakologija

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To my parents

1. INTRODUCTION

1.1 Paraventricular nucleus of the hypothalamus

Mille via educunt homines per saecula Romam
(All roads lead to Rome)

The paraventricular nucleus (PVN) is a bilateral structure located in the medial hypothalamus that borders third ventricle.

Although PVN constitutes only 1% of the brain (Swanson, 1995), this morphologically and functionally heterogeneous nucleus is crucially involved in an assortment of functions including parturition, lactation and reproductive behavior, maintenance of body fluid balance, eating, behavior, nociception, cardiovascular regulation, and stress response.

Cytoarchitecture of the PVN

Based strictly on morphological features of the rat brain structure, the PVN may be parceled into two divisions – *magnocellular*and *parvocellular*, the former subdivided into three regions (medial, anterior and posterior) and the latter comprising five regions (periventricular, medial, dorsal, anterior and lateral) (Picture 1). Having in mind that this kind of structural organization of the PVN has never been firmly confirmed in other mammals, including humans, the PVN can also be divided into three major parts based on structure-function grounds (Simmons and Swanson, 2009):

- **Magnocellular neuroendocrine PVN** consists mainly of two types of neurons-oxytocinergic and vasopressinergic. These neurons project to posterior

pituitary, from where vasopressin and oxytocin are released into the systemic circulation

- **Parvocellular neuroendocrine PVN** is made up of separate neuronal populations that project to the median eminence and synthesize corticotropin-releasing hormone (CRH), vasopressin, thyrotropin-releasing hormone (TRH), growth hormone-releasing hormone (GHRH), somatostatin and dopamine.
- **Parvocellular descending part of PVN** contains neurons (also called pre-autonomic neurons) which are involved in autonomic control of bodily functions.

Apart from aforementioned neuronal populations, the PVN and region surrounding this hypothalamic nucleus contain significant number of glutamate and gama-aminobutyricacid (GABA) interneurons (Ferguson *et al*, 2008) and glial cells, the exact roles of which in complex intranuclear interplay are yet to be unveiled.

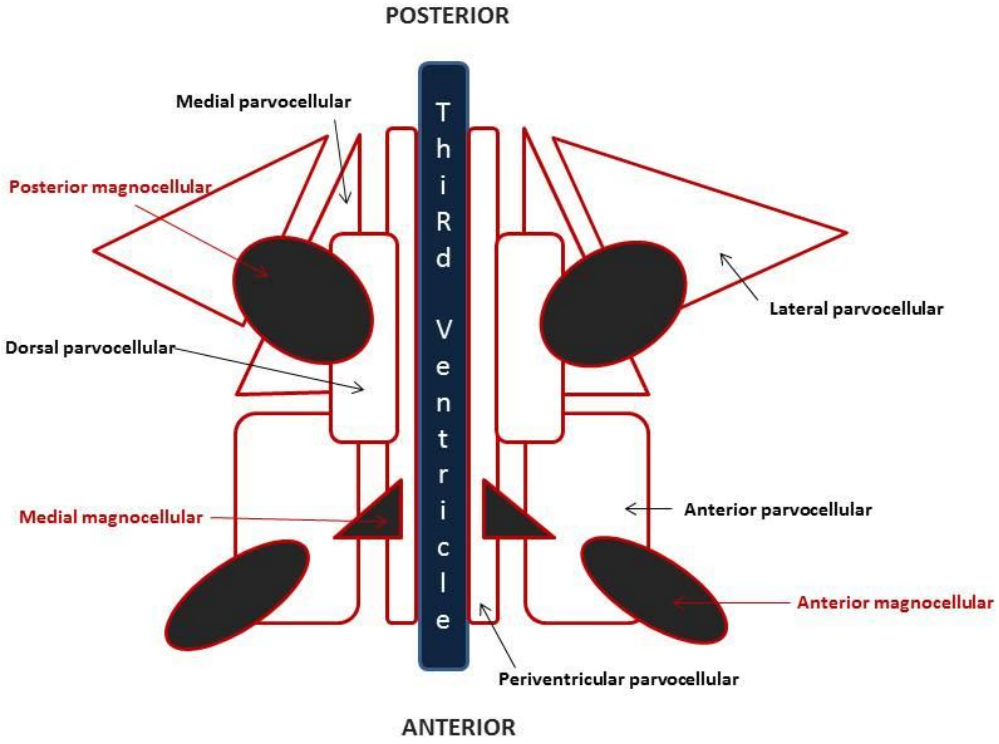

Figure 1. Schematic presentation of magnocellular and parvocellular subdivisions of the paraventricular nucleus of the hypothalamus (modified from Pyner, 2009 and Swanson et al., 1981)

Afferent inputs to the PVN

The PVN receives afferent inputs from various brain regions, such as limbic system (especially lateral septal nucleus and the ventral portion of the subicular cortex), circumventricular organs- the subfornical organ and the organum vasculosum of the lamina terminalis (OVLT), many important integrative centers of the hypothalamus (medial and lateral preoptic areas, suprachiasmatic nucleus, ventromedial nucleus, arcuate nucleus, retrochiasmatic and lateral hypothalamic areas), pons (lateral parabrachial nucleus), and medulla (nucleus tractus solitarii-NTS, dorsal motor nucleus of the vagus, and the ventrolateral medulla).

Cardiovascular afferents relaying information about pressure, volume and oxygen saturation terminate mainly within the NTS (Coote, 2005), and signals from each of these cardiovascular sensory inputs can exert different effects on PVN neurons. Afferents from the NTS target at least four types of PVN-associated neurons (Affleck *et al*., 2012)., i.e. presympathetic and putative magnocellular (neuronal nitric oxide synthase (nNOS)-positive) neurons lying within the PVN, and GABA and nNOS-positive neurons surrounding the PVN. It is noteworthy that the PVN also receives inputs from the contralateral PVN and ipsilateral SON (Silverman *et al*, 1981).

Efferent outputs from the PVN

This highly integrated nucleus sends projections to the neurohypophysis, median eminence and to the centers controlling autonomic functions, proving it right to be called the sentinel of homeostasis. The magnocellular neurons project to the posterior pituitary and secrete vasopressin and oxytocin into the blood stream.

Descending projections from the PVN innervate the dorsal motor nucleus of the vagus, NTS, nucleus ambiguus, central gray matter, Edinger-Westphal nucleus, pedunculopontine tegmental nucleus, nucleus of the locus coeruleus and parabrachial nucleus (Zheng *et al*., 1995).

Efferents to vasomotor centers are elaborated further in the text.

PVN in autonomic control of the cardiovascular system

For years it had been considered that the pivotal role in central regulation of blood pressure belonged to the rostral ventrolateral medulla (RVLM) (Dampney, 1994) and the PVN was thought to contribute to the cardiovascular homeostasis merely by influencingblood volume.Undoubtedly, the RVLM is an

important region of central nervous system (CNS) for tonic regulation of arterial blood pressure(Coote, 2007),butduring last fifteen years, as knowledge about the PVN grew, it became more evident that the PVN is the key integrative site ofautonomic cardiovascular regulation, and also the site which puts together emotional, autonomic and endocrine responses to stress .

Parvocellular pre-autonomic neurons are mutually connected to the NTS (Swanson and Sawschwnko, 1983), the nucleus that provides the PVN with the information coming from the peripheral cardiovascular receptors. In order to maintain cardiovascular homeostasis, the PVN adjusts sympathetic outflow according to the information delivered by the NTS.Neurons located in the lateral, ventral and dorsal subdivisions of the parvocellular part of the PVN project to the pressor region of the RVLM and sympathetic pre-ganglionic neurons inintermediolateral (IML) cell column of the thoraco-lumbal part of the spinal cord. Parvocellularneurons affect sympathetic nerve activity in three ways (Picture 2): directly projecting to the IML, projecting indirectly via RVLM and through a collateral projection to both IML and RVLM (Coote et al.,1998; Pyner and Coote, 1999).

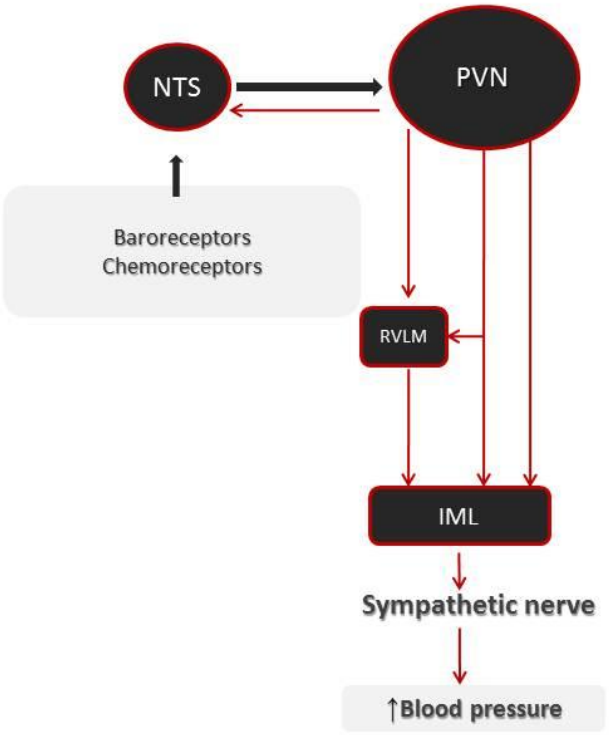

Figure 2. Schematic illustration of the pathways by which the paraventricular nucleus of the hypothalamus affects sympathetic activity.NTS-nucleus tractussolitarius, PVN-paraventricular nucleus of the hypothalamus, RVLM-rostroventrolateral medulla, IML-intermediolateral cell column of spinal cord.

1.2 Oxytocin

Discovery

In 1906 **Sir Henry Hallett Dale** discovered that the extract of human posterior pituitary affects uterine contractility of pregnant cats. Not only did this English

Nobel Prize laureate discover the novel substance, but he also coined the name for it -oxytocin-finding inspiration in Greek words meaning "quick/swift birth".

Soon after the findings of oxytocin, Ott and Scott (1910) and Schäfer and Mackenzie (1911) described its milk ejection properties. During the first hundred years since its discovery, oxytocin has gone a long way from a hormone of maternity to a substance with numerous roles in both central nervous system and periphery.

Biochemistry of oxytocin

Another Nobel laureate, **Vincent du Vigneaud**, won his Prize in Chemistry in 1955 for the isolation, structural identification and synthesis of the oxytocin. Thanks to du Vigneaud, who described his efforts in understanding the structural features of oxytocin in his Nobel lecture called "A trail of sulfa research: from insulin to oxytocin", today we know that the oxytocin is a cyclic peptide which contains nine amino acids (Figure 3) and its systematic name is **cysteine-tyrosine-isoleucine-glutamine-asparagine-cysteine-proline-leucine-glycine-amide.** Oxytocin shares very similar structure to another nonapeptide-vasopressin. Their structures differ in only two aminoacids in positions 3 and 8 (phenylalanine and arginine in vasopressin instead of isoleucine and leucine in oxytocin).

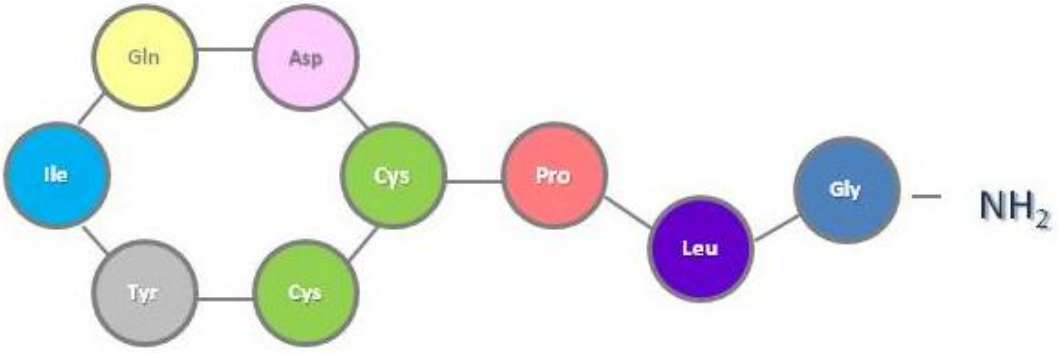

Figure 3. Schematic presentation of oxytocin structure. Cys-cysteine; Tyr-tyrosine; Ile-isoleucine; Gln-glutamine; Asp-asparagine; Pro-proline; Leu-leucine, Gly-glycine

Apart from having similar structure, these two neuropeptides are also evolutionarily well conserved (Acher et al, 1995), share the same peptide ancestor and their genes, which probably originate from the same gene that duplicated due to irradiation 500 million years ago, are located on the same chromosome in mice (Hara et al, 1990), rats (Ivell and Richter, 1984) and humans (Sausville et al, 1985). Table 1 summarizes landmark studies in oxytocin discovery.

Table 1. Landmark studies in oxytocin discovery

Dale, 1906	Discovery of uterine-contracting properties of the extract of the posterior pituitary
Ott and Scott, 1910 **Schäfer and Mackenzie, 1911**	Milk ejecting properties of oxytocin
du Vigneaud, 1953 **Acher, 1953**	Determination of nine amino acid sequence
du Vigneaud, 1954	Synthesis of oxytocin
Brownstein et al., 1980	Discovery of precursor which cleaves into oxytocin and neurophysin during axonal transport to neurohypophysis
Ivell and Richter, 1984	Cloning of oxytocin gene
Pow and Morris, 1989	First notice of dendritic release of oxytocin in SON using electron microscopy
Kimura *et al*, 1992	Cloning of oxytocin receptor gene

Ludwig M *et al*, 2002	First description of mechanisms of dendritic release of oxytocin

Regulation of oxytocin release from the PVN and SON

Oxytocin is mainly synthesized in magnocellular neurons of supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus that project to the posterior pituitary. In response to numerous physiological stimuli, oxytocin is released from axonal endings into the systemic circulation where it acts as a hormone.Oxytocin primarily acts upon the uterus during to initiate labour, and upon myoepitelial cells to induce milk ejection during breastfeeding. In the periphery, oxytocin has been reported to produce natruiresis in the kidneys by enhancing the release of natriuretic peptide in the atria, to produce vasoconstriction and to increase the force of the contraction of the myocardium. Oxytocin is also found to participate in heart development during embryogenesis and heart heeling.

Besides having properties of a "classical hormone", oxytocin is also aneurotransmitter and neuromodulator.Released from the axons of parvocellular neurons of the PVN, oxytocin reaches other brain regions like the eminentia mediana, limbic system, brain stem and spinal cord and participates in various complex processes which include stress response, emotionality, autonomic regulation of bodily functions, *etc*.

Apart from being released from axons, oxytocin is released from somata and dendrites of the magnocellular neurons. Magnocellular neurons typically have two or three long dendrites, and one dendrite contains about 11000 vesicles of the peptide. Somato-dendritically released oxytocin exerts autocrine and paracrine control of neurons in the PVN and SON. They can exert both positive and negative feed-back on oxytocin axonal release and also synchronize the bursting activity of the local population of magnocellular neurons. For instance,

it has been shown that oxytocin released somato-dendritically act upon oxytocin receptors on magnocellular neurons to synchronize the firing rate to produces surges of oxytocin relased in blood stream to boost milk ejection during breast feeding (Ludwig and Leng, 2006).

In addition to being synthesized in the PVN and SON, it is worth mentioning that oxytocin is also (depending on species) produced in the bed nucleus of stria terminalis (BNST), lateral amygdala and medial preoptic area and released within the brain (Young and Gainer, 2003).

1.3 Oxytocin receptor

Structure

The structure and expression of the human OTR was first reported byKimura *et al.*, who described OTR as a 389- amino-acid polypeptide encoded by cDNA that is 4.1 kb in length. The OT receptor is a typical member of the rhodopsin-type (class A) GPCR family (Alexander et al., 2013) with seven hydrophobic transmembrane domains folded in an α-helical conformation and connected by extracellular and intracellular hydrophilic loops (Figure 4).

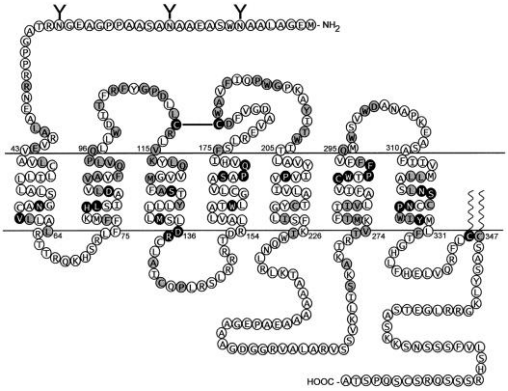

Figure 4. Schematic presentation of oxytocin receptor (from Gimpl and Fahrenholz, 2001).

OTR has high homology (approximately 30-40%) with the receptors for other related nonapeptide hormones such as the vasopressin V1a, V1b, V2 receptors and the teleost fish Arg^8-vasotocin receptor, but the highest degree of sequence similarity (70%) OTR shares with toad mesotocin receptor (Gimpl and Fahrenholz, 2001; Akhundova *et al*., 1996).

OTR cDNAs have been obtained from pig, rat, sheep, bovine and mouse and all of them showed more than 90% amino acid homology with the human OTR (Kimura and Saji, 1995).

Signal tranduction

The principal transduction pathway (Figure 5) of OTR is through $G_{\alpha q/11}$coupled to phospholipase C (PLC), which controls the generation of inositol-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). Activation of IP_3 induces discharge of Ca^{2+} from intracellular stores, whereas DAG stimulates protein kinase C (PKC).

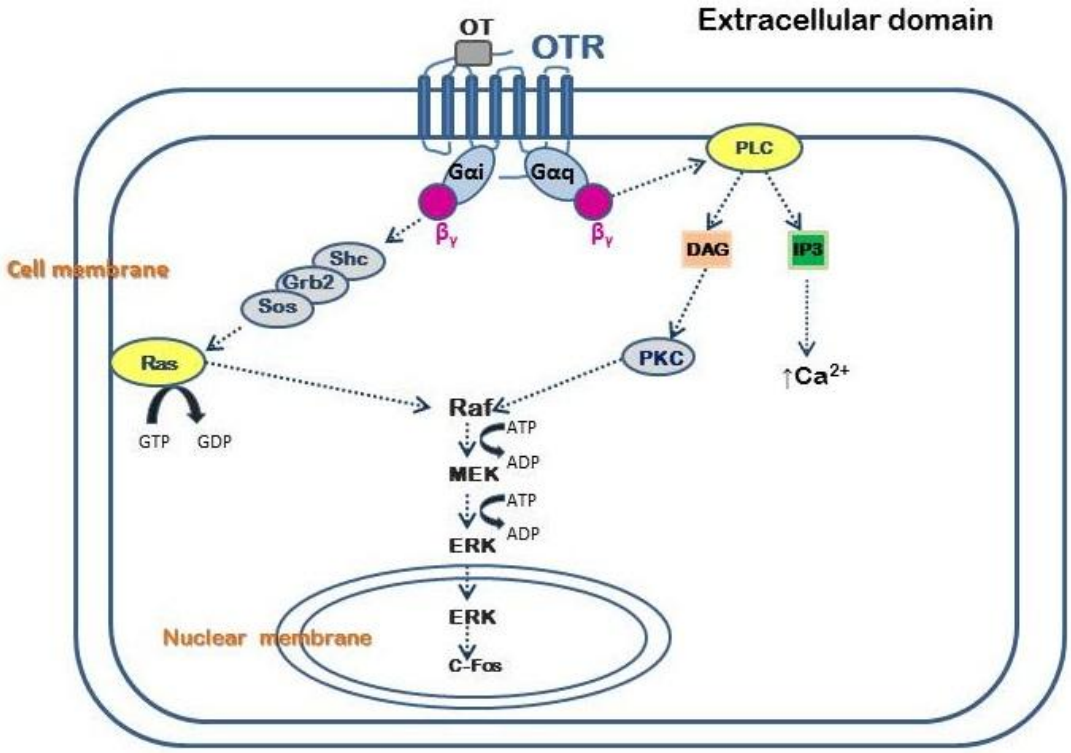

Figure 5. Principal OTR signaling pathways (Modified from Favre *et al.*, 1995)

The rise of intracellular Ca^{2+} is important for initiation of variety of physiological processes. In smooth muscle cells (e.g. myometrial or mammary myoepithelial cells), formation of Ca^{2+}-calmodulin complex leads to contraction, while the rise of Ca^{2+} in neurosecretory cells induces neurotransmitter release.

Several intracellular signaling pathways are reported to be activated *via* OTR coupling to G_q. Both proliferative and anxiolytic effects (Neumann *et al.*, 2007) of oxytocin appear to be G_q-linked and likely involve mitogen-activated protein kinases (MAPK) activation.OTR activation leads to the stimulation of phospholipase A2 production and an increase in cyclooxygenase 2 (COX-2) levels which results in the increase of prostaglandin production and potentiation of smooth musclecontraction, especially of the uterus.

OTR also couples to other G proteins- $G_{\alpha i}$ (leads to inhibition of protein kinase A-PKA signaling pathway) and $G_{\alpha s}$(similar to vasopressin V2 receptor, stimulates PKA signaling pathway, Gimpl and Fahrenholz, 2001).Inhibition of cell growth has been reported to be G_i-mediated (Rimoldi *et al.*, 2003), while the transcriptional upregulation of Gs signaling pathway was found in children with autism and it probably reflects compensatory mechanism for another (unidentified) downstream defect in autistic spectrum disorder (Jacobson *et al.*, 2014).

It seems like the oxytocin-mediated contractile, proliferative, trophic, and antiproliferative effects are supported by complex networks of signaling pathways. The existence of parallel signaling pathways, which are not active at the same time and in the same cell types, may be important in situations when the initial signal needs to be amplified or in order to provide an additional cascade in case the main cascade is impaired.

Peripheral oxytocin receptors

OTRs are widely expressed in peripheral tissues (Figure 6)where they are included in myriad of physiological and pathophysiological processes.

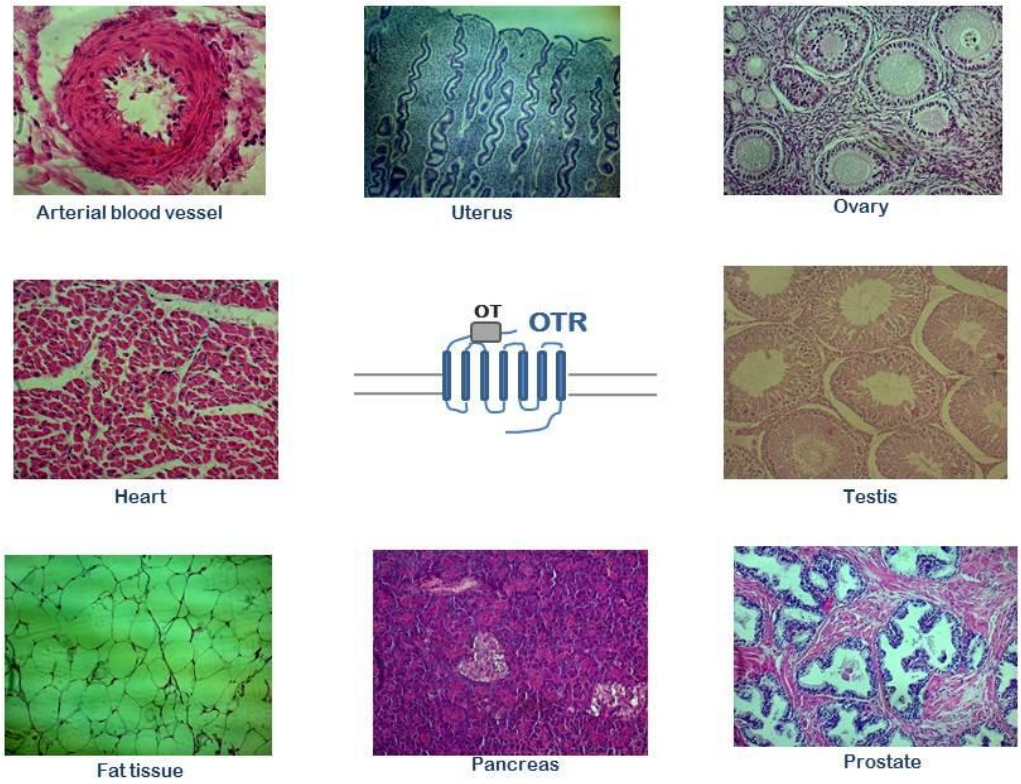

Figure 6. Distribution of peripheral oxytocin receptors (Histological preparations kindly provided by Professor Gordana Kuburović, Department of Histology, School of Dentistry, University of Belgrade).

The first described localization of OTRs was in gravid uterus. During pregnancy and labor OTRs are found in both endometrium and myometrium and their number during early stages of labor increases up to 200-fold compared to non-pregnant state, indicating the important role of sex-hormones in regulation of the OTR expression. In non-pregnant uterus, OTRs are primarily localized in endometrium but their exact role is not completely understood.

The oxytocin receptor mRNA is localized in placental tissue and this finding indicates that there is an oxytocinergic autocrine/paracrine circuit in chorio-decidual layer.

Oxytocin in mammary gland binds to OTRs on myoepithelial cells and stimulates milk ejection during breastfeeding. Recent studies have shown potential involvement of OTRs in the pathogenesis of breast cancer, since OTRs have been described in number of human breast tumor cell lines, e.g. MCF-7 or Hs578T.

OTRs have been detected in ovary, where their density increases during luteal phase.There is also an oxytocinergic autocrine and/or paracrine system in the ovary that regulates steroidogenesis and the early stages of fertilization.

Activation of OT binding sites in male reproductive system leads to contraction of seminiferous tubules and steroidogenesis in testis and contraction of prostate and expulsion of prostatic secretion at ejaculation.

According to Gutkowska and Jankowski (2012), OT and OTRs are present in the cardiovascular system in rat, especially in atria, aorta and vena cava, where they regulate secretion of ANP.

OTRs are also found in kidneys, in fat tissue where OT shows insulin-like activity, in pancreas where OT stimulates release of both insulin and glucagon, in thymus where OT system is involved in immune system functioning.

Central oxytocin receptors

According to Nancy Ostrowski`s work (Ostrowski, 1992) which tackles the distribution of brain OTR *m*RNA, the OTR system in CNS can be divided into four groups:

1. OTRs in brain regions implicated in reproductive (steroid-sensitive) behavior
2. OTRs in brain regions responsible for maternal behavior

3. OTRs involved in cognitive functions-learning and memory
4. OTRs in brain regions involved in reinforcement

OTRs in brain regions implicated in reproductive (steroid-sensitive) behavior. Expression of oxytocin and OTRs, their distribution throughout the brain along with central effects of oxytocin are proven to be gender-dependent and steroid-dependent (Insel*et al*., 1991; Carter, 2007). Not all the brain regions show sexual dimorphism in OTR expression, but regions included in sexual behavior and reproduction show different, often estrogen-dependent levels of expression and patterns of distribution of OTRs. These regions are the ventromedial nucleus of hypothalamus (VMH) and the PVN, the former crucial in lordosis behavior and the latter involved in penile erection. VMH projects to the amygdala, lateral septum and locus coeruelus where OT and its receptors are also detected.

*OTRs in brain regions responsible for maternal behavior.*Two brain regions are important for the initiation of maternal behavior- the PVN and ventral tegmental area (VTA). Interesting findings of Pedersen and coworkers (Pedersen et al., 1992) show the presence of OTRs in VTA only during parturition and lactation, while OTR *m*RNA seems to persist in VTA throughout adulthood. These results indicate the possible involvement of estrogen in translation of OTRs in VTA. OTRs are also found in regions important in full expression in parental behavior-SON, amygdala, lateral septum, BNST and frontal cortex.

OTRs involved in cognitive functions-learning and memory. Although regarded as amnesic, the exact role of oxytocin in memory and learning seems to be unclear. The fact is that oxytocin and its receptor are present in hippocampus and lateral septum, regions involved in the process of learning.

OTRs in brain regions involved in reinforcement. All throughout the dopamine mesolimbic-mesocortical pathway (cingulated and frontal cortex, olfactory tubercle, amygdala, lateral septum, ventral pallidum, nucleus accumbens) engaged in the system of reward it is possible to detect OTR *m*RNA.

The question rises about the expression of OTRs in the brain regions involved in cardiovascular regulation. Today we know that OTRs in humans and rats may be found in almost all important autonomic cardiovascular regions-preganglionic sympathetic and parasympathetic neurons, NTS, RVLM, SON, PVN, VMH and in more rostral neural structures known to be involved in complex autonomic response patterns-amygdala and cingulated cortex (Tribollet E, 1992).

Oxytocin system and cardiovascular homeostasis

An independent oxytocin system has been discovered in the heart and the blood vessels, 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 action 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, oxytocin exerts endocrine and neuromodulator influences on the circulation (Haanwinckel*et al*.,1995; Randolph *et al*., 1998). Oxytocin neurons located in the parvocellular part of the PVN project to the brainstem vagal nuclear complex (nucleus tractussolitarius - NTS, nucleus ambiguus - NA and dorsal vagal nucleus – DVN), rostroventrolateral medulla (RVLM) and the intermediolateral column of the spinal cord (IML) where oxytocin 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 oxytocin mediates the heart rate response to exercise (Martins *et al*., 2005) and heart rate adjustment to stress (Wsol*et al*., 2008). Reduction in oxytocin mRNA in the PVN and oxytocin receptormRNAs in the brainstem were described in

genetically hypertensive rats (Martins *et al*., 2005), while failure of central oxytocin was associated with increased cardiovascular reactivity to stress in rats survivors of myocardial infarction (Wsol*et al*., 2009).

1. IZVOD IZ UVODA

Oksitocin je ciklični neuropeptid koji se sastoji od 9 aminokiselina dobro poznate uloge u reprodukciji i laktaciji. Za otkriće, izolaciju i sintezuoksitocina, 1955. godine Nobelovu nagradu dobio je Vincent du Vigneaud. Glavni izvor oksitocina u organizmu su neuroni paraventrikularnog (PVN) i supraoptičkog jedra (SON) hipotalamusa.

Poslednjih decenija, pokazano je da oksitocin učestvuje i u regulaciji kardiovaskularnog sistema, kako na periferiji tako i u centralnom nervnom sistemu.

U srcu i u krvnim sudovima, otkriven je nezavisni oksitocinski sistem neophodan za normalan razvoj srca, regeneraciju i izlučivanje natrijuma putem bubrega. Oksitocin deluje direktno na sam srčani mišić negativno inotropno i negativno hronotropno, kao i na krvne sudove, izazivajući vazokonstrikciju. Vazokonstrikcija koju izaziva oksitocin je blaga, osim u umbilikalnoj arteriji kod porođaja. U nekim vaskularnim koritima, kao na primer u bazilarnoj arteriji, oksitocin prouzrokuje NO-zavisnu vazodilataciju.

U centralnom nervnom sistemu, oksitocinski neuroni lokalizovani u magnocelularnom delu PVN-a, završavaju se u neurohipofizi odakle se oksitocin otpušta u krv i ostvaruje periferne efekte. Iz parvocelularnog dela PVN-a oksitocinski neuroni se projektuju u produženu moždinu, do jedra vagalnog jedarnog kompleksa, rostroventrolateralne medule i intermediolateralne kolumne kičmene moždine. Na tim mestima oksitocin, stimulacijom specifičnih receptora, modulira i parasimpatičku i simpatičku kontrolu cirkulacije.

Oksitocin ostvaruje svoje efekte stimulacijom specifičnog oksitocinskog receptora koji spada u klasične transmembranske, G-protein vezane receptore (OTR). OTR su široko rasprostranjeni na periferiji i u centralnom nervnom

sistemu. Posebno je pokazana velika gustina OTR kolokalizovanih sa neuronima u PVN-u za koje se smatra da imaju važnu ulogu u autoregulaciji oksitocinskih neurona u magnocelularnom delu PVN-a.

2. HYPOTHESIS &AIMS

The focus of present work is the role of OTRs found in PVN, which have been reported to play an important role in autoregulation of magnocellular neuronal activity (Richard *et al*., 1997).

I hypothesized that, by increasing the number of OTRs located in PVN, and by selectively blocking their activity, it might be possible to modulate PVN neuronal activity involved in autonomic cardiovascular control.

To test the hypothesis, I used genetic tools, microinjection of adenoviral vectors (Ads) carrying the tagged gene for OTR into the PVN to induce over-expression of OTR and pharmacological tools, microinjections of selective OTR antagonist in the PVN of conscious transfected and non-transfected wild type rats, both under baseline conditions and stress.

The aims of the study were to investigate:

1. the effects of over-expression of OTR in the PVN of freely moving rats, on blood pressure, heart rate, their short-term variability and baroreceptor reflex functioningboth under baseline conditions and during exposure to emotional stress.

2. the effects of selective pharmacological blockade of OTR in the PVN of freely moving wild type rats, on blood pressure, heart rate, their short-term variability and baroreceptor reflex functioning both under baseline conditions and during exposure to emotional stress..

3. the effects of selective blockade of OTR in the PVN of freely moving ratsover-expressing OTR, on blood pressure, heart rate, their short-term variability and baroreceptor reflex functioning both under baseline conditions and during exposure to emotional stress.

2. HIPOTEZA I CILJEVI ISTRAŽIVANJA

Pretpostavili smo da će selektivna blokada OTR, ili pak povećanje gustine OTR u PVN-u, značajno menjati autonomnu kontrolu krvnog pritiska i srčane frekvencije, kako pod bazalnim fiziološkim uslovima, tako i prilikom izlaganja emocionalnom stresu.

Cilj istraživanja

Da bismo ispitali postavljenu hipotezu, koristićemo kombinovani genetski i farmakološki metodološki pristup: *in vivo* genski transfer oksitocinskih receptora u PVN-u pacova i mikroinjekcije selektivnog antagoniste oksitocinskih receptora, kod normalnih i transgenih pacova.

Pod bazalnim fiziološkim uslovima i nakon izlaganja akutnom emocionalnom stresu ispitaćemo efekte:

1. povećanja ekspresije OTR u PVN-u hipotalamusa na kratkoročni varijabilitet srčanog pritiska i frekvencije i na funkcionisanje baroreceptorskog refleksa pacova u stanju mirovanja i tokom izlaganja akutnom emocionalnom stresu

2. selektivne farmakološke blokade OTR u PVN-u hipotalamusa na kratkoročni varijabilitet srčanog pritiska i frekvencije i na funkcionisanje baroreceptorskog refleksa pacova u stanju mirovanja i tokom izlaganja akutnom emocionalnom stresu

3. selektivnefarmakološke blokade OTR u PVN-u hipotalamusa pacova sa povećanom ekspresijom OTR na kratkoročni varijabilitet srčanog pritiska i frekvencije i na funkcionisanje baroreceptorskog refleksa u stanju mirovanja i tokom izlaganja akutnom emocionalnom stresu

3. METHODS

All experimental procedures in this study conformed to European Communities Council Directive of November 24, 1986 (86/609/EEC). The experimental protocol was approved by the School of Medicine University of Belgrade Ethics review board and and the results of the study are reported in accordance with The ARRIVE Guidelines.

3.1 Animals

Experiments were performed in male, twelve week old male Wistar rats weighing 310-360g bred at the local animal facility. 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, Veterinarskizavod, 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/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 a combination of three anaesthetics (0.1 ml, i.p. of T61® solution).

3.2 Implementation of radiotelemetry device

Under combined ketamine (100 mg·kg^{-1}, i.m.) and xylazine (10 mg·kg^{-1}, i.m.) anaesthesia, 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 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. To prevent bacterial infection neomycin and bacitracin were sprayed topically, and the rats were treated with gentamicin (25 mg·kg^{-1}, i.m.) three 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 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.

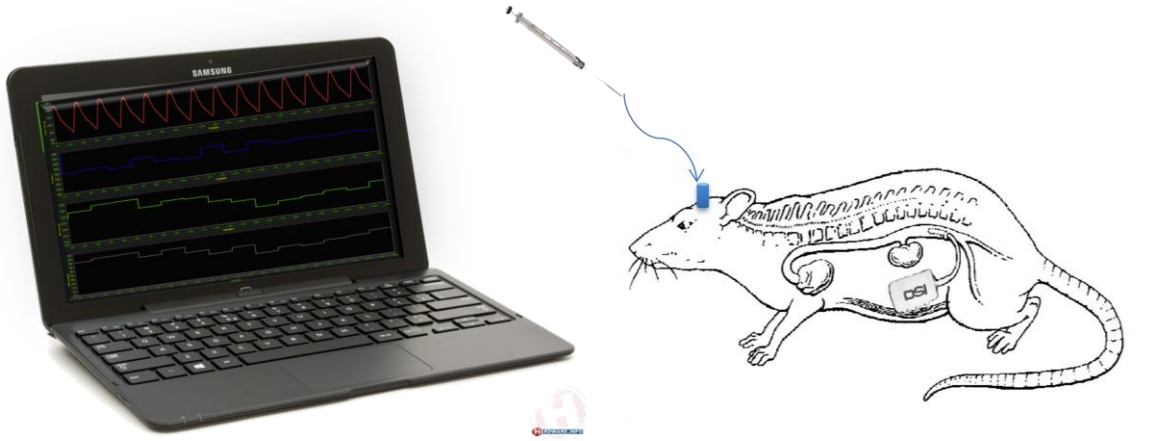

Figure 7. Registration of cardiovascular parameters of male Wistar rat equipped with radiotelemetric device implanted in abdominal aorta and treated with a drug through PVN cannula.

3.3 Adenoviral vector production

The cDNA clone of the rat OTR in pcD2 was generously provided by Dr. Stephen Lolait, University of Bristol (Jeng*et al*., 1996). The OTR was amplified

from pcD2 using Phusion High-Fidelity DNA polymerase (New England Biolabs) and primers OTR_F (5′-GTCTCGAGCATGGAGGGCACGCCAGCA-3′) and OTR_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). 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 calcium phosphate method in accordance with manufacturer's guidelines (Cell Biolabs). 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 titres were determined in triplicate by standard plaque assay.

3.4 *In vivo* gene transfer

Ten days after fitting the telemetry device, 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 (Figure)and the skin was incised 3 mm to expose the skull.

Figure 8. Stereotaxic frame and stereomicroscope

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(titre $4 \cdot 10^{10}$ pfu $\cdot mL^{-1}$) 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, the skin above 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 microinfusion of OTR antagonist. In the post-operative period rats were treated with gentamicin (25 mg $\cdot kg^{-1}$i.m.) one day before, on the day of surgery and 2 days after surgery and carprofen (5 mg $\cdot kg^{-1}$ daily, s.c.) on the day of surgery and two days after. Rats were left to recover for seven days, time necessary for maximal expression of transfected gene(Lonergan T *et al*., 2005).

3.5 Evaluation of OTR expression

Tissue preparation and collection

Following sacrifice, brains were carefully removed and snap-frozen with powdered dry ice. Hypothalamic paraventricular nuclei 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 (Watson &Paxinos, 2004). Following identification, 60µm caudal-rostral slices were taken from PVN using a cryostat (Leica Microsystems CM1900, Leica Microsystems Nussloch GmbH, Nussloch, Germany) maintained between -18 ^{o}C and -20 ^{o}C.Bilateral tissue punches of Left and Right PVN were obtained using a micro-punch (0.1mm 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 -80 ^{o}C, until extraction.

RNA extraction

All procedures were carried out in an RNase-free environment and all solutions made up using RNase-free water/reagents. To each sample tube, 1ml TRIzolLysis Reagent (Life Technologies, Paisley, UK; Cat No. 15596-018) was added and samples were mixed by briefly vortexing. Samples were then allowed to stand at room temperature (RT) for 5min prior to centrifugation (10,300 rpm, 4 ^{o}C) for 10min, in order to pellet any cellular debris. The resulting supernatant was collected, and added into a new microcentrifuge tube containing200ul of chloroform (with amylenes as stabilizer ≥99% (Sigma Aldrich). Samples were then mixed by vortex for 20sec and then allowed to stand for 5min at room temeperature. To separate organic and aqueous phases, samples were centrifuged for 15min (11,200 rpm, 4 ^{o}C), and the aqueous phase (350µl from approx. total 500µl) was collected and added into a new

microcentrifuge tube containing 1 volume (350µl) of 70% (v/v) EtOH, in order to precipitate total RNA. Further purification was performed using the RNeasy Mini Kit according to manufacturer's protocol (Qiagen; Qiagen LTD., Manchester, UK; Cat No. 74104). Purified RNA was then quantitatified using an ImplenGeneflowNanophotometer, and then stored at -20 ^{o}C until cDNA synthesis.

cDNAsynthesis

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

Real Time qRT-PCR and data 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 primers for OTR(Rn_OTR_1_SGl, Quantitect Primer Assay, Qiagen, Qiagen Ltd., Manchester, UK). . Expression via RT-qPCR was analysed for all genes ona 96 well PCR plate (MicroAmp Fast 96-Well Reaction Plate (0.1mL), Ref; 4346907, Applied Biosystems, Foster City, CA, USA), with each well containing; 2ng of *c*DNA (1µl per well) along with 11µl 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 (MicroAmp Optical Adhesive Film, Ref; 210404056, Applied Biosystems) and centrifuged for 30sec to ensure proper mixing of reagents and to remove air bubbles. RT-qPCR analysis was performed using anAppliedBiosystemsStepOnePlus Real Time PCR System in conjunction with theStepOne v2.2.2 software. RAW data were firstly exported andintoExcelwhere'Delta-Ct' (Target gene Ct – Rpl19 Ct) was calculated for each sample tested. Following this, an exponentiation of 'Power2' against the negative of Delta-Ct was applied, and finally any difference in expression was assessed by normalising Left PVN to 1, and dividing the 'Power2' of Right PVN by Left PVN. Specific statistical analysis was undertaken usingGraphPad Prism (Version 6) Software to apply a Wilcoxon-Signed Rank test to determine if significant expression differences between Left & Right PVN existed for eGFP and the gene of overexpression.

TISSUE PREPARATION AND IMMUNOHISTOCHEMISTRY

Tissue perfusion and preparation for immunohistochemistry

At the end of experiments rats were anesthetized and transcardially perfused with 100 ml of 0.1 M phosphate-buffered saline (PBS pH 7.4) at room temperature followed by 300 ml of 4% (w/v) ice-cold 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 (35µm) 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 OTRs.

Tissue staining

For immunohistochemical detection of OTRcommercially available goat polyclonal anti-OTR antibody was used (1:100, Santa-Cruz, USA, catalogue n^{o} sc-8103). Free-floating rat hypothalamic sections were incubated for 30 minutes in a blocking solution comprising 10% normal horse serum (NHS; Sigma-Aldrich Co. Ltd., Poole, Dorset, UK), and 0.3% (v/v) Triton X-100 (Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) in 0.1 M PBS followed by rinses (3x10min) in PBS. Sections were then incubated in goat anti-OTR primary antibody (dilution 1:100) in PBS containing 1% (v/v) NHS and 0.3% (v/v) Triton X-100 overnight. After the primary antibody incubation, sections were rinsed in PBS (3x10min) before a 1-hr incubation in PBS containing donkey Alexa Fluor 594 anti-goat IgG (dilution 1:100, Abcam, UK), 10% (v/v) NHS and 0.3% (v/v) Triton X-100 at room temperature. Following rinses in PBS (3x10min) sections were mounted onto glass microscope slides with 0.5% (w/v) gelatin and allowed to air dry for several minutes. Once dry, the slides were dehydrated in ethanol (75%, 85%, 96% v/v), cleared in Histoclear (RA Lamb, UK), and cover slipped in DPX mountant (VWR, UK).

3.6 Pilot experiments

Pilot experiments were performed to determine the selective dose of OTR antagonist. Six rats equipped with radiotelemetric device and intrahypothalamic cannula was used. Following vehicle application (200 nL $\cdot$min^{-1} 0.9% NaCl), increasing doses of oxytocin (30 ng, 100 ng and 300 ng) in a volume of 200 nL were microinfused for 1 minute in PVN of conscious rats, at 2 hours interval. Cardiovascular parameters were recorded between OT administrations for 60 minutes. Five days later, non-peptide OTR antagonist

(OTX) and OT were co-administered to rats to test OTX blocking efficacy. Subsequently, cardiovascular parameters were recorded for 60 minutes.

3.7 Experimental design

Seven days post-transfection, rats were subjected to experimentation. All experiments started around 10 a.m. in quiet surroundings under controlled environmental conditions, following 60-minute-long baseline recordings of 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 (n=6) to stress and during recovery period until normalization of BP and heart rate (HR). Stress was induced by directing air-jet (bottle-compressed under 1 bar) to the top of rats' head avoiding its snout. A separate group of wild type rats (n=6) and transfected rats (n=6) equipped with radiotelemetric device and intrahypothalamic cannula for drug injection, were subjected to microinfusion of OTX (300 ng·200 nL^{-1}; n=6) or saline (200 nL·min^{-1}; n=6) to be recorded under baseline conditions for 20 minutes and 10 minutes during exposure to stress.

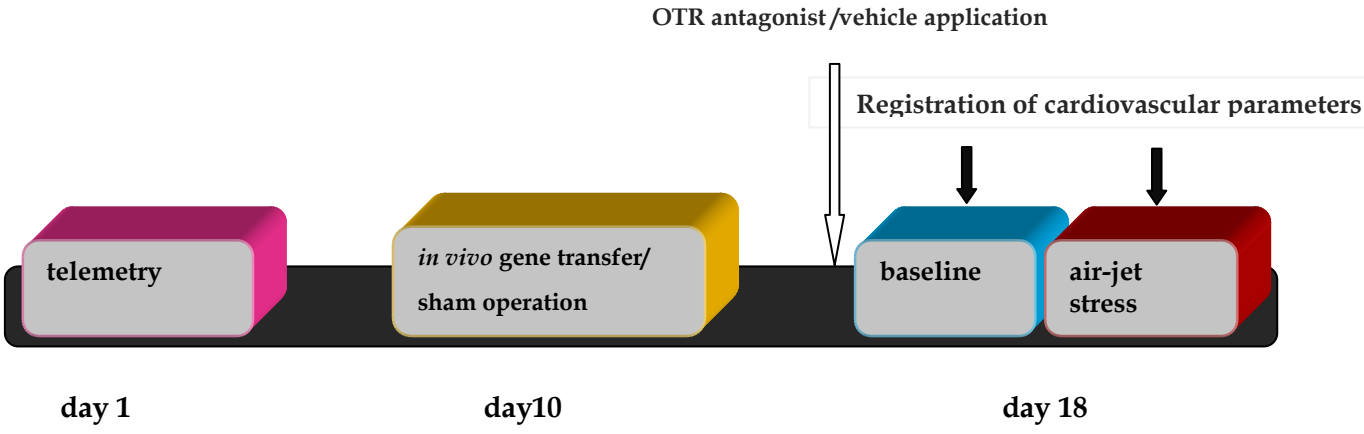

Figure 9. Timeline of experimental protocol

3.8 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, 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.

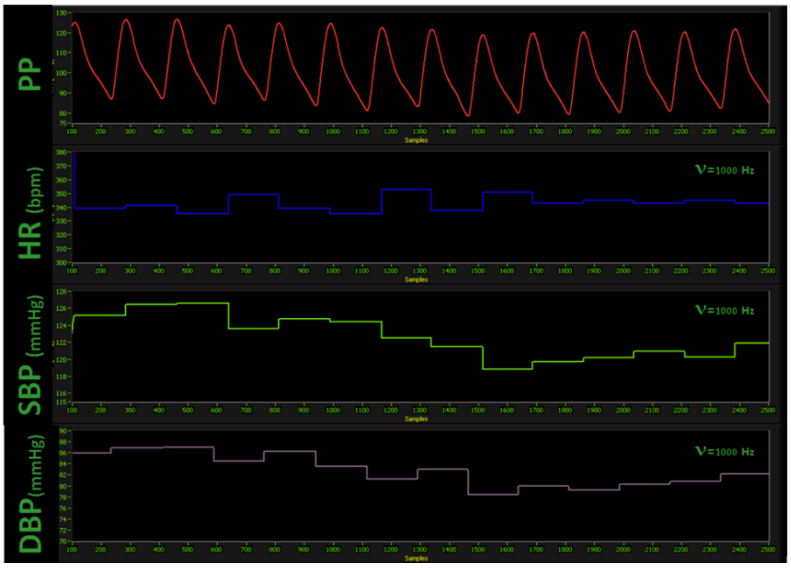

Figure 10. **Pulse pressure recording.**PP-pulse pressure; HR-heart rate; SBP-systolic blood pressure; DBP-diastolic blood pressure

Evaluation of the spontaneous baroreceptor reflex by sequence method

The spontaneous baro-receptor reflex sequence (Bajić*et al*., 2010)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 baro-receptor reflex [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).

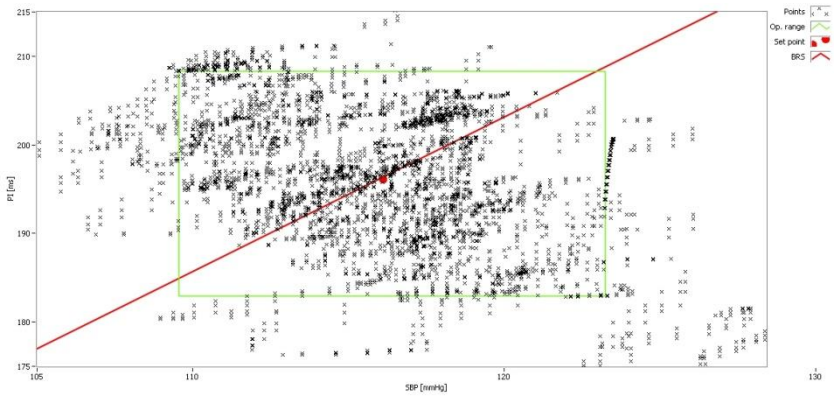

Figure 11. Parameters of spontaneous baroreceptor reflex in Wistar rat

Spectral analysis of blood pressure and heart rate

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 (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 (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 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-Žigon, 1998).

3.9 DRUGS

Non-peptide and selective OTR 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. Oxytocin acetate was purchased from Sigma Aldrich (UniChem, Belgrade, RS). Ketamine, xylazine, carprofen (Rimadyl®) and combination of embutramide plus mebezonium plus tetracaine (T61®) injections were purchased from MarloFarma (Belgrade, RS). Gentamicin (Gentamicin®) injections and bacitracin plus neomycin spray (Bivacyn®) were purchased from Hemofarm (Vršac, RS).

3.10 STATISTICS

Cardiovascular parameters are presented as mean ± standard error of the mean. Multiple comparisons of hemodynamic parameters between experimental groups were performed by ANOVA for repeated measures followed by *posthoc*Bonferroni test using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). Wilcoxon signed rank test was used to evaluate mRNA expression data. Statistical significance was considered at $p<0.05$.

4. RESULTS

4.1 Verification of microinfusion sites and Ad expression

The position of the guide cannula in the PVN was confirmed histologically and is shown in Figure 12. The efficacy of Ad transfection in PVN at the microinfusion site is illustrated by eGFP fluorescence (Figure 13A, C), OTR immunostaining (Figure 13B, C) and increased *m*RNA of OTR in PVN at Ad virus infusion site (Figure 13D).

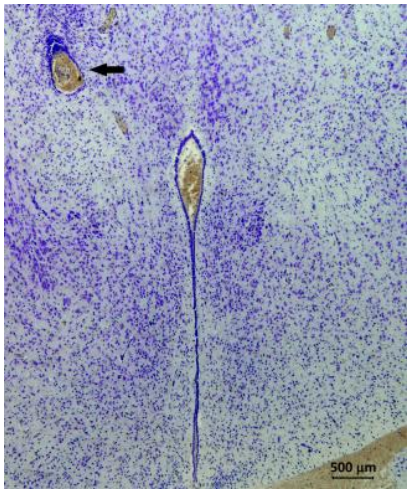

Figure 12.Microinfusion site in PVN (-1.8 mm from Bregma). Representative picture.The arrow points to the lesion made by chronic cannulation. Magnification 4x.

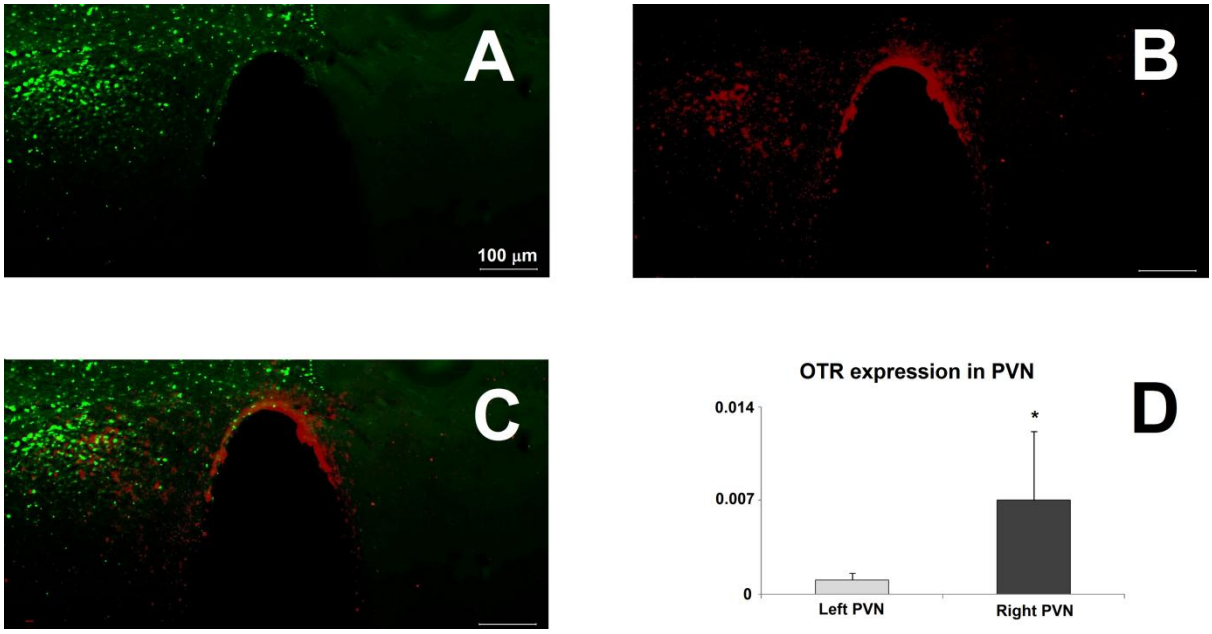

Figure 13.Adenoviral vector transfections site in PVN (-1.8 mm from Bregma). eGFP fluorescence (A), immunostaining to OTRs (B) merged A and B (C), and relative mRNA expression of OTR in PVN (D). Magnification 10 x.

4.2 Pilot experiments

The dose response with OT shown in table 2 was performed in n=6 conscious Wild-type rats. OT microinfused in 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 minutes. The hypertensive effect and tachycardia induced by 100 ng of OT was prevented by pre-treatment of rats by OTX in a dose of 300 ng. OT infused alone in a dose of 100 ng increased SBP from 133 ± 2 mmHg in saline treated rats to 152 ± 1 mmHg (p<0.01) or to 135 ± 4 mmHg (non significant, p>0.05) in 300 ng OTX pre-treated 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 pre-treated 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 pre-treated rats

(non significant, $p>0.05$). HR increased from 363 ± 14 bpm to 446 ± 18 bpm in 100 ngOT treated rats ($p<0.01$) or to 395 ± 31 bpm in OTX pre-treated rats (non significant, $p>0.05$).

Table 2 Effects of OT microinjected in the PVN on blood pressure and heart rate of conscious Wt rats

	SBP **(mmHg)**	MBP **(mmHg)**	DBP **(mmHg)**	HR **(bpm)**
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 6 rats ± s.e.m. SBP: systolic blood pressure; MBP: mean blood pressure; DBP: diastolic blood pressure; HR: heart rate. $^{**}p<0.01$; $^{***}p<0.001$ *vs.* saline.

4.3 Cardiovascular parameters in the rats over-expressing OTRs 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 3). In contrast, rats over-expressing OTRs in the PVN (OTR group) exhibited increased values of SBP, MBP and enhanced BRS compared to 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 whilst in OTR rats BRS did not decrease (table 3).

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 14). However, in OTR rats, reduction of SBP and DBP total variability due to the statistically significant decrease of VLF variability was observed. Concomitantly, HF-SBP and HF-DBP variability increased.

When rats were exposed to air-jet stress, BP variability increased due to the increase of variability in all spectral bands. However, the increase of LF and HF variability in SBP and LF in DBP spectra was statistically significantly smaller in OTR rats as compared to both eGFP and Wt groups (Figure 14).

Under baseline physiological conditions, HR variability in rats over-expressing OTRs in PVN did not differ from eGFP or Wt controls (Figure 15). However, when rats were exposed to acute stressful conditions, OTR rats exhibited an increase of HR variability in all spectral bands without changes in LF to HF ratio (Figure 15) suggesting a concomitant sympathetic and vagal stimulation of the heart.

Table 3 BP, HR and BRS in rats over-expressing OT receptors in thePV

		SBP (mmHg)	MBP (mmHg)	DBP (mmHg)	HR (bpm)	BRS (ms/mmHg)
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 6 rats ± s.e.m..Wt: wild-type rats; eGFP: rats transfected with enhanced green fluorescent protein; OTR: rats over-expressing oxytocin receptors; SBP: systolic blood pressure; MBP: mean blood pressure; DBP: diastolic blood pressure; HR: heart rate; BRS: baro-receptor reflex sensitivity. *p<0.05; **p<0.01; ***p<0.001 *vs.* baseline; †p<0.05; ††p<0.01 *vs.*eGFP-transfected rats; ‡p<0.05; ‡‡p<0.01 *vs.* wild-type rats.

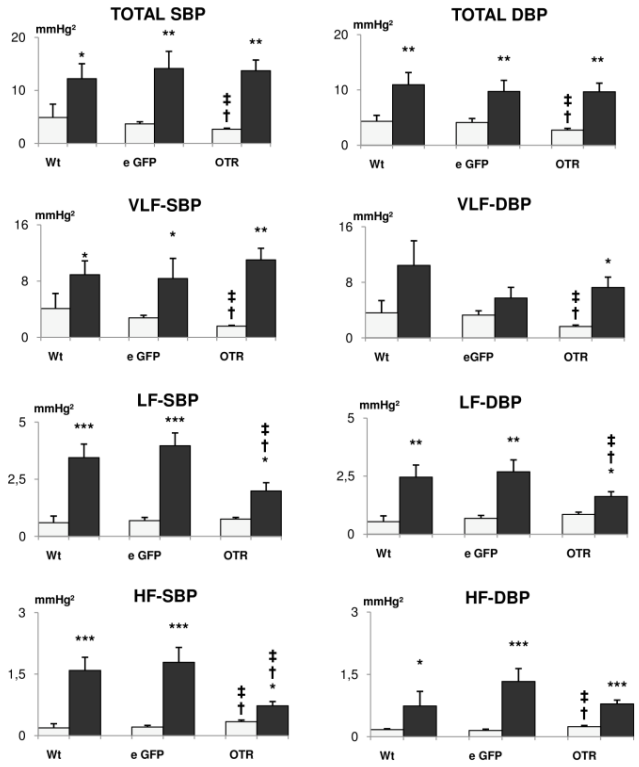

Figure 14. **Components of BP short-term variability in rats over-expressing OTRs in PVN**
Empty bars indicate baseline values, black bars indicate stress values. Wt: wild-type rats; eGFP: rats transfected with enhanced green fluorescent protein; OTR: rats over-expressing oxytocin receptors. TOTAL-SBP: total systolic blood pressure variability; VLF-SBP: very low-frequency systolic blood pressure variability; LF-SBP: low-frequency systolic blood pressure variability; HF-SBP: high-frequency systolic blood pressure variability; TOTAL-DBP: total diastolic blood pressure variability; VLF-DBP: very low-frequency diastolic 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; †$p<0.05$ *vs.*eGFP; ‡$p<0.05$ *vs.* Wt.

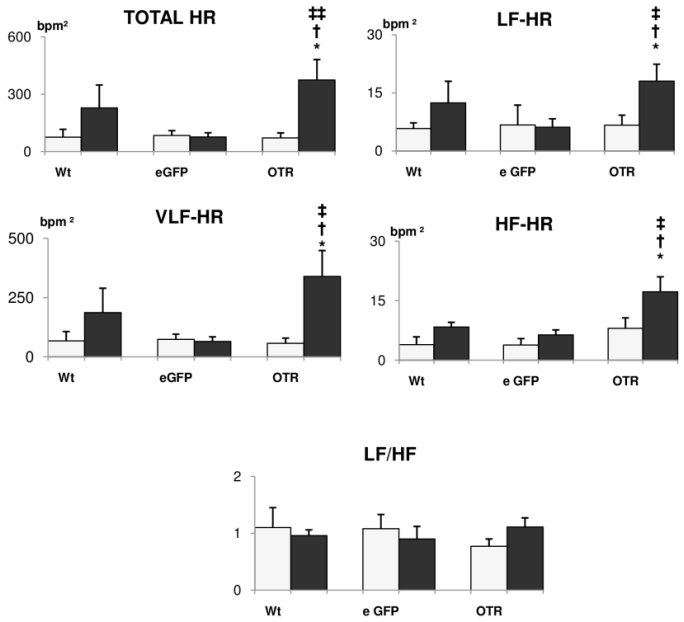

Figure 15.Components of HR short-term variability in rats over-expressing OTRs in PVN

Empty bars indicate baseline values, black bars indicate stress values. Wt: wild-type rats; eGFP: rats transfected with enhanced green fluorescent protein; OTR: rats over-expressing oxytocin receptors; TOTAL-HR: total heart rate variability; VLF-HR: very low-frequency heart rate variability; 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; †p<0.05 *vs.*eGFP rats; ‡p<0.05; ‡‡p<0.01 *vs.* Wt.

4.4 Effects of OTX on cardiovascular parameters in wild-type rats and rats over-expressing OTRs in the PVN

In wild-type rats, under baseline physiological conditions, microinfusion of OTX into the PVN significantly reduced BRS in respect to non-treated rats, and had no effect on mean levels of SBP, DBP, MBP and HR (table 4). In these rats, LF-SBP LF-DBP and HF-SBP spectral domains increased (Figure 16). HR variability was not affected significantly by OTX under baseline physiological conditions (Figure 17).

Wild type rats pre-treated by OTX and exposed to stress exhibited similar increase of SBP as non-treated rats, but the increase in MBP, DBP and HR were smaller and the BRS remained reduced (table 4). In these rats SBP and DBP variability (Figure 16) and HR variability increased in all spectral domains, as well as the ratio LF/HF-HR (Figure 17).

In rats over-expressing OTR, microinfusion of OTX under baseline conditions reduced BRS in comparison to both non-treated OTR rats and Wt rats (table 4). OTX had no effect on mean values of SBP, DBP, MBP and HR of OTR rats in respect to OTR non-treated rats, but SBP and MBP remained higher in respect to Wt rats (table 4). OTR rats pre-treated 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 4).

In OTX-treated OTR rats, both under baseline and stressful conditions, SBP, DBP (Figure 16) and HR variability (Figure 17) increased in all spectral domains. Also, in rats over-expressing OTRs in PVN and pre-treated with OTX, the increase of LF/HF-HR occurred both under baseline and stressful conditions (Figure 17) in respect to non-treated OTR rats and in respect to Wt OTX treated rats.

Table 4 **Effects of selective OTR antagonist microinfused in PVN on BP, HR and BRS of Wild-type rats and rats over-expressing OT receptors**

		SBP (mmHg)	MBP (mmHg)	DBP (mmHg)	HR (bpm)	BRS (ms/mmHg)
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 ‡@@
	Stress	151 ± 6**	111 ± 5 ** ‡	94 ± 1** ‡	414 ± 5 ** ‡	1.7 ± 0.6 @@

Values are mean of 6 rats ± s.e.m..Wt: wild-type rats; OTX_{Wt}: Wild type ratsmicroinfused with oxytocin receptor antagonist in PVN; OTR: rats over-expressing oxytocin receptors; OTX_{OTR}: OTR rats microinfused with oxytocin receptor antagonist in PVN; SBP: systolic blood pressure; MBP: mean blood pressure; DBP: diastolic blood pressure; HR: heart rate; BRS: baro-receptor reflex

sensitivity. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ *vs.* baseline; $^{‡}p<0.05$; $^{‡‡}p<0.01$ *vs.* wild-type rats; $^{@@}p<0.01$ *vs.* OTR rats.

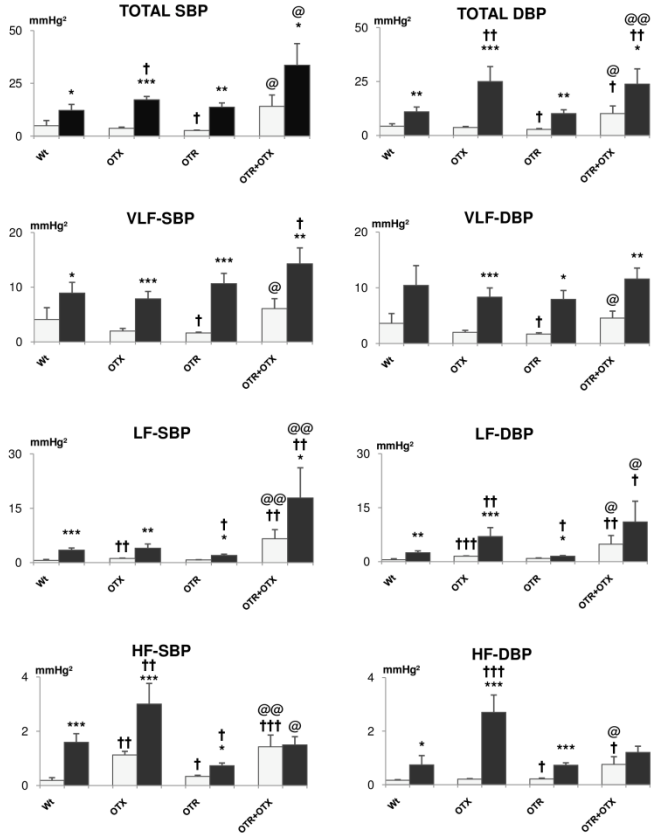

Figure 16.Effects of selective OTR antagonist (OTX) in PVN on the components of BP short-term variability of Wild type rats and rats over-expressing OT receptors.

Empty bars indicate baseline values, black bars indicate stress values. Wt: wild-type rats; eGFP: rats transfected with enhanced green fluorescent protein; OTR: rats over-expressing oxytocin receptors. TOTAL-SBP: total systolic blood pressure variability; VLF-SBP: very low-frequency systolic blood pressure variability; LF-SBP: low-frequency systolic blood pressure variability; HF-SBP: high-frequency systolic blood pressure variability; TOTAL-DBP: total diastolic blood pressure variability; VLF-DBP: very low-frequency diastolic 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; $^{†}p<0.05$, $^{††}p<0.01$, $^{†††}p<0.001$*vs.*Wt; $^{@}\underline{\boldsymbol{p}}<0.05$; $^{@@}\underline{\boldsymbol{p}}<0.01$ *vs.* OTR rats.

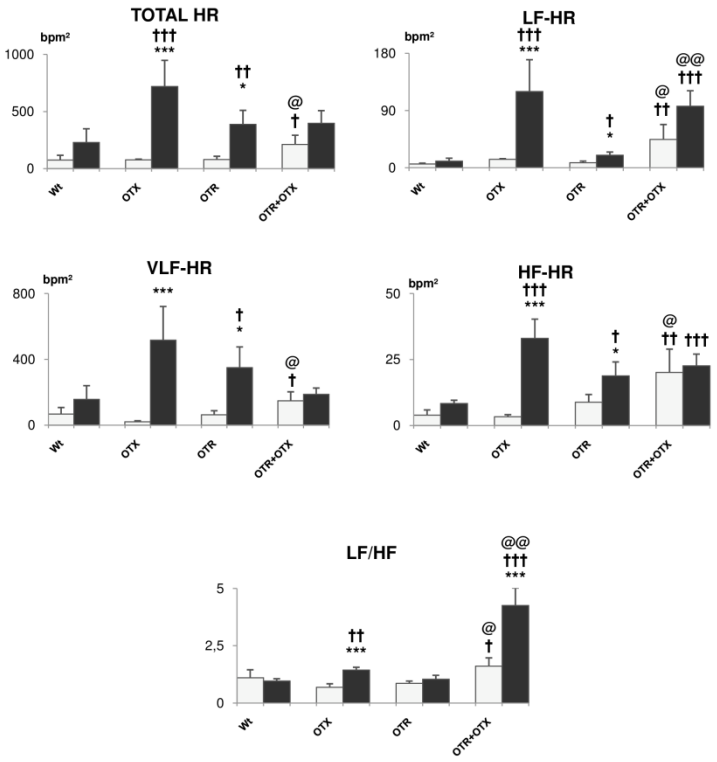

Figure 17. Effects of selective OTR antagonist (OTX) in PVN on the components of HR short-term variability of Wild type rats and rats over-expressing OT receptors.

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. Empty bars indicate baseline values, black bars indicate stress values. Wt: wild-type rats; eGFP: rats transfected with enhanced green fluorescent protein; OTR: rats over-expressing oxytocin receptors; TOTAL-HR: total heart rate variability; VLF-HR: very low-frequency heart rate variability; LF-HR: low-frequency heart rate variability; HF-HR: high-frequency heart rate variability. Values are mean of 6 rats ± s.e.m..$^{*}p<0.05$; $^{***}p<0.001$ *vs.* baseline; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$*vs.*Wt; $^{@}\underline{p}<0.05$; $^{@@}\underline{p}<0.01$ *vs.* OTR rats.

4. REZULTATI

-sažeti prikaz-

Rezultati istraživanja pokazali su da su efekti oksitocina primenjenog u paraventrikularno jedru hipotalamusa pacova porast arterijskog krvnog pritiska i srčane frekvencije. Ovaj efekt je najverovatnije rezultat aktivacije oksitocinskih receptora na nivou hipotalamičkog paraventrikularnog jedra, s obzirom da prethodna blokada oksitocinskih receptora selektivnim antagonistom nije dovela do navedenih promena kardiovaskularnih parametara.

Povećanje ekspresije oksitocinskih receptora u paraventrikularnom jedru hipotalamusa je dovelo do povećanja vrednosti sistolnog, srednjeg arterijskog krvnog pritiska i senzitivnosti baroreceptorskog refleksa tokom perioda mirovanja u grupi transfeciranih životinja u odnosu na kontrolne. Akutni emocionalni stres, u vidu izlaganja mlazu vazduha pod pritiskom, je uzrokovao povećanje vrednosti krvnog pritiska (sistolnog, dijastolnog i srednjeg), kao i porast vrednosti srčane frekvencije u eksperimentalnoj i kontrolnim grupama životinja.. Pad osetljivosti baroreceptorskog refleksa u kontrolnim grupama životinja, nije registrovan kod životinja sa povećanim brojem oksitocinskih receptora u paraventrikularnom jedru. Spektralna analiza kratkoročnog varijabiliteta je pokazala da je pod bazalnim fiziološkim uslovima u grupi transfeciranih životinja varijabilitet krvog pritiska snižen, prvenstveno usled sniženja u VLF-BP spektralnom domenu. Akutni stres je uzrokovao značajan porast srčanog varijabiliteta, kao i varijabiliteta krvnog pritiska u svim grupama životinja, ali je u grupi transfeciranih životinja porast u LF-BP bio manje izražen nego kod kontrola, sugerišući manje izražen uticaj simpatikusa na krvne sudove.

Primena selektivnog antagoniste u grupi životinja sa vektorski-posredovanim povećanjem broja oksitocinskih receptora je dovela do smanjenja osetljivosti baroreceptorskog refleksa i povećanja kratkoročnog varijabiliteta tokom bazalnih uslova i prilikom izlaganja stresu, čime je pokazana funkcionalnost transfeciranih receptora.

5. DISCUSSION

The central neural control of the cardiovascular system by the oxytocinergic system in the PVN was studied by microinfusions of exogenous oxytocin, selective antagonists of oxytocin receptors, and a gain-of-function approach.The findings of the present work show for the first time that OTRs in the PVN are able to tonically modulate autonomic cardiovascular control, both under baseline and stressful physiological conditions.

Over-expression of OTRs in the PVN causes increased mean values of SBP, MBP and BRS and decreased VLF-BP and total BP variability during baseline conditions. The most striking result in animals with higher density of OTRs in the PVN exposed to stressful conditions is significantly higher value of BRS compared to controls along with decrease in LF-SBP and LF-DBP and increased overall HR variability. These findings suggest that ectopic OTRs in the PVN are functional and that the increase of their number may potentiate the physiological effects of naturally occurring endogenous ligand at physiological concentrations.

Administration of selective antagonist in animals over-expressing OTRs confirmed the functionality of ectopic OT receptors by reducing BRS and de-buffering cardiovascular short-term variability under baseline and stressful conditions, but also raised the question of potential constitutive activity of virally delivered OTRs since the pretreatment with OTX in animals over-expressing OTRs failed to induce changes in mean values of SBP and MBP, which remained significantly higher compared to controls.

Results in wild-type rats demonstrate that administration of OTX into the PVN during baseline physiological conditions reduces BRS without affecting mean values of BP and HR and causes un-buffering of BP variability manifested as an increase in LF and HF domains. Exposure to acute emotional stress shows that pretreatment of wild-type rats with OTX induces less pronounced elevation of mean values of MBP,

DBP and HR, prevents decrease of BRS and increases HR variability, especially in LF frequency domain, pointing to the domination of sympathetic control of the heart.

5.1 Over-expression of OTR in the PVN on autonomic cardiovascular control

It is well established that OT receptors are normally expressed in the PVN (Van Leeuwe*et al.,* 1985; Freund-Mercier *et al*., 1987; Tribollet*et al*., 1988; Yoshimura *et al.,* 1993; Adan *et al*., 1995) where they have an important function as a part of an endogenous autocontrol mechanism (Richard *et al*., 1997). Electrophysiological studies showed that during suckling somato-dendritically released OT stimulates OTRs 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 IP_3-sensitive intracellular stores (Inenaga and Yamashita, 1986; Moos and Richard, 1989; Richard *et al*., 1997; Ludwig and Leng, 2006).

As previously mentioned, neurons originating from the PVN project to NTS, DVN, NA, RVLM and IML column of the spinal cord (Sawchenko and Swanson, 1982; Lang *et al*., 1982; 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. Anatomical and electrophysiological studies indicate a potentially important role of the paraventricular oxytocinergic system in autonomic cardiovascular control, since approximately 40% of the spinally projecting PVN neurons contain mRNA for OT (Pyner, 2009). Our results provide the evidence that a change of expression of OTRs in the PVN in conscious rats can modulate neurogenic control of the circulation.

Functional studies by Russ and Walker (1994) revealed that peripherally applied exogenous OT significantly augmented the bradycardic response to intravenous bolus doses of selective α_1 agonist in conscious rats, thus enhancing the BRS. Microinjections of oxytocin in the region which contains specific and dense

concentration of OT-immunoreactive terminals such as solitary-vagal complex (NTS/DVN) facilitate reflex bradycardia via the stimulation of OT receptors (Higa *et al*., 2002). Using a loss-of-function approach, Michelini and collaborators (2003) reported blunted BRR in response to pressure changes in oxytocin-deficient mice. In our experiments over-expression of OTR in the PVN enhanced BRS, and this effect on baroreceptor reflex was unmasked by OTX administration.

It should be noted that our experiments cannot incontrovertibly identify the ligand, since both oxytocin and vasopressin (as well as their receptors) display a high degree of sequence homology, and both peptides can potentially activate both receptors (Chini and Manning, 2007), there is a possibility that OTRs 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 axonal release of OT in the vicinity of NTS, where OTRs have been shown to increase BRS. Alternatively, over-expression of ectopic OTRs could have occurred on any other neuron in the PVN that is integrated in 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 multiple neuronal activity in the PVN (Tasker *et al*., 2012). Future experiments in which the expression of OTRs is directed to specific cell-types could address these issues.

Our results further show that up-regulation of OTRs in the PVN reduces BP variability in the VLF domain during baseline physiological conditions. VLF-BP oscillations contribute most to overall BP short-term variability (Japundzic-Žigon, 1998), and dominate under basal conditions. They are created by multiple mechanisms acting in concert and opposition. Oscillations at ~0.1 Hz in rat originate from spontaneous myogenic activity of blood vessels (Stauss *et al*., 2009), these can be enhanced by rennin-angiotensin system activation (Ponchon and Elhgozi, 1996) and counteracted by baro-receptor reflex, as suggested in experiments with surgical or pharmacological opening of the baro-receptor reflex loop (Japundzic *et al*., 1990;

Cerutti *et al*., 1994). Therefore, in our experiments, the reduction of VLF variability could be related to the enhanced BRS.

Constitutive activity of GPCRs was first described in β_2-adrenoceptors and later also in adrenergic α_{1b}, vasopressin V_2, muscarinic M_1 and histamine H_2 receptors (Gimpl and Fahrenholz, 2001; Favre *et al*., 2005) unraveling the ability of receptors to show activity without being occupied by an agonist. Administration of OTX in the PVN of rats over-expressing OTRs failed to induce lowering of SBP and MBP, an effect that could be ascribed to potential constitutive activity of OTRs.

It is well recognized that the PVN is a major site where integration of neuroendocrine and behavioural response to stress occurs (Herman and Cullinan, 1997; Dampney and Horiuchi 2003; Benarroch, 2005). Rats exposed to jet of air pointed to the top of their head exhibited a startle reaction followed by elevation of blood pressure and tachycardia (Blanc J *et al*., 1994). Using microdialysis Nishioka and co-workers (1998) found that shaker stress increases the content of OT in the PVN. It was also reported that specific PVN lesions, or microinjection of antagonists intracerebroventricularly or OT antisense oligonucleotides into the PVN (Callahan *et al*. 1989; 1992) attenuated the HR response to stress. In our experiments, up-regulation of OTRs in the PVN did not modulate the HR response to stress, but provoked the increase in HR variability. The increase of HR variability is both sympathetically and vagally mediated. Pre-treatment of these rats with OTX in the PVN revealed that ectopic OTRs buffer BP and HR variability and favour vagal control of the heart (according to changes in LF/HF–HR ratio).

I also observed that over-expression of OTRs in the PVN buffers the stress-induced BP variability response mediated by increased sympathetic outflow to blood vessels (LF) and stimulation of respiration (HF).

The buffering effect on BP variability could be mediated via neural pathways crossing ventrolateral medulla, since it was reported that OT-containing neurons of the hypothalamic paraventricular nucleus (PVN) project to the pre-Bötzinger

complex (pre-BötC) in ventrolateral medulla of the brainstem (Mack *et al*., 2002; 2007) controlling both respiratory and cardiovascular functions.

Another possibility is that stress-induced axonal release of OT in amygdale activates a subpopulation of GABA interneurons that inhibit neurons in 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 pacify respiration, as reflected in HF and LF BP short-term variability.

Altogether, our results suggest that OTRs in the PVN activate downstream signaling pathways in neighbouring cells leading to neurotransmitter release in brainstem targets that increase BRS. They also suggest that the number of OTRs expressed in PVN may alter the level of tonic input of PVN in neurogenic cardiovascular control without alteration in ligand release.

5.2 Effects of OTR antagonist injected in the PVN on autonomic cardiovascular control

According to the literature, both hypotensive and hypertensive effects of exogenously applied OT have been reported, depending on species and the route of administration.

Subcutaneous injections of oxytocin induced short-lasting increase in blood pressure that could not be fully antagonised by oxytocin antagonist (Petterson *et al*, 1999). This effect is probably due to activation of vasopressin V1a receptors, since oxytocin in high doses shows affinity for vasopressin receptors.

However, short-lasting increase in blood pressure was followed by long-term hypotension in conscious male and female rats (Petterson *et al*., 1996) systemically treated with oxytocin during five-day period. Interestingly, systemically administered oxytocin in humans showed opposite effects. Ronald Katz (1964)

described transient hypotension followed by secondary rise in blood pressure after injection of synthetic oxytocin in human studies.

Oxytocin applied intrathecally, in large doses brought to significant pressor response in rats and appreciable, but inconsistent changes in heart rate reflected as both tachycardia and decreased number of beats per minute (Riphagen C, Pittman Q, 1986), while intracerebroventricular administration of OT was reported to decrease BP (Petterson *et al*., 1996, Petterson and Uvnäs-Moberg, 2007). It was further suggested that the central hypotensive effects of OT is mediated through axonal release in locus coeruleus where OT increases the density and the affinity of α-2 adrenoceptors known to reduce sympathetic outflow (Petterson *et al*., 2005).

Our experiments indicate that pharmacological effects of OT microinfused in the PVN are hypertension and tachycardia, which is in concert with results obtained after OT microinjections in brainstem nuclei involved in cardiovascular regulation, namely RVLM, NTS and DVN (Mack *et al*., 2002; Vela *et al.,* 2010).

Several studies suggested that endogenous oxytocin is involved in tonic maintenance of blood pressure. Oxytocin-deficient mice show lower values of mean blood pressure compared to wild-type controls (Michelini *et al.,* 2003*)*, while Nissen and collaborators (1996) demonstrated that in lactating women blood pressure falls in response to each nursing session.

In the PVN, NO, GABA and glutamate are regarded as crucial, while oxytocin, vasopressin, dopamine and angiotensin II are found to selectively modulate tonic PVN signal in autonomic cardiovascular control (Pyner, 2009). Results from our study confirm that OT in the PVN has no tonic physiological influences on mean levels of BP and HR. OTX administered in the region of PVN failed to modulate BP and HR under baseline conditions in wild-type rats, but it caused unbuffering of BP variability and desensitization of baroreceptor reflex functioning. These results further confirm those obtained in OTR over-expressing rats and indicate that by reducing BP variability and increasing sensitivity of BRR, markers correlated with positive clinical outcome (Mancia *et al*., 1994; Narkiewicz and Grassi, 2008), OT plays an important part in maintenance of cardiovascular health. In this context it is

important to mention that better understanding of central mechanisms that alter BRS, BP and HR variability is of interest.

In our experiments microinfusion of OTX in the PVN of non-transfected rats prevented stress-induced DBP increase, reduced but did not prevent tachycardia and provoked the increase in HR variability.The increase of vagal influences to the heart during stress was reported to be useful in protecting the heart against sympathetic over-stimulation involving cholinergic NO synthesis in ventricles(Brack*et al*., 2012). This protective effect of the vagus is lifesaving during cardiac ischemia, when sympathetic over-stimulation triggers life threatening arrhythmias and sudden death. This assumption is further supported by the work of Wsol and associates (2009). In a series of experiments in rats that survived myocardial infarction after ligation of coronary arteries, authors reported reduced survival due to the failure of brain OT to attenuate cardiovascular response to stress.

Our findings are in line with a number of animal studies that suggest that OT activates an anti-stress response (Grippo*et al*., 2009; Lee *et al*., 2005; Windle *et al*., 1997; 2004). For instance OT is found to decrease cardiovascular response to isolation in a monogamous praire voles, that represent excellent model for studying social behavior (Grippo*et al*., 2009). Oxytocin blunts restraint-induced hypothalamo-pituitary axes activation (Windle*et al*., 1997; 2004) by reducing stress-induced elevation of corticosterone levels in ratsand promotes social interactions (Lee *et al*., 2005). Rats centrally-treated with oxytocin showed higher proportion of open-arms entry and spent more time in open arms in elevated plus maze, expressing signs of reduced anxiety-like behaviour (Windle*et al*., 1997). In OT knock-out mice, Bernatova and co-workers (2004) described accentuated BP and corticosterone response during exposure to acute stress. In line with their finding Wsol and colleagues (2008) reported that central application of OTR antagonist enhanced 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.

6. CONCLUSIONS

1. Under basal physiological conditions, SBP of rats with over-expression of OTR in PVN was slightly but significantly increased in respect to SBP of non-transfected wild-type rats. Mean BP and diastolic BP did not differ between groups. Both under basal conditions and stress,BP variability in these rats was reduced, probably due to the concomitant increase of barorefelx sensitivity. HR and HR variability was similar in rats over-expressing OTR and wild-type rats under basal conditions whereas during exposure to stress, HR and HR variability increased more in rats over-expressing OTR than in wild-type rats.

2. Selective pharmacological blockade of OTR in the PVN of wild-type rats under basal physiological conditions did not affect BP, HR and increased BP variability due to the decrease in barorefelx sensitivity. Exposure to stress of rats under blockade of OTR triggered an increase of BP and HR variability more than in non-treated, control rats.

3. Both under basal physiological conditions and stress, selective pharmacological blockade of OTR in the PVN of rats over-expressing OTR, increased BP variability, HR variability and decreased the sensitivity of baroreceptor reflex in respect to wild-type rats.

4. **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 baroreceptor function and cardiovascular short-term variability. OTRs in the PVN enhance the sensitivity of the baroreceptor reflex and buffer blood pressure and heart rate short-term variability favouring vagal control of the heart. These effects are more pronounced in rats over-expressing OTR in the PVN than in wild-type rats.**

6. ZAKLJUČCI

1. Kod pacova sa hiperekspresijom oksitocinskih receptora u PVN-usistolni arterijski krvni pritisak je nešto povišen u odnosu na netransfecirane pacove ali ne i srednji i dijastolni krvni pritisak. Varijabilitet krvnog pritiska snižen je pod bazalnim uslovima i ispoljava manji porast prilikom izlaganja ovih pacova stresu u odnosu na netransfecirane pacove. Srčana frekvecija i varijabilitet srčane frekvencije transfeciranih pacova pod bazalnim uslovima se ne razlikuje od netransfeciranih pacova. Medjutim, prilikom izlaganja stresu srčana frekvencija i njen varijabilitet značajno su viši nego kod netransfeciranih pacova. Senzitivnost baroreceptorskog refleksa je povećana kod pacova sa hipereksperesijoim OTR kako pod bazalnim uslovima tako i prilikom izlaganja stresu.
2. Selektivna farmakološka blokada oksitocinskih receptora u PVN-u netransfeciranih pacova ne utiče na vrednost arterijskog krvnog pritiska i srčane frekvencije pod bazalnim uslovima ali povećava varijabilitet krvnog pritiska najverovatnije zbog smanjenja osetljivosti barorefleksa. Izlaganje akutnom stresu dovodi do povećanja kratkoročnog varijabiliteta krvnog pritiska i srčane frekvence više nego kod netretiranih kontrolnih pacova izoloženih stresu.
3. Pod bazalnim fiziološkim uslovima i prilikom izlaganja stresu, selektivna farmakološka blokada oksitocinskih receptora u PVN-u pacova sa hipereksprimiranim oksitocinskim receptorima, povećava varijabilitet krvnog pritiska i srčane frekvencije i smanjuje osetljivosti barorereceptroskog refleksa u odnosu na netransfecirane, netretirane pacove.
4. **Naši rezultati prvi put pokazuju da su OT receptori u PVN-u uključeni u lokalnu regulaciju (autokrinu i/ili parakrinu) neurona uključenih u toničku kontrolu senzitivnosti barorefleksa i kardiovaskularnog kratkoročnog varijabiliteta. OTR u PVN-u povećavaju senzitivnost barorefleksa i smanjuju varijabilitet arterijskog krvnog pritiska i srčane frekvencije favorizujući vagalnu kontrolu srčanog rada. Ovi efekti su izraženiji kod pacova sa hiperekspresijom OTR u PVN-u nego kod netransfeciranih pacova.**

7. LITERATURE

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8. List of abbreviations

BP- blood pressure;

BPV- blood pressure variability;

BRS- baroreceptor reflex sensitivity;

DBP- diastolic arterial blood pressure;

DVN- dorsal nucleus of vagus;

GABA- gamma-aminobutyric acid;

GPCR- G-protein coupled receptor;

HR- heart rate;

HRV- heart rate variability;

HF- high-frequency short-term variability;

IML- intermediolateral column of the spinal cord;

LF- low-frequency short-term variability;

MBP- mean arterial blood pressure;

Nam- nucleus ambiguus;

NO- nitric oxide;

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;

SON-supraoptic nucleus;

VLF- very low-frequency short-term variability

9. Biography

Maja Lozić Đurić is a teaching and research assistant working at the Institute of Pharmacology, Clinical Pharmacology and Toxicology, School of Medicine, Belgrade. She was born on 25th May 1982 in Belgrade, where she finished elementary and high school as first in her class. After obtaining her MD at the University of Belgrade School of Medicine (average grade 9.34/10), Maja Lozić Đurić started working at the Laboratory for Cardiovascular Pharmacology. During her doctoral studies she spent several months in the Molecular Neueroendocrinology Research Group, University of Bristol, lead by Professor David Murphy. She specialized in clinical pharmacology in January 2015.

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