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NEURÁLNÍ MECHANISMY PATOGENEZE SPONTÁNNÍ HYPERTENZE U POTKANA

NEURAL MECHANISMS IN THE PATHOGENESIS OF SPONTANEOUS HYPERTENSION IN THE RAT

Disertační práce

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PROHLÁŠENÍ

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ABSTRACT

Both sympathoneural and sympathoadrenal systems are involved in the regulation of arterial blood pressure and in the pathogenesis of hypertension. Spontaneously hypertensive rats (SHR), the mostly used animal model of genetic hypertension, is characterized by multiple molecular, morphological and functional alterations at different levels of sympathoneural and sympathoadrenal systems. The study of young prehypertensive SHR allows to reveal the abnormalities preceding hypertension development, whereas adult SHR with established hypertension offers a better model for the treatment of human essential hypertension. The aim of my PhD Thesis was to describe abnormalities in sympathoneural and sympathoadrenal systems in SHR under different conditions. Firstly, ontogenetic differences which might contribute to hypertension development were determined. Secondly, the effect of chemical sympathectomy induced by guanethidine in adulthood on cardiovascular parameters and on the compensatory mechanisms counteracting the reduction of blood pressure were studied. Thirdly, stress-induced cardiovascular response and stress-induced changes of sympathoneural and sympathoadrenal systems were described in adult SHR. My Thesis brought several important results. The increased adrenal catecholamine content and the increased density of sympathetic innervation observed in prehypertensive SHR compared to age-matched normotensive WKY rats could be involved in the pathogenesis of high blood pressure. The downregulation of the expression of genes involved in catecholamine biosynthesis (*Th*, *Ddc*, *Dbh*, *Pnmt*) is probably a compensatory mechanism counteracting the hyperfunction of the sympathoneural system. The suppression of catecholamine biosynthesis develops concurrently with the progress of hypertension in SHR. It results in the lower catecholamine content in the adrenal glands but not in the lower vascular sympathetic innervation of adult SHR. A greater role of sympathetic nervous system in blood pressure maintenance was documented in adult SHR compared to WKY rats. However, chronic sympathectomy by guanethidine is not an effective method for pernament blood pressure lowering in adult SHR with established hypertension. This might be explained by the involvement of compensatory mechanisms in sympathectomized rats, such as the

enhanced blood presure sensitivity to catecholamines and the increased plasma levels of adrenaline. Adult SHR showed an exaggerated cardiovascular response and excessive activation of sympathoneural and sympathoadrenal systems during the acute restraint compared to WKY rats. Furthermore, SHR subjected to restraint exhibited the overactivation of hypothalamic-pituitary-adrenal axis which might intensify sympathetically mediated rise in peripheral vascular resistance and stress-induced cardiovascular response. In line with sympathetic hyperactivity, a greater elevation of mRNA expression of *Th* gene was observed in the adrenal medulla of stressed SHR compared to WKY rats. In contrast, the mRNA expression of other genes involved in catecholamine biosynthesis (*Ddc*, *Dbh*, *Pnmt*) was lower in adrenal medulla of stress-naive as well as stressed SHR in comparison to WKY. This finding suggests the involvement of other mechanisms in the regulation of these enzymes. The possible cause might be a lower stimulation of adrenal chromaffin cells by angiotensin II resulting from the attenuated plasma renin activity and the decreased mRNA expression of adrenal angiotensin II receptors observed in SHR. In conclusion, the data presented in my PhD Thesis confirmed that the sympathetic nervous system contributes to the development and maintenance of high blood pressure in SHR. Its effects on cardiovascular system might be potentiated by the excessive activation of hypothalamic-pituitary-adrenal axis observed in this rat strain. Similar mechanisms are involved in the development and maintenance of high blood pressure in humans. Therefore, the investigation of abovementioned phenomena in SHR can contribute to a better understanding and treatment of human essential hypertension. The resistance of adult SHR to the treatment targeting the peripheral sympathetic nervous system can provide an insight into the compensatory mechanisms which counteract the effective treatment of high blood pressure. Therefore, the drugs affecting central regulation of cardiovascular system (e.g. ACE inhibitors or angiotensin receptor blockers) might be better for the effective lowering of blood pressure in hypertension.

Key words: adrenal medulla, catecholamines, hypertension, stress, sympathetic nervou system

ABSTRAKT

Sympatický nervový systém a dřeň nadledvin se účastní regulace arteriálního krevního tlaku a hrají významnou úlohu v patogenezi hypertenze. Spontánně hypertenzní potkani (SHR), kteří jsou nejvíce používaný model genetické hypertenze, se vyznačují mnoha molekulárními, morfologickými i funkčními změnami sympatického nervového systému a dřeně nadledvin. Cílem této disertační práce bylo popsat tyto abnormality u SHR potkanů za různých podmínek. Zaprvé byly zkoumány ontogenetické rozdíly, které mohou přispívat k rozvoji hypertenze. Zadruhé byly studovány účinky chemické sympatektomie (indukované guanetidinem podávaným v dospělosti) na kardiovaskulární parametry a zapojení kompenzačních mechanismů, které působí proti snížení krevního tlaku. Zatřetí jsme u dospělých SHR popsali stresovou kardiovaskulární odpověď a stresem indukované změny v sympatickém nervovém systému a dřeni nadledvin. Moje dizertační práce přinesla několik důležitých nálezů. Zvýšený obsah katecholaminů v nadledvině a vyšší hustota sympatické inervace cév pozorované u prehypertenzních SHR v porovnání se stejně starými normotenzními WKY potkany mohou přispívat k patogenezi hypertenze. Snížená mRNA exprese genů zapojených do biosyntézy katecholaminů (*Th*, *Ddc*, *Dbh*, *Pnmt*) je pravděpodobně kompenzační mechanismus působící proti zvýšené aktivitě sympatického nervového systému. Útlum biosyntézy katecholaminů se u SHR rozvíjí souběžně s rozvojem hypertenze. Důsledkem je snížení obsahu katecholaminů v nadledvině, ale ne v sympatické inervaci cév dospělých SHR. Výraznější úloha sympatického nervového systému při udržování krevního tlaku byla prokázána u dospělých SHR v porovnání s WKY potkany. Nicméně chronická sympatektomie guanetidinem není efektivní metodou pro dlouhodobé snížení krevního tlaku u dospělých SHR s rozvinutou hypertenzí. Vysvětlením může být zapojení kompenzačních mechanismů u sympatektomovaných zvířat jako jsou zvýšená citlivost odpovědi krevního tlaku na katecholaminy a vyšší plazmatické hladiny adrenalinu. Ve srovnání s WKY potkany měli dospělí SHR výraznější kardiovaskulární odpověď a nadměrnou

aktivaci sympatického nervového systému a dřeně nadledvin během stresu vyvolaného akutním omezením pohybu (restraint). Stresovaní SHR potkani navíc vykazovali větší aktivaci osy hypotalamus-hypofýza-nadledviny, což může vést k dalšímu zesílení sympaticky řízené periferní vasokonstrikce a tedy i stresem indukované kardiovaskulární odpovědi. V souladu s hyperaktivitou sympatického nervového systému bylo ve dřeni nadledvin stresovaných SHR potkanů pozorováno výraznější zvýšení exprese *Th* genu v porovnání s WKY potkany. Naopak exprese ostatních genů účastnících se biosyntézy katecholaminů (*Ddc*, *Dbh*, *Pnmt*) byla nižší ve dřeni nadledvin nestresovaných i stresovaných SHR v porovnání s WKY potkany. Tento nález naznačuje, že regulace zmíněných enzymů se účastní jiné mechanismy. Možnou příčinou může být menší stimulace chromafinních buněk dřeně nadledvin angiotensinem II, která je způsobena utlumenou aktivitou reninu v plasmě a sníženou mRNA expresí receptorů pro angiotensin II u SHR potkanů. Závěrem, data v mé disertační práci potvrzují, že sympatický nervový systém přispívá k rozvoji a udržování vysokého krevního tlaku u SHR potkanů. Jeho vliv na kardiovaskulární systém může být potencován nadměrnou aktivací osy hypotalamus-hypofýza-nadledviny, kterou jsme také pozorovali u tohoto kmene. Protože podobné mechanismy se podílejí na vzniku a udržování vysokého krevního tlaku u lidí, jejich výzkum u SHR mohou přispět k lepšímu porozumění a léčbě lidské esenciální hypertenze. Odolnost dospělých SHR potkanů k léčbě zaměřené na periferní sympatický nervový systém upozorňuje na existenci kompenzačních mechanismů, které působí proti efektivní léčbě vysokého krevního tlaku. Pro efektivní snížení krevního tlaku tedy mohou být lepší volbou léky působící na centrální regulaci kardiovaskulárního systému, např. ACE inhibitory nebo blokátory angiotensinových receptorů.

Klíčová slova: dřeň nadledvin, katecholaminy, hypertenze, stres, sympatický nervový systém

LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
Ang II	angiotensin II
ANOVA	analysis of variance
b.w.	body weight
BP	blood pressure
cAMP	cyclic adenosine monophosphate
CRH	corticotropin-releasing hormone
C_t	cycle of treshold
CTRL	control
DBH	dopamine β-hydroxylase
DDC	L-DOPA decarboxylase
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immuno sorbent assay
HF SBPV	high-frequency component of systolic blood pressure variability
HPA	hypothalamic-pituitary-adrenocortical
HR	heart rate
LF SBPV	low-frequency component of systolic blood pressure variability
MAP	mean arterial pressure
mRNA	messenger RNA
n	number
ND	not determined
NS	not significant
NTS	*nucleus tractus solitarii*
PCR	polymerase chain reaction
PNMT	phenylethanolamine N-methyl transferase
POMC	pro-opiomelanocortin
PRA	plasma renin activity
PVN	paraventricular nucleus of hypothalamus
RAS	renin-angiotensin system
RNA	ribonucleic acid
RVLM	rostral ventrolateral medulla

SEM	standard error of the mean
SHR	spontaneously hypertensive rats
SYMPX	sympathectomized
TBS-T	Tris-buffered saline with Tween
TH	tyrosine hydroxylase
WKY	Wistar-Kyoto

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1. INTRODUCTION

Arterial blood pressure (BP) is the pressure of circulating blood exerted on the large arteries in the systemic circulation. This parameter depends on cardiac output and total peripheral resistance. The arterial BP is maintained within an optimal range which represents a compromise between the need to ensure a sufficient tissue perfusion and the effort to minimize the risk of structural damage of the heart and blood vessels. The sympathetic nervous system is involved in the regulation of both arterial BP and regional vascular resistance (in cooperation with humoral and local factors), under various circumstances, such as postural change, physical exercise, stress, etc. (Silverthorn and Johnson, 2010). Since human essential hypertension is one of the major risk factors for cardiovascular complications, understanding the mechanisms involved in the regulation of arterial pressure is desirable. Various animal models with experimental hypertension have been developed to study genetic and environmental factors contributing to the pathophysiology of high BP (reviewed in Lin *et al.*, 2016). This thesis is focused on the neural mechanisms involved in the pathogenesis and maintenance of high BP in rats with genetic hypertension.

1.1. Spontaneously hypertensive rat

Spontaneously hypertensive rats (SHR) are the mostly used animal model of genetic hypertension. This inbred strain was developed in 1960s in Kyoto (Japan) from outbred Wistar rats by the selective breeding for high BP (Okamoto and Aoki, 1963). The inbred Wistar-Kyoto (WKY) rats, established later from the same Wistar colony, are used as normotensive controls for SHR (Aoki *et al.*, 1972). The SHR develop hypertension spontaneously without physiological, pharmacological or surgical intervention at the age of 5–12 weeks and their mean arterial pressure in adulthood achieves 160-180 mm Hg (in contrast to 120 mm Hg in adult WKY rats) (Behuliak *et al.*, 2015; Judy and Farrell, 1979). Numerous functional and structural abnormalities were described in SHR including abnormal neurohumoral regulation, vascular hypertrophy, impaired endothelium-dependent relaxation, renal dysfunction etc. (Zicha and Kunes, 1999). However, some of the described changes may be causal to hypertension in SHR, while the others may be the result or compensation of high blood pressure in this model. The sympathetic nervous system is considered to be involved in the pathogenesis of hypertension in SHR since the sympathetic activity rises dramatically concomitantly as high BP develops (Judy and Farrell, 1979). Moreover, the development of hypertension in SHR can be attenuated by neonatal sympathectomy (destruction of sympathetic nervous system, e.g. by guanetidine

administration), but even in sympathectomized SHR moderately elevated BP still persists (Lee *et al*., 1987). The residual BP difference can be abolished by a combination of sympathectomy with adrenal demedullation (Lee *et al*., 1991a) or with α_1-adrenergic blockade (Korner *et al*., 1993). On the other hand, the sympathectomy performed in adult SHR is markedly less efficient in reducing BP (Ferrari *et al*., 1991; Yamori *et al*., 1972). It is possible that physiological changes associated with the maturation and hypertension development, e.g. vascular remodeling (Intengan and Schiffrin, 2001; Martinez-Quinones *et al*., 2018), can cause the resistance of adult SHR to guanethidine treatment. Moreover, after the disruption of sympathetic innervation in the vasculature, vascular tone could be maintained by several compensatory mechanisms, such as by the increased catecholamine release from adrenal medulla (Lee *et al*., 1991a), by the augmented activation of renin-angiotensin system (RAS) (Lo *et al*., 1991) or by the enhanced responsiveness of vascular smooth muscle cells to available vasoconstrictors (Fleming, 1981). Thus, the mechanisms participating in the pathogenesis of spontaneous hypertension development in young animals and high BP maintenance in adult SHR may differ. The study of young prehypertensive SHR allows to reveal the abnormalities preceding hypertension development. On the other hand, the study of adult SHR with established hypertension offers a better model for the treatment of human essential hypertension.

1.2. Sympathetic nervous system

The sympathetic nervous system (together with the parasympathetic nervous system) is a part of the autonomic nervous system that regulates involuntary physiologic processes including BP, heart rate (HR), respiration, etc. The activation of the sympathetic nervous system is associated with a so-called “fight or flight” response, which is a state of overall elevated activity and attention, accompanied by the increased BP and HR. On the other hand, the parasympathetic system promotes the “rest and digest” state characterized by the reduced HR. The sympathetic and parasympathetic nervous systems typically function in a reciprocal manner, e.g. an increase in the sympathetic activity is associated with a decrease in the parasympathetic activity (Altimiras, 1999; Karemaker, 2017)

1.2.1. *The functional anatomy of sympathetic nervous system*

Essentially, the sympathetic nervous system consists of sensory pathways, central region of autonomic control and efferent pathways. The signals from the visceral receptors (e.g., from the arterial baroreceptors) are conveyed by the afferent sympathetic fibers into the central nervous system. The information from periphery is integrated and modulated by a neuronal

network, located mainly in the spinal cord, brainstem and hypothalamus, which determines the activity of efferent sympathetic preganglionic neurons (Dampney *et al*., 2002, Pintérová *et al*., 2011). These preganglionic neurons form cholinergic synapses with postganglionic neurons or chromaffin cells of adrenal medulla (sympathoneural and sympathoadrenal subdivision of sympathetic nervous system). Particular preganglionic neurons are differentially activated under various conditions allowing the appropriate activation of their targets, such as vascular smooth muscles, heart, kidney or adrenal medulla (Guyenet, 2006). Although the sympathetic postganglionic neurons and chromaffin cells of adrenal medulla are developmentally related cells, they differ in morphology, area of their action and also in the signaling molecules (Anderson and Axel, 1986). The nerve endings of postganglionic sympathetic neurons release primarily noradrenaline which acts directly on the respective cardiovascular targets (Brock and Cunnanne, 1993). On the other hand, adrenaline released by the adrenal medulla into the blood stream can act on various distant targets (Flatmark, 2000).

The alterations at almost all levels of the peripheral sympathetic nervous system were reported in SHR (for review see Head, 1989; Head, 1991; Pintérová *et al*., 2011). The sympathetic activity is increased in splanchnic and renal nerves of SHR (Judy et al., 1979; Judy and Farrell, 1979; Okamoto *et al.*, 1967) and also the preganglionic activity is more effectively transmitted through sympathetic ganglia in these rats (Magee and Schofield,1992). Moreover, the denser sympathetic innervation (Mangiarua and Lee, 1990; Scott and Pang, 1983) and greater noradrenaline content (Cassis *et al*., 1985; Donohue *et al*., 1988; Head *et al*., 1985) were described in the arteries from SHR. Accordingly, *in vitro* electrical field stimulation leads to a greater noradrenaline release and an augmented pressor response in vessels from SHR than in those from WKY rats (Tsuda *et al*., 1984; Westfall *et al*., 1984). This might be caused by the altered density or function of presynaptic adrenergic receptors involved in the regulation of neurotransmitter release (Tsuda and Masuyama,1991). It was proposed that adrenaline released by adrenal medulla might have a facilitatory role in sympathetic neurotransmitter release and thus contribute to the development of hypertension in SHR (Borkowski, 1991; Lee *et al*., 1991a; Lee *et al*., 1991b). Adrenal glands of SHR had a greater content of catecholamines (Kumai *et al*., 1994) and acetylcholine- or potassium-stimulated catecholamine release from adrenal gland is also enhanced in SHR (Lim *et al*., 2002; Miranda-Ferreira *et al*., 2009).

1.2.2. *The central regulation of sympathetic nervous system*

The sympathetic outflow is determined by a tonic activity of neurons located in the rostral ventrolateral medulla (RVLM). RVLM integrates the information coming from various peripheral receptors (vestibular receptors, skeletal muscle receptors, nociceptors etc.), *nucleus tractus solitarii* (NTS; mediating baroreceptor and chemoreceptor reflexes), paraventricular nucleus of hypothalamus (PVN; involved in fluid, metabolism and temperature regulation) and higher brain regions (limbic, cortical and midbrain structures) (Dampney, 2016; Guyenet, 2006). The sympathetic vasomotor activity is regulated in a short-term (i.e. seconds to minutes) as well as in a long-term manner (i.e. over hours or days). The short-term changes in sympathetic outflow comprise cardiovascular reflexes (which compensate external disturbances threatening cardiovascular homeostasis, such as postural changes) and centrally generated cardiovascular responses (being a part of more complex behavioral responses, e.g. at the onset of physical exercise). The long-term changes in sympathetic outflow can also be induced by external stimuli (e.g., by enhanced salt intake) or they can accompany certain disease states (such as heart failure) (Dampney *et al*., 2002).

The major short-term mechanism regulating arterial BP is the baroreceptor reflex. Baroreceptors are tonically active stretch-sensitive mechanoreceptors located in the carotid sinus and aortic arch that fire action potentials continuously at normal values of BP. The increased BP in the arteries stretches the baroreceptor membrane and thus firing rate of the receptor increases. The signals from the baroreceptors travel via sensory neurons to the central nervous system, where sensory inputs are integrated and an appropriate response is initiated rapidly. The activation of parasympathetic efferents and the concurrent inhibition of sympathetic efferents (the inverse response occurs when BP is lowered) lead to a decrease in cardiac output and arteriolar resistance and to a subsequent restoration of BP (Guyenet, 2006; Pinterova et al., 2011, Swenne, 2013). The major function of baroreflex is to dampen short-term fluctuations of BP. On the long-term scale, the reflex can be reset according to the actual conditions in order to change the operating range of BP and to preserve the baroreflex sensitivity. The impaired baroreflex function was observed in various forms of human and experimental hypertension (Head, 1994) and it is associated with increased mortality in humans (De Ferrari *et al*., 2007; Gerritsen *et al*., 2001). The attenuated baroreflex sensitivity was described in SHR with established hypertension (Behuliak *et al*., 2018; Struyker-Boudier et al., 1982), the parasympathetic component being particularly impaired (Head, 1992).

Although the neural mechanisms were considered to be involved solely in the short-term regulation of BP, it has become increasingly evident that the sympathetic nervous system also plays a role in the long-term control of cardiovascular system (Guyenet, 2006). Arterial baroreceptors influence sympathetic nerve activity only in a short-term manner while the long-term regulation of sympathetic system is baroreceptor-independent (Osborn, 2005). The inhibition of RVLM neurons in chronically baroreceptor-denervated rats results in a BP fall which is equivalent to that produced in baroreceptor-intact rats (Sved *et al*., 2003). The alterations of biochemical milieu in the RVLM (e.g., increased gamma-aminobutyric acid (GABA) levels) can chronically alter sympathetic activity, BP and HR in rats (Kishi *et al*., 2001). The existing evidence suggests that RVLM neurons do not generate a pacemaker potential but they seem to be regulated exclusively by excitatory and inhibitory synaptic currents (Sved *et al*., 2003). Moreover, numerous humoral factors (especially those involved in the control of extracellular fluid volume and osmolality) alter the influence of RVLM on the sympathetic nervous system (Ferguson and Bains, 1997; Sved *et al*., 2003). Taken together, the exact role of the central nervous system in the long-term BP control is not well understood yet. However, the enhanced tonic activity of brain centers involved in cardiovascular regulation (including RVLM, NTS, PVN) was described in various hypertensive models and the reduction of neural activity in these areas decreased the peripheral sympathetic activity and BP (Geraldes *et al*., 2014; Matsuura *et al*., 2002; Sato 2002; Sved *et al*., 2003; Stern *et al*., 2012).

A less negative membrane potential and a faster firing rate was described in RVLM neurons of SHR. The hyperactivity of RVLM neurons in SHR was associated with the augmented signaling via angiotensin II receptors subtype 1 (Matsuura *et al*., 2002) which might be caused by the increased density of these receptors in RVLM of SHR (Hu *et al*., 2002). The activation of angiotensin II receptors subtype 1 in RVLM neurons causes closing of potassium channels and thereby depolarization of these neurons (Li and Guyenet, 1996). The overexpression of human Kir2.1-potassium channel (inward-rectifier potassium channel causing hyperpolarization) in RVLM neurons of SHR lead to a reduction of sympathetic outflow accompanied by BP decrease (Geraldes *et al*., 2014). RVLM neurons receive signals from PVN and NTS. PVN neurons are more excitable in SHR and their firing activity is augmented in comparison with that of WKY rats (Li *et al*., 2008; Stern *et al*., 2012). Similarly to RVLM, the overexpression of Kir2.1-potassium channel in PVN neurons lead to a BP decrease in SHR (Geraldes *et al*., 2016). The firing activity of NTS neurons is also augmented in SHR (Abdel-Rahman abd Tao, 1996) and the inhibition or electrolytic lesion of NTS neurons caused a

reduction in BP and sympathetic nerve activity in SHR (Sato *et al*., 2002; Sato *et al*., 2001). Taken together, peripheral sympathetic hyperactivity observed in SHR is triggered by an increased excitability and activity of brain areas including RVLM, PVN and NTS.

1.2.3. *Synthesis and metabolism of catecholamines*

The catecholamines noradrenaline and adrenaline are released from sympathetic postganglionic neurons and chromaffin cells of adrenal medulla, respectively. The pathway of catecholamine biosynthesis, storage, release, reuptake and degradation in chromaffin cells is shown in the Fig. 1.

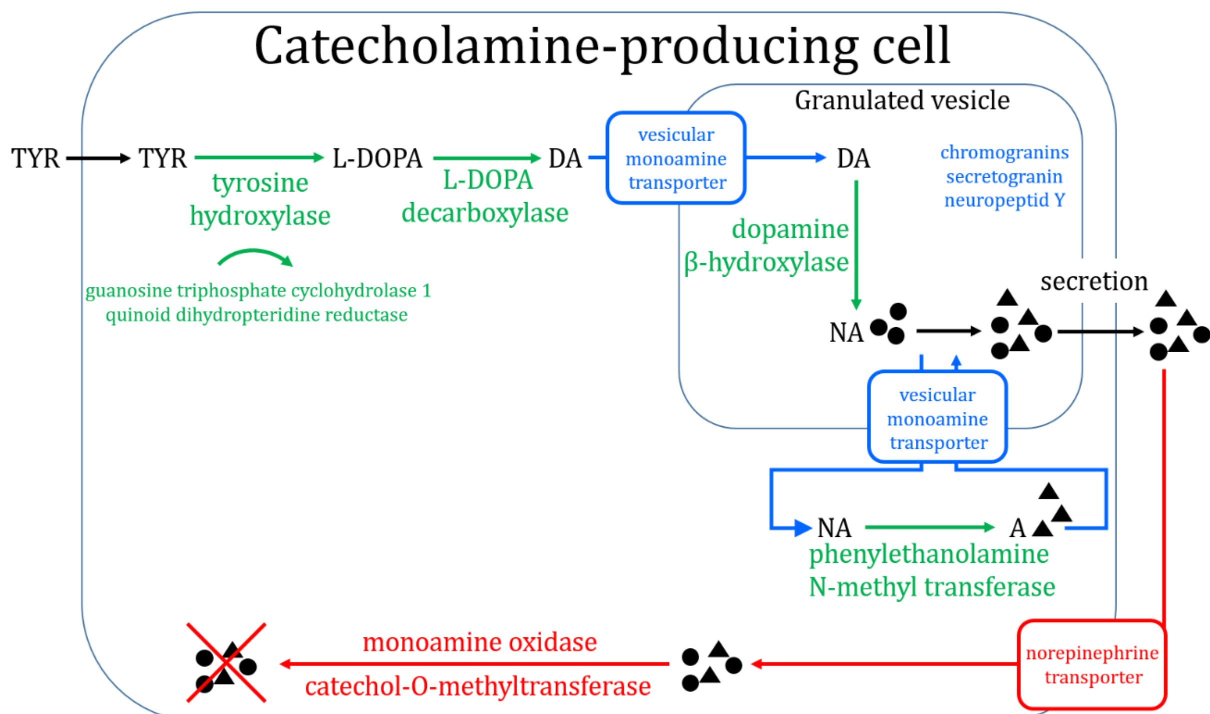

Figure 1. The pathway of catecholamine biosynthesis, storage, release, reuptake and degradation in catecholamine-producing cell. A, adrenaline; DA, dopamine; L-DOPA, l-3,4-dihydroxyphenylalanine; NA, noradrenaline.

The catecholamine biosynthesis in adrenaline or noradrenaline producing cells starts with an import of aminoacid L-tyrosine and its hydroxylation by the enzyme tyrosine hydroxylase (TH, encoded by *Th* gene; Nagatsu *et al*, 1964). This step of catecholamine synthesis is considered to be a rate-limiting and it is a subject of complex regulation (Tekin *et al*., 2014). TH enzyme requires for catecholamine biosynthesis tetrahydrobiopterin (Nagatsu *et al*, 1964), the regulatory cofactor, which is synthesized by guanosine triphosphate cyclohydrolase 1 (encoded by *Gch1* gene) and recycled by quinoid dihydropteridine reductase (encoded by *Qdpr* gene; Thöny *et al*., 2000). The second enzyme involved in catecholamine synthesis is L-DOPA decarboxylase (DDC, encoded by *Ddc* gene) which converts L-DOPA to dopamine (Blaschko,

1942). Subsequently, dopamine is converted by enzyme dopamine β-hydroxylase (DBH, encoded by *Dbh* gene) to form noradrenaline (Friedman and Kaufman, 1965). The enzyme phenylethanolamine N-methyl transferase (PNMT, encoded by *Pnmt* gene) is found primarily in adrenal medulla where it synthesizes adrenaline from noradrenaline (Wong *et al.*, 1987).

The catecholamine synthesis is regulated by multiple short-term and long-term physiological mechanisms, e.g. by the state of activation or the rate of expression or degradation of the abovementioned enzymes (Flatmark, 2000). In adrenals of SHR, both increased or decreased expression of catecholamine biosynthetic enzymes were demonstrated (Friese *et al.*, 2005; Kumai *et al.*, 1994; Moura *et al.*, 2005; Nguyen *et al.*, 2009). The catecholamine content in the adrenal gland was reported to be similar or lower in SHR as compared to WKY rats (Lee *et al.*, 1991a; Moura *et al.*, 2005). However, the catecholaminergic system is extremely susceptible to the stressful conditions (Kvetnansky *et al.*, 2004) and indeed the exaggerated stress-induced increase in tyrosine hydroxylase mRNA expression was reported in adrenal gland of SHR (Grundt *et al.*, 2009). On the other hand, the expression of genes involved in catecholamine synthesis in sympathetic ganglia of SHR has not been documented in such detail. However, greater vascular noradrenaline content and denser vascular sympathetic innervation were reported in various vascular beds of SHR (Cassis *et al.*, 1985; Donohue *et al.*, 1988; Head, 1989; Scott and Pang, 1983). The plasma levels of noradrenaline and adrenaline in SHR were described to be similar or higher (Kvetnansky *et al.*, 1979a; Moura *et al.*, 2005; Szemeredi *et al.*, 1988) than those in WKY rats. Apart from the rate of catecholamine synthesis which was studied frequently, their availability in target tissues can also be influenced by the changes in their storage, release, reuptake and degradation. The augmented catecholamine release was described in adrenal glands as well as in blood vessels of SHR (Bomfim *et al.*, 2017; Lim *et al.*, 2002; Tsuda and Masuyama,1993; Westfall *et al.*, 1984). Catecholamine storage vesicles of the adrenal medulla contain remarkably high concentrations of chromogranins (encoded by *Chga* and *Chgb* genes) and secretogranin (encoded by *Scg2* gene), which stabilize the vesicle core osmotically and which are also involved in the regulation of exocytosis (Zhang *et al.*, 2011). In the adrenal medulla of SHR, either augmented (O'Connor et al., 1999) or unchanged (Friese *et al.*, 2005; Jirout *et al.*, 2010) mRNA expression of *Chga* gene was demonstrated, while *Scg2* gene was reported to be underexpressed (Friese *et al.*, 2005). The mRNA expression of vesicular monoamine transporter 1 (encoded by *Slc18a1* gene, alias *Vmat1*), involved in the filling of catecholaminergic vesicles was shown to be unchanged (Jirout *et al.*, 2010) or decreased (Friese *et al.*, 2005) in adrenal medulla of SHR. On the other hand, vesicular monoamine transporter 2

(encoded by *Slc18a2* gene, alias *Vmat2*) was reported to be overexpressed in SHR (Friese *et al*., 2005).

Catecholamine uptake at the neuroeffector junction is an important mechanism for the regulation of the synaptic noradrenaline concentrations. The catecholamine reuptake by noradrenaline transporter (encoded by *Slc6a2* gene, alias *Net*) was demonstrated to be enhanced in blood vessels of SHR (Hano and Rho,1989; Rho *et al*., 1981; Whall *et al*., 1980) because it possibly compensates greater noradrenaline release from the sympathetic nerve endings. The mRNA expression of *Net* gene was shown to be higher (Reja *et al*., 2002b) or unchanged (Friese *et al*., 2005) in adrenal medulla of SHR. Catecholamines are degraded by enzymes monoamine oxidases (encoded by *Maoa* and *Maob* genes) or catechol-O-methyltransferase (encoded by *Comt* gene). Adrenaline is converted by catechol-O-methyltransferase to metadrenaline. Noradrenaline can be converted by both monoamine oxidase and catechol-O-methyltransferase and several metabolites can be produced, e.g. 3,4-dihydroxymandelic acid, normetadrenaline, vanillylmandelic acid etc. Sympathetic nerves contain only monoamine oxidase, while adrenal medulla and other non-neural tissues contain both enzymes monoamine oxidase and catechol-O-methyltransferase (Eisenhofer *et al.*, 2004). The decreased catecholamine degradation was observed in neural and non-neural tissues of SHR (Masuda *et al*., 2006; Tsunoda *et al*., 2003). The mRNA expression of *Comt* gene was reported to be decreased (Jirout *et al*., 2010) or increased (Friese *et al*., 2005) in adrenal medulla of SHR. The mRNA expression of *Maob* gene was shown to be downregulated (Friese *et al*., 2005) or unchanged (Jirout *et al*., 2010). Taken together, the studies concerning catecholamine storage, release, reuptake and degradation are not numerous and often yielded contradictory results.

1.2.4. *Adrenergic receptors*

Physiological actions of catecholamines in the body are realized through their interaction with adrenergic receptors which belong to a G-protein-coupled receptor superfamily. Adrenergic receptors can be divided into several distinct classes depending on their pharmacological specificity, coupling to different second messenger systems and various physiological actions (α_1-, α_2- and β-classes). α-adrenergic receptors have similar affinity for adrenaline and noradrenaline, while β-adrenergic receptors have stronger affinity to adrenaline than noradrenaline (Saunders and Limbird, 1999). The physiological effect depends on the actual ratio of adrenaline and noradrenaline and the quantity of particular types present in the cardiovascular target. The α_1-adrenergic receptors activate primarily $G_{q/11}$ pathway stimulating the

phospholipase C which leads to a generation of diacylglycerol and inositol trisphosphate and to a mobilization of intracellular calcium (Wecker *et al*., 2010). The α_2-adrenergic receptors activate G_i-proteins leading to the inhibition of adenylate cyclase and to the decrease of intracellular cAMP. In contrast, the activation of the β-adrenergic receptors leads mainly to the activation of G_S-proteins, the activation of adenylate cyclase and the increase of intracellular cAMP (Guimarães and Moura, 2001; Raymond et al., 1990). The receptor classes include receptor subtypes encoded by particular genes (e.g. α_{1A}, α_{1B}, etc.) which can differ in their efficiency to activate the downstream signaling cascade and in the susceptibility to desensitization (Guimarães and Moura, 2001; Piascik and Perez, 2001; Raymond et al., 1990).

Vascular smooth muscle contraction is triggered predominantly by α_1-adrenergic receptors, the involvement of α_{1A}- and/or α_{1D}-subtypes was implicated depending on species and vascular bed studied (Chen and Minneman, 2005; Guimarães and Moura, 2001; Piascik and Perez, 2001). Moreover, the α_1-adrenergic receptors stimulate a proliferation of vascular smooth muscle cells (Chen *et al*., 1995). The α_2-adrenergic receptors also contribute to a vasoconstriction (Chen and Minneman, 2005; Guimarães and Moura, 2001; Piascik and Perez, 2001). On the contrary, the β_2-adrenergic receptors mediate smooth muscle relaxation (Chruscinski *et al*., 2001). In addition, α_2- and β_2-adrenergic receptors can be found on endothelium and their stimulation leads to release of nitric oxide and vasodilation (Guimarães and Moura, 2001). An augmented vascular response to noradrenaline was described in prehypertensive SHR (Lais and Brody, 1978) and α-adrenergic receptor-mediated vasoconstriction was proposed to be involved in the pathogenesis of hypertension. Moreover, the enhanced proliferation of smooth muscle cells in blood vessels of SHR can be reversed by chronic α_1-adrenergic blockade (Kuriyama *et al*., 1991). However, radio-ligand binding studies have not provided a convincing evidence about the increased α-adrenergic receptor density or affinity in SHR (Michel *et al*., 1990, Takata and Kato, 1996). By contrast, the impaired β-adrenergic receptor-mediated relaxation in SHR is generally accepted, although the density of β-adrenergic receptors was shown to be either decreased, unchanged or increased in blood vessels of SHR (Asano *et al*., 1991; Bruschi *et al*., 1984, Kwan and Lee, 1990). The decreased β-adrenergic receptor responsiveness in SHR may rather be associated with the reduced function of G_S-protein and downstream signaling cascade (Asano *et al*., 1991; Masuzawa *et al*., 1989).

β_1-adrenergic receptors are the predominating type which control cardiac contractility and relaxation (Madamanchi, 2007). In the rat heart, α_1-adrenergic receptors appear to be important, although in other species their influence is quite small (Michel *et al*., 1990). Cardiac

β_1-adrenergic receptors were reported to be downregulated in SHR (Castellano et al., 1993; Michel *et al*., 1987; Yamada *et al*., 1984). On the other hand, cardiac β_2-adrenergic receptors were reported to be increased in SHR (Michel *et al*., 1987; Yamada *et al*., 1984), but they are not localized on ventricular myocytes (Takata and Kato, 1996) but rather on the sympathetic nerve endings (Michel *et al*., 1987) and in the atria (Juberg *et al*., 1985). β_2-adrenergic receptors on the sympathetic nerve endings might be involved in the facilitation of noradrenaline release (Michel *et al*., 1987). The functional role of atrial β_2-adrenergic receptors remained to be established, but they are not involved in the regulation of heart rate or cardiac contractility (Juberg *et al*., 1985). Although, studies on the density of cardiac α-adrenergic receptors in SHR provided inconsistent results, it was proposed that they can be involved in the development of cardiac hypertrophy (Takata and Kato, 1996).

Adrenergic receptors are involved in the feedback regulation of catecholamine release. The activation of α_2-adrenergic receptors inhibits catecholamine release from sympathetic terminals, adrenal medulla and neurons in the brainstem (Brede *et al*., 2003; Gilsbach et al., 2009; Urban *et al*., 1995), thus exhibiting a hypotensive effect. In contrast, β-adrenergic receptors facilitate noradrenaline release from sympathetic terminals (Guimarães and Moura, 2001). Presynaptic α_2-adrenoceptor inhibition of noradrenaline and adrenaline secretion was found to be less effective in SHR (Berg and Jensen, 2013; Zugck *et al*., 2003). Accordingly, the α_2-adrenoceptor expression was lower in the brain, sympathetic ganglia and adrenal medulla (Carrettiero *et al*., 2012; Moura *et al*., 2012; Zugck *et al*., 2003) while the density of β_2-adrenergic receptors was reported to be increased in sympathetic ganglia of SHR (Saavedra *et al*., 1990; Pinto *et al*., 1991).

Taken together, numerous studies demonstrated the alterations in the function of adrenergic receptors in blood vessels, heart and neurons in SHR which resulted from the altered receptor density and/or abnormal intracellular signal transduction. Thus the altered function of adrenergic receptors can play an important role in the pathophysiology of hypertension in SHR.

1.3. Stress and cardiovascular system

Stress can be defined as a state when homeostasis is disrupted or it is perceived to be threatened. The stress-induced physiological and behavioral changes (increased cardiovascular tone and respiratory rate; augmented gluconeogenesis and lipolysis; increased alertness and vigilance; improved cognition; enhanced analgesia; etc.) aim to re-establish the homeostasis and to maintain the integrity of the organism (Charmandari *et al*., 2005). The central components of

stress system are located in the hypothalamus and in the brainstem and comprise many areas involved in the regulation of BP including NTS, PVN and RVLM. The major components of the stress system are the autonomic nervous system and the hypothalamic-pituitary-adrenocortical (HPA) axis (Herman *et al.*, 2003; Ulrich-Lai and Herman, 2009).

The activation of HPA axis triggers the secretion of corticotropin-releasing hormone (CRH) from the PVN of hypothalamus into hypophyseal portal circulation. CRH induces the subsequent secretion of adrenocorticotropic hormone (ACTH) from anterior pituitary gland. ACTH acts on the adrenal cortex and initiates the synthesis and release of glucocorticoid hormones (corticosterone in rats, cortisol in humans) from zona fasciculata (Chrousos, 2009; Ulrich-Lai and Herman, 2009). Glucocorticoids regulate basal activity of the HPA axis and also terminate the stress response by a negative feedback inhibition of CRH and ACTH release (Sapolsky *et al.*, 1986).

The cardiovascular response during acute stress (characterized by the increased BP and HR) is based upon the activation of sympathoneural and sympathoadrenal systems (Dos Reis *et al.*, 2014). The parasympathetic system facilitates the stress response by withdrawing its inhibitory effects (Porges, 1995). Glucocorticoids potentiate numerous sympathetically mediated effects, including peripheral vasoconstriction (Ulrich-Lai and Herman, 2009). The activation of HPA axis and autonomic nervous system is highly coordinated and their regulation is interconnected. In the brain, CRH mediates sympathetic arousal (Sapolsky *et al.*, 2000), while noradrenaline promotes CRH expression in the PVN (Ma and Morilak, 2005). In the periphery, glucocorticoids activate the enzymes of catecholamine biosynthesis in the adrenal medulla (Kvetnansky *et al.*, 1995), whereas adrenal cortex is directly innervated by the sympathetic nervous system, which can regulate corticosteroid release (Chrousos, 2009; Ulrich-Lai and Herman, 2009).

Both glucocorticoids and catecholamines have short-term beneficial effects but they can also have deteriorating effects in the long-term period, especially during frequent or prolonged stress exposure. The adaptation (i.e. progressive reduction) of physiological and behavioral responses to the repeated exposures to stressor is an important process that attenuates the deleterious consequences of long-term stress on the organism (Benini *et al.*, 2019; McEwen, 1998). Chronic stress or the inappropriate reaction of the stress system can participate in the pathogenesis of cardiovascular diseases (Herman, 2013; McEwen, 1998; McEwen and Stellar, 1993). It was shown that repeated administration of ACTH or corticosterone lead to the

development of high BP in rats (Mangos *et al*., 2000; Turner *et al*., 1998; Whitworth *et al*., 1990) but the mechanism remains still unknown. The abnormal HPA function might also be related to the hypertension development in SHR. Hypophysis and adrenal glands are greater in SHR than in normotensive controls (Aoki *et al*., 1963; Aoki *et al*., 1973). Hypophysectomy as well as adrenalectomy in prehypertensive SHR prevented the development of high BP (Aoki, 1963; Aoki *et al*., 1973), while corticosterone but not aldosterone replacement restored hypertension in adrenalectomized SHR (Aoki, 1964; Hashimoto *et al*., 1989; Yagil *et al*., 1989). The decreased CRH content was observed in hypothalamus and hypophysis of young SHR (Hattori *et al*., 1986b). Moreover, lower content of pro-opiomelanocortin (POMC, ACTH precursor) in the hypophysis (Braas and Hendley, 1994) and an impaired ACTH response to CRH administration were described in young SHR (Hasmimoto *et al*., 1985; Hattori *et al*., 1986b). On the other hand, plasma ACTH levels induced by ether exposure (Häusler *et al*., 1983), cold exposure or immobilization were reported to be enhanced in SHR compared to WKY rats (Djordjevic *et al*., 2007). Basal levels of circulating corticosterone were shown to be unchanged (Gómez *et al*., 1998; Häusler *et al*., 1983; Kvetnansky *et al*., 1979a) or increased (Ardekani et al., 1989; Hattori *et al*., 1986a) in SHR.

SHR exhibit exaggerated cardiovascular responses (BP increase and HR acceleration) to various stress stimuli, such as restraint, handling, open-field or air-jet (Ely *et al*., 1985; McDougall *et al*., 2005; van den Buuse *et al*., 2001). Moreover, the pressor response of SHR to various types of stressor is prolonged compared with the normotensive WKY rats (McDougall *et al*., 2005) and the BP response declines with repeated exposures to the same stressor down to the levels similar to those observed in the normotensive controls (McDougall *et al*., 2005; McDougall *et al*. 2000). In accordance with the above mentioned hyperactivity of sympathoadrenal and sympathoneural systems, SHR have higher plasma levels of noradrenaline and adrenaline during the acute exposure to the immobilization as compared to WKY rats (Kvetnansky *et al*., 1979a). Furthermore, plasma corticosterone levels are also higher in SHR subjected to ether exposure or immobilization (Häusler *et al*., 1983; Kvetnansky *et al*., 1979a). The stress-induced increase in plasma adrenaline, noradrenaline and corticosterone levels decline in SHR, which were repeatedly exposed to the same stressor (Kvetnansky *et al*., 1979a), this being in line with the cardiovascular adaptation (McDougall *et al*., 2005; McDougall *et al*., 2000). Taken together, the altered regulation, function and adaptation of HPA axis and/or sympathoadrenal system might be involved in the exaggerated response of SHR to stressor exposure as well as in the pathogenesis of hypertension in this rat strain.

2. AIMS OF THE THESIS

The spontaneously hypertensive rats (SHR) are a model of human essential hypertension characterized by multiple molecular, morphological and functional alterations at many different anatomical sites of sympathoneural and sympathoadrenal systems including sympathetic ganglia, vascular innervation and adrenal medulla. Some abnormalities are already present in the prehypertensive SHR and might contribute to the hypertension development, whereas the other changes can rather be consequences of long-term high blood pressure or compensations aiming to reduce deteriorating effects of hypertensive state. The general goal of the thesis was to study the role of sympathoadrenal and sympathoneural systems in the development and maintenance of high blood pressure in spontaneously hypertensive rat. Specifically, these aims were addressed in three projects, which were focused on:

2.1. Project 1 - the comparison of sympathoneural and sympathoadrenal abnormalities in young prehypertensive and adult spontaneously hypertensive rats

The aim of this project was to describe ontogenetic differences in the sympathoneural and sympathoadrenal systems between SHR and WKY rats. A comparison of prehypertensive and hypertensive animals (4 and 24 weeks of age) may reveal some important abnormalities underlying the development of hypertension in this model.

2.2. Project 2 - the effects of sympathectomy on cardiovascular system: a comparison of adult normotensive and spontaneously hypertensive rats

The aim of this project was to compare the effect of chemical sympathectomy induced by guanethidine on cardiovascular parameters in adult SHR and WKY rats. The compensatory role of adrenal hormones, renin-angiotensin system and blood pressure sensitivity to vasoconstrictors was studied.

2.3. Project 3 - the comparison of stress-induced cardiovascular and hormonal responses in adult normotensive and spontaneously hypertensive rats

The aim of this project was to compare stress-induced cardiovascular response of adult SHR and WKY rats evoked by the acute restraint and to study stress-induced changes of sympathoneural and sympathoadrenal systems in normotensive and hypertensive rats.

The study concerning changes in sympathoneural and sympathoadrenal systems in the spontaneously hypertensive rats under different conditions (young vs. adult rats, destruction of sympathetic terminals, stress) can provide useful information on the role of both systems in the development and maintenance of high blood pressure.

3. METHODS

The methods are described in detail in the particular publications, which are in Attachments.

3.1. Animals

The experiments were performed in male spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats (bred at the Institute of Physiology, Czech Academy of Sciences). The rats were housed under standard laboratory conditions: temperature 23 ± 1°C, 12 h light–dark regime, free access to water and chow (Altromin 1324, Altromin, Lage, Germany). At the end of experiments, the rats were sacrificed by an overdose of anaesthetic isoflurane and cardiac exsanguination. All experimental procedures were approved by the Ethical Committee of the Institute of Physiology, Czech Academy of Sciences and conform to the European Convention on Animal Protection and Guidelines on Research Animal Use.

3.2. Experimental groups and procedures

Project 1 - Comparison of sympathoneural and sympathoadrenal abnormalities in young prehypertensive and adult spontaneously hypertensive rats

Male SHR rats aged 4 weeks (prehypertensive) and 24 weeks (with established hypertension) were used for the experiments. WKY rats were used as a normotensive controls. **Series 1** (n=8 in each group) was used for direct BP measurement. **Series 2** (n=8 in each group) was utilized for the collection of plasma and tissue samples.

Project 2 - The effects of sympathectomy on cardiovascular system: a comparison of adult normotensive and spontaneously hypertensive rats

Chemical sympathectomy was performed in adult SHR and WKY rats (20-22 weeks) by a daily intraperitoneal administration of guanethidine hemisulfate (30 mg/kg of body weight; b.w.) for two weeks. Control rats were injected with saline. There were four series of sympathectomized (SYMPX) and control rats. **Series 1** (n=7 rats in each group) was used for BP

and HR measurement by radiotelemetry in freely moving and restrained rats. **Series 2** (n=6-8 rats in each group) was on the 14th day of guanethidine treatment used for a measurement of mean arterial pressure (MAP) response to catecholamine administration and MAP response to ganglionic blocker pentolinium. **Series 3** (n=9-11 rats in each group) was used on the 14th day of guanethidine treatment for a measurement of MAP response to angiotensin II (Ang II) administration and MAP response to angiotensin-converting enzyme inhibitor captopril. **Series 4** (n=8 in each group) was utilized on the 14th day of guanethidine treatment for a collection of tissue and plasma samples.

Project 3 - Comparison of stress-induced cardiovascular and hormonal responses in adult normotensive and spontaneously hypertensive rats

Adult SHR and WKY rats (18-24 weeks) were used for the experiment. There were three series of rats. **Series 1** (n=7 rats in each group) was used for BP and HR measurement by radiotelemetry in freely moving animals and under the conditions of restraint stress (120 min). The rats in **series 2** were subjected to either no stressor exposure (stress-naive rats), 10 min of restraint, 120 min of restraint or 120 min of restraint followed by 120 min recovery period (n=8 in each group) and utilized for a collection of tissue and plasma samples. **Series 3** (n=6-10 rats in each group) was analogical to series 2, blood was collected and processed in a manner appropriate for determination of plasma renin activity.

3.3. Radiotelemetric measurement of blood pressure and heart rate

In Projects 2 and 3, the rats were implanted with telemetry devices (model HD-S10, Data Sciences International, New Brighton, USA) under isoflurane anaesthesia (5 % for induction and 2.5 % for maintenance; Forane, AbbVie, USA) as previously described (Behuliak *et al*., 2018; Vavřínová *et al*., 2019b). Following a 10-day recovery period, basal BP and HR were measured in freely moving rats for three days (5-min intervals were recorded four times per hour). The

next day, the effect of restraint stress on cardiovascular parameters was evaluated (Projects 2 and 3).

In Project 2, the rats were left to rest for three days after single restraint stress exposure and guanethidine treatment was started. After 14-day of guanethidine administration, the effect of restraint stress on cardiovascular parameters was evaluated sympathectomized rats.

3.4. Spectral analysis of systolic blood pressure variability and baroreflex function

In Projects 2 and 3, power spectral analysis of systolic blood pressure variability (SBPV) was done by dr. Behuliak and dr. Bencze to evaluate the low-frequency (LF; 0.2 - 0.75 Hz), and high-frequency (HF; 0.75 - 4 Hz) components of SBPV as previously described (Behuliak *et al*., 2018). These two frequencies were reported to reflect vascular and cardiac sympathetic activity, respectively (Yoshimoto *et al*., 2011). Baroreflex function was evaluated in freely-moving animals by the spontaneous sequence technique (Bertinieri *et al*., 1985) using Hemolab software (ver. 21.0) programmed by Harald Stauss.

3.5. Direct blood pressure measurement

In Projects 1 and 2, one day before BP measurement, catheters were inserted into the left carotid artery (PE-50 for BP measurements) and jugular vein (PE-10 for infusion of drugs) of rats under the isoflurane anaesthesia. After 24-h recovery, BP and HR were measured between 08:00 and 11:30 AM in conscious rats placed in small transparent cages (partially restrained) using PowerLab system (ADInstruments, Bella Vista, Australia) (Behuliak *et al*., 2018; Kunes *et al*., 2002).

3.6. Cardiovascular response to vasoactive agents

In Project 2, BP and HR responses to intravenous administration of vasoactive agents were measured in conscious cannulated control and sympathectomized SHR and WKY rats. In one series of rats, noncumulative doses of noradrenaline (0.001-10 μg/kg b.w.) or adrenaline

(0.001-10 µg/kg b.w.) were administered. Thereafter, the extent of sympathectomy was verified by the BP response to intravenous administration of ganglionic blocker pentolinium (5 mg/kg b.w.) as well as to catecholamine-releasing agent tyramine (100 µg/kg b.w.). In the second series of rats, a single dose of angiotensin-converting enzyme inhibitor captopril (10 mg/kg b.w.) was injected intravenously to conscious SHR and WKY rats to determine the contribution of endogenous RAS (Zicha *et al*., 2014) and to prevent the interference of endogenous RAS with Ang II administered exogenously. Then, a single dose of pentolinium (5 mg/kg b.w.) was injected and noncumulative doses of Ang II (0.1-100 ng/kg b.w.) were administered. Finally, the extent of sympathectomy was verified by tyramine administration (100 µg/kg b.w.).

3.7. Restraint stress

The rats subjected to restraint were horizontally placed into transparent plastic cylinders (6.5 cm inner diameter; adjustable in length depending on animal size) equipped with ventilation holes.

3.8. Tissue sampling

In all projects, rats designated to sample collection were anesthetized by isoflurane (5 % for induction and 2.5 % for maintenance). Blood was collected into S-Monovette® K_3EDTA tubes (SARSTEDT AG & Co. KG, Nümbrecht, Germany) and centrifuged for 10 min (3000 g, in room temperature for determination of plasma renin activity or in 4 °C for other hormone assays).

In Project 1, one adrenal gland was taken for protein and catecholamine measurements. Adrenal medulla from the contralateral adrenal gland and superior cervical ganglia were used for mRNA measurements. Femoral arteries were immediately used for histochemical visualization of monoamines by the SPG method.

In Project 2, one adrenal gland was taken for catecholamine measurements. The contralateral adrenal gland was embedded in Tissue-Tek O.C.T. (Sakura, Tokyo, Japan), processed by laser capture microdissection and used for mRNA measurements in adrenal medulla. Vascular smooth muscle samples from the endothelium-denuded thoracic aorta were snap-frozen in liquid nitrogen. Femoral arteries were used for histochemical visualization of monoamines by SPG method.

In Project 3, adrenal gland was embedded in Tissue-Tek O.C.T. (Sakura, Tokyo, Japan) processed by laser capture microdissection and used for mRNA measurements.

3.9. Histochemical visualization of monoamines (SPG method)

In Projects 2 and 3, histochemical visualization of monoamines in femoral arteries was performed according to the protocol of de la Torre and Surgeon (1976) as described previously (Bencze *et al*., 2016; Vavřínová *et al*., 2019a). The arteries were dipped in the glyoxylic acid solution (1% glyoxylic acid (Sigma-Aldrich), 236 mM KH_2PO_4 and 200 mM sucrose), mounted on glass slides, dried by air cooler and the slides were heated on a hot plate (80 °C, 5 min). Thereafter, Mineral Oil (Sigma-Aldrich) and cover glass were added and the slides were heated again (80 °C, 90 s). The specimens were observed using fluorescent microscope Leica LMD6000 with DAPI filter cube. The quantification was done using ImageJ 1.4v software (Schindelin *et al*., 2012).

3.10. Hormone assays

All hormones were measured by commercial kits according to the manufacturer`s instructions. The concentration of catecholamines was measured in plasma and adrenal homogenates using 3-CAT Research ELISA (LDN, Nordhorn, Germany). Plasma normetadrenaline and metadrenaline were measured with 2 MET Plasma ELISA Fast Track (LDN). Adrenal glands were homogenized in phosphate buffer saline (Sigma-Aldrich) with

EDTA and sodium metabisulfite (1 mM and 4 mM, respectively) using MagNA Lyser Instrument and MagNA Lyser Green Beads (Roche, Basel, Switzerland). Plasma ACTH was measured by ACTH ELISA (MD Bioproducts, Zurich, Switzerland). Plasma corticosteroids were measured using Corticosterone rat/mouse ELISA (LDN) and Aldosterone ELISA (LDN). Plasma renin activity was measured by PRA ELISA (Crystal Chem, USA) based on the measurement of the amount of angiotensin I generated over a specific period (90 min). Microplate reader Tecan Infinite M200 (Tecan Group Ltd., Männedorf, Switzerland) was used for reading the absorbance at 450 nm.

3.11. Laser capture microdissection

In the Projects 2 and 3, adrenal medulla for mRNA expression measurement was dissected by laser capture microdissection. Adrenal glands embedded in Tissue-Tek O.C.T. were tempered to -19 °C and serially cut into 20 μm thick sections using a cryostat Leica CM 1850 (Leica Microsystems, Wetzlar, Germany). The slices were transferred to polyethylene-naphtalate membrane slides (Leica Microsystems), dehydrated in 95 % ethanol, stained with 1 % cresyl violet acetate (75 % ethanol, pH=6.92) and washed in 95 % ethanol for 10 s. Total area 1 mm^2 of adrenal medulla was dissected using the Leica LMD 6000 Laser Microdissection System (Leica Microsystems) into the microcentrifuge tubes with RNA Lysis Buffer (Zymo Research, Irvine, USA).

3.12. RNA isolation, reverse transcription and quantitative real-time PCR

Smooth muscles from aorta (in Project 2), adrenal medulla (macrosample) and sympathetic ganglia (in Project 1) were homogenized by MagNA Lyser Instrument and MagNA Lyser Green Beads (Roche, Basel, Switzerland). Total RNA isolation was performed by commercial kits according to the manufacturer`s instructions. The total RNA of vascular smooth muscles was isolated using RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). The

total RNA of adrenal medulla and sympathetic ganglia was isolated using a Genelute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St Louis, MO, USA).

Laser capture microdissected samples of adrenal medulla (Projects 2 and 3) were isolated with Quick-RNA™ MicroPrep kit (Zymo Research). In all isolated RNA samples, genomic DNA was removed with RNase-free DNase I (Qiagen). The integrity of RNA was tested by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The quantity of RNA was checked by spectrophotometer NanoDrop ND 1000 (NanoDrop Products, Wilmington, DE, USA) or by QuantiFluor® RNA System kit using Quantus™ Fluorometer (Promega, Madison, USA). RNA from macrosamples was transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA), whereas RNA from microdissected samples was transcribed using a more sensitive SuperScript® VILO™ cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA).

The gene expression was determined on the LightCycler® 480 System (Roche) using HOT FIREPol® Probe qPCR Mix Plus (SolisBioDyne, Tartu, Estonia) and TaqMan® Gene Expression Assays (Life Technologies). The expression of following genes was determined: genes involved in catecholamine biosynthesis (enzymes *Th*, *Ddc*, *Dbh*, *Pnmt* and cofactors quinoid dihydropteridine reductase*, Qdpr* and GTP cyclohydrolase 1, *Gch1*), genes related to catecholamine vesicles (*Vmat1*, *Vmat2*, *Chga*, *Chgb*, *Scg2*, *Npy*), genes involved in catecholamine reuptake or degradation (*Net*, *Maoa*, *Maob*, *Comt*), genes for adrenergic receptors (adrenoceptor alpha 1A, *Adra1a*; adrenoceptor alpha 1B, *Adra1b*; adrenoceptor alpha 1D, *Adra1d*; adrenoceptor alpha 2A, *Adra2a*; adrenoceptor alpha 2B, *Adra2b*; adrenoceptor alpha 2C, *Adra2c*; adrenoceptor beta 1, *Adrb1*; adrenoceptor beta 2, *Adrb2*) and particular G-proteins (G protein subunit alpha q, *Gnaq*; G protein subunit alpha 11, *Gna11*), genes involved in corticosteroid signaling (glucocorticoid receptor, *Nr3c1*; mineralocorticoid receptor, *Nr3c2*; Fkbp prolyl isomerase 5, *Fkbp5*; 11β-hydroxysteroid dehydrogenase type 1 and 2, *Hsd11b1* and

Hsd11b2; glucose-6-phosphate dehydrogenase, *H6pd*) and genes for Ang II receptors (angiotensin II receptor subtype 1A, *Agtr1a*; angiotensin II receptor subtype 1B, *Agtr1b*; angiotensin II receptor subtype 2, *Agtr2*). The list of all TaqMan® Gene Expression Assays is available in Tables 1 and 2.

Group of genes	Gene symbol	Alias	Gene name	Assay ID
Catecholamine biosynthesis	*Th*		tyrosine hydroxylase	Rn00562500_m1
	Ddc	AADC	DOPA decarboxylase	Rn00561113_m1
	Dbh		dopamine beta-hydroxylase	Rn00565819_m1
	Pnmt		phenylethanolamine-N-methyltransferase	Rn01495588_m1
	Qdpr		quinoid dihydropteridine reductase	Rn00574367_m1
	Gch1		GTP cyclohydrolase 1	Rn00577450_m1
Vesicle-related	*Vmat1*	Slc18a1	solute carrier family 18, member 1	Rn00461866_m1
	Vmat2	Slc18a2	solute carrier family 18, member 2	Rn00564688_m1
	Chga		chromogranin A	Rn00572200_m1
	Chgb		chromogranin B	Rn01514853_m1
	Scg2	Chcg	secretogranin II	Rn02042961_s1
	Npy		neuropeptide Y	Rn01410145_m1
Catecholamine removal from synaptic cleft	*Net*	Slc6a2	solute carrier family 6, member 2	Rn00580207_m1
	Maoa		monoamine oxidase A	Rn01430950_m1
	Maob		monoamine oxidase B	Rn00566203_m1
	Comt		catechol-O-methyltransferase	Rn00561037_m1
Adrenergic receptors	*Adra1a*		adrenoceptor alpha 1A	Rn00567876_m1
	Adra1b		adrenoceptor alpha 1B	Rn01471343_m1
	Adra1d		adrenoceptor alpha 1D	Rn00567876_m1
	Adra2a		adrenoceptor alpha 2A	Rn00562488_s1
	Adra2b		adrenoceptor alpha 2B	Rn00593312_s1
	Adra2c		adrenoceptor alpha 2C	Rn00593341_s1
	Adrb1		adrenoceptor beta 1	Rn00824536_s1
	Adrb2		adrenoceptor beta 2	Rn00560650_s1
G-proteins	*Gnaq*		G protein subunit alpha q	Rn00578978_m1
	Gna11		G protein subunit alpha 11	Rn00578959_m1

Table 1. TaqMan® Gene Expression Assays for measurements of mRNA expression of genes involved in catecholamine biosynthesis, genes related to catecholamine vesicles, genes involved in catecholamine reuptake or degradation and genes for adrenergic receptors.

Exported raw data were analyzed by software LinRegPCR (version 2013.0; Ruijter *et al*., 2009) for determination of C_t values (number of cycles needed to reach the threshold) and mean PCR efficiencies per amplicon (averaged efficiencies of individual samples amplified with particular TaqMan Gene Expression Assay). The obtained values were used for relative quantification by a modified $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), where PCR efficiency was used as a base of exponentiation. The data from particular tissues and under particular experimental conditions were normalized to the best combination of two reference genes which were selected by NormFinder software (Andersen *et al*., 2004) as described in Vavřínová *et al*. (2016), see Attachments.

Group of genes	Gene symbol	Alias	Gene name	Assay ID
Corticosteroid signaling	*Nr3c1*	Gr	nuclear receptor subfamily 3, group C, member 1; glucocorticoid receptor	Rn00561369_m1
	Nr3c1	Mr	nuclear receptor subfamily 3, group C, member 2; mineralocorticoid receptor	Rn00565562_m1
	Fkbp5		FKBP prolyl isomerase 5	Rn01768371_m1
	Hsd11b1		hydroxysteroid 11-beta dehydrogenase 1	Rn00567167_m1
	Hsd11b2		hydroxysteroid 11-beta dehydrogenase 2	Rn00492539_m1
	H6pd		hexose-6-phosphate dehydrogenase	Rn01519771_m1
Ang II receptors	*Agtr1a*	At1A	angiotensin II receptor subtype 1A	Rn00578456_m1
	Agtr1b	At1B	angiotensin II receptor subtype 1B	Rn02132799_s1
	Agtr2	At2	angiotensin II receptor subtype 2	Rn00560677_s1
Reference	*18S*	Rn18s	18S ribosomal RNA	Rn03928990_g1
	Gapdh	Gapd	glyceraldehyde-3-phosphate dehydrogenase	Rn01775763_g1
	Hprt1	Hprt	hypoxanthine phosphoribosyltransferase 1	Rn01527840_m1
	Sdha		succinate dehydrogenase complex flavoprotein subunit A	Rn00590475_m1
	Ywhaz	14-3-3z	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	Rn00755072_m1

Table 2. TaqMan® Gene Expression Assays for measurements of mRNA expression of genes involved in corticosteroid signaling, genes for Ang II receptors and reference genes.

3.13. Western blot analysis

Frozen adrenal glands or sympathetic ganglia were homogenized in RIPA buffer (Sigma-Aldrich) using MagnaLyser Green Beads (Roche). The homogenate was centrifuged for 20 min at 14000 g and 4 °C. The reducing SDS polyacrylamide gel electrophoresis was performed using Optiblot precast 4-20 % gradient gels (Abcam, Cambridge, UK) at 100 V for 120 min. Semi-dry transfer of proteins to Polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) was performed with a Tris Glycine Buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing 10 % methanol at 25 V for 30 min. Ponceau-S (0.1 % Ponceau S in 5 % acetic acid) was used for control of the transfer efficiency. Subsequently, membranes were washed from the dye and blocked with 3 % milk diluted in Tris-buffered saline with Tween (TBS-T; 137 mM NaCl, 20 mM Trizma® base and 0.1 % Tween® 20, Sigma-Aldrich) at room temperature for 60 min. Membranes were incubated with primary antibody diluted in 3 % milk TBS-T at 4 °C overnight. Primary antibodies used: Anti-Tyrosine hydroxylase (1:4000, Abcam, ab112, LOT: GR265840-3), Anti Dopamine beta hydroxylase (1:2000, Abcam, ab43868, LOT: GR110853-8), Anti DOPA decarboxylase (1:2000, Abcam, ab3905, LOT: GR2164-10), Anti PNMT (1:4000, Abcam, ab69579, LOT: GR129455-1), Anti-HPRT (1:5000, Abcam, ab109021, LOT: GR153613-1) and Anti-GAPDH (1:8000, Cell Signaling Technology, Inc., Danvers, MA, United States, #2118, LOT: 2118S). Subsequently, the membranes with samples of adrenal gland were incubated with Peroxidase-Conjugated Goat Anti-Rabbit secondary antibody (1:5000, Thermo Fisher Scientific) in 3 % milk TBS-T for 60 min at room temperature. For the signal enhancement in samples of sympathetic ganglia, a biotinylated secondary antibody was used in a concentration of 1:4000 in 3 % milk TBS-T for 60 min and then Avidin and Biotinylated Horseradish Peroxidase 1:400 in 3 % milk TBS-T for 60 min at room temperature was added (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). The horseradish peroxidase was detected using a SuperSignal West Femto reagent (Thermo Fisher Scientific)

and the emitted light was captured with a chemiluminescence imaging analyzer LAS 1000 (Fujifilm, Tokyo, Japan). We used the same conditions for each run of particular antibody in a given tissue (the same concentration, time with horseradish peroxidase substrate, exposure time etc.). The obtained images were analyzed using software ImageJ 1.4v (Schindelin *et al*., 2012). Protein expression of the genes of interest was normalized to the expression of HPRT or GAPDH in adrenal glands or sympathetic ganglia, respectively.

3.14. Statistics

The data are expressed as the means ± SEM. Statistical significance of the differences between experimental groups was determined by Student's t test, two-way analysis of variance (ANOVA), two-way repeated measures ANOVA or three-way ANOVA as appropriate for the respective data. For details see the publications (Attachments) or the legends of the figures or tables. The differences were considered to be significant at $p < 0.05$.

4. RESULTS

4.1. *Project 1 – The comparison of sympathoneural and sympathoadrenal abnormalities in young prehypertensive and adult spontaneously hypertensive rats*

4.1.1. *Physiological parameters*

Mean arterial pressure (MAP) was similar in 4-week-old SHR and WKY rats, but HR was higher in prehypertensive SHR (Table 3). Both MAP and HR were higher in 24-week-old SHR than in WKY rats. The body weights of SHR were lower than those of WKY rats. The relative weight of adrenal glands was greater in both prehypertensive and adult SHR compared to the age-matched WKY rats.

	WKY 4 weeks	**SHR 4 weeks**	**WKY 24 weeks**	**SHR 24 weeks**
Mean arterial pressure (mm Hg)	89 ± 2	92 ± 4	109 ± 2 †	177 ± 3 * †
Heart rate (beat/min)	398 ± 12	476 ± 6 *	317 ± 6 †	361 ± 8 * †
Body weight (g)	102 ± 3	71 ± 1 *	329 ± 4 †	315 ± 3 * †
Absolute adrenal weight (mg)	21.0 ± 0.6	22.5 ± 0.4	35.8 ± 0.7 †	40.8 ± 0.7 * †
Relative adrenal weight (mg/100g)	20.4 ± 0.34	31.6 ± 0.70 *	10.9 ± 0.3 †	13.0 ± 0.2 * †

Table 3. Physiological parameters and weight of 4-week-old and 24-week-old WKY and SHR rats. Values are expressed as mean ± SEM, n = 8 for each group. The effects of strain and age were analyzed by two-way ANOVA, Bonferroni *post-hoc* test was performed. * $p < 0.05$ vs. age-matched WKY; † $p < 0.05$ vs. 4-week-old rats of the same strain. Vavřínová *et al*., 2019a

4.1.2. *The expression of genes of catecholaminergic system in adrenal glands*

For a reliable comparison of mRNA expression, the stability of the expression of 12 reference genes was tested as described in Vavřínová *et al*., 2016, see Attachments. The best combination of two reference genes *Hprt1* and *Ywhaz* was used for normalization of mRNA expression data in adrenal medulla of 4- and 24-week-old SHR and WKY rats.

The mRNA expression of genes involved in catecholamine biosynthesis *Dbh* and *Pnmt* was found to be lower in the adrenals of 4-week-old SHR, while the expression of *Th* and *Ddc* was unchanged compared to the age-matched WKY rats (Fig. 2A). Protein expression of DDC and DBH enzymes was reduced, while there was no change in protein expression of TH and PNMT enzymes in prehypertensive SHR (Fig. 2B).

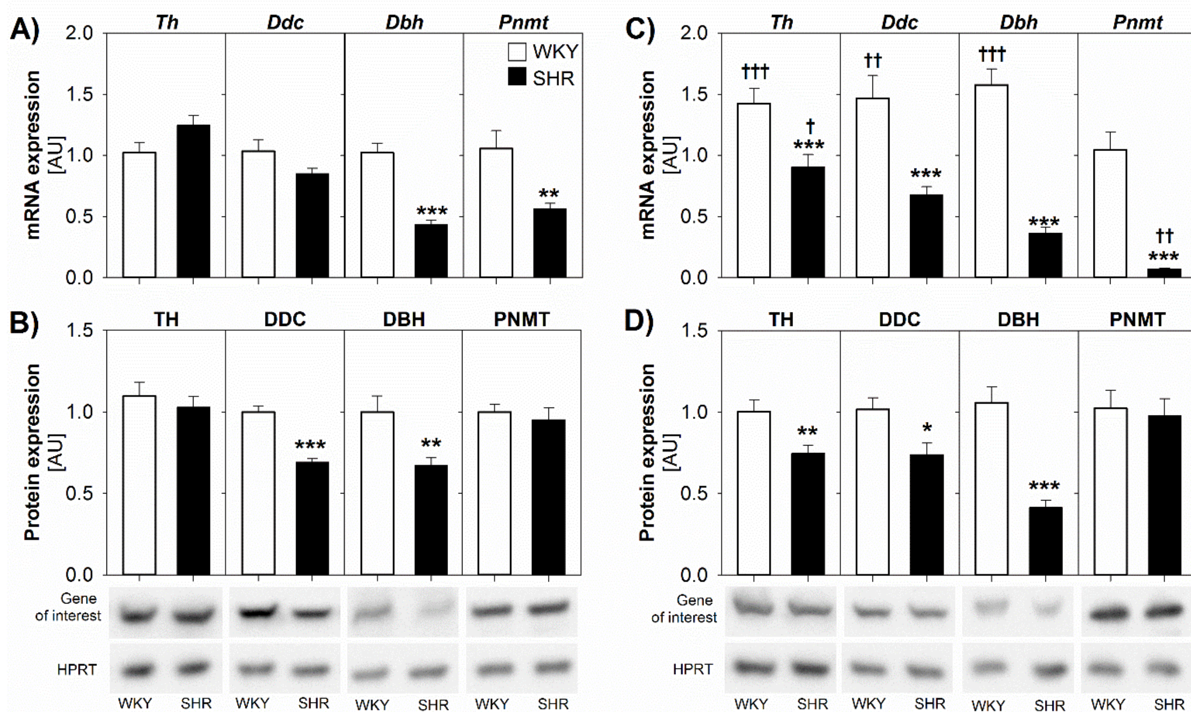

Figure 2. mRNA (A, C) and protein (B, D) expression of genes involved in catecholamine biosynthesis in the adrenal glands of prehypertensive 4-week-old (A, B) or hypertensive 24-week-old (C, D) SHR and age-matched WKY rats. The mRNA expression was standardized to the best combination of reference genes *Hprt1* and *Ywhaz*. The protein expression was standardized to HPRT. Data are plotted relatively to WKY rats as mean ± SEM, n = 8 for each group. The effect of strain and age on mRNA expression was analyzed by two-way ANOVA, Bonferroni *post-hoc* test was performed. * $p<0.05$ vs. age-matched WKY; † $p<0.05$ vs. mRNA expression in 4-week-old rats of the same strain. The statistical significance of Western blot data was determined with Student's t test separately for either age. * $p<0.05$ vs. age-matched WKY. Vavřínová *et al*., 2019a

Furthermore, a higher mRNA expression of *Gch1* gene was found in the adrenal glands of prehypertensive SHR while the mRNA expression of *Vmat2* and *Npy* genes was reduced compared to age-matched WKY rats (Table 4).

In the adrenals of adult SHR, the mRNA expression of *Th*, *Ddc*, *Dbh* and *Pnmt* was lower than in age-matched WKY rats (Fig. 2C). Accordingly protein expression of TH, DDC and DBH enzymes was also reduced in adult SHR, but PNMT protein was unchanged (Fig. 2D). Furthermore, the mRNA expression levels of almost all measured genes were reduced in the adrenals of adult SHR, with the exception of overexpressed *Maob* and unchanged *Comt* (Table 4).

Taken together, the mRNA and protein expression of genes involved in catecholamine biosynthesis was downregulated in adrenal medulla of prehypertensive as well as of adult SHR.

Group of genes	**Gene symbol**	**4 weeks**		**24 weeks**	
		WKY	**SHR**	**WKY**	**SHR**
Catecholamine biosynthesis	*Qdpr*	1.02 ± 0.08	0.89 ± 0.04	1.16 ± 0.04	0.91 ± 0.05 *
	Gch1	1.03 ± 0.09	1.52 ± 0.09 *	1.36 ± 0.16	0.92 ± 0.11 * †
Vesicle-related	*Vmat1*	1.03 ± 0.10	1.35 ± 0.06	1.88 ± 0.14 †	1.31 ± 0.15 *
	Vmat2	1.06 ± 0.12	0.54 ± 0.03 *	1.39 ± 0.17 †	0.33 ± 0.04 *
	Chga	1.05 ± 0.12	1.05 ± 0.04	1.42 ± 0.10 †	0.85 ± 0.11 *
	Chgb	1.05 ± 0.13	1.03 ± 0.07	1.85 ± 0.24 †	1.02 ± 0.12 *
	Scg2	1.03 ± 0.10	1.06 ± 0.09	1.58 ± 0.13 †	0.89 ± 0.10 *
	Npy	1.04 ± 0.10	0.48 ± 0.04 *	1.95 ± 0.17 †	0.72 ± 0.07 *
Catecholamine removal from synaptic cleft	*Net*	1.05 ± 0.12	0.79 ± 0.04	1.05 ± 0.15	0.62 ± 0.07 *
	Maoa	1.01 ± 0.06	0.91 ± 0.04	1.37 ± 0.09 †	1.02 ± 0.06 *
	Maob	1.06 ± 0.13	0.93 ± 0.06	3.92 ± 0.27 †	4.88 ± 0.33 *
	Comt	1.01 ± 0.05	0.98 ± 0.05	1.58 ± 0.09 †	1.44 ± 0.10 †

Table 4. mRNA expression of genes of catecholaminergic system in adrenal medulla of 4-week-old and 24-week-old WKY rats and SHR. The mRNA expression was standardized to the best combination of reference genes (*Hprt1* and *Ywhaz*). Values are expressed relatively to 4-week-old WKY rats as mean of group ± SEM, n = 8 for each group. The effects of strain and age were analyzed by two-way ANOVA, Bonferroni *post-hoc* test was performed. * $p < 0.05$ vs. age-matched WKY; † $p < 0.05$ vs. 4-week-old rats of the same strain. Vavřínová *et al*., 2019a

4.1.3. *Catecholamine content in the adrenal glands*

The amounts of dopamine, noradrenaline and adrenaline were greater in the adrenal glands of 4-week-old SHR than in those of WKY rats (Table 5). In contrast, the amounts of dopamine and noradrenaline were decreased in the adrenals of adult SHR, while the amount of adrenaline was similar in comparison to adult WKY rats.

Greater content of catecholamines in adrenal glands of adult rats was partially related to the increase of adrenal size during aging. Considering the adrenal weights, the adrenal contents of dopamine, noradrenaline and adrenaline were ~4-fold higher in adult WKY rats compared to young WKY rats. In SHR, the relative adrenal content of adrenaline was 2.5-fold greater in adult than in prehypertensive SHR, while adrenal dopamine and noradrenaline content did not increase during the aging of SHR.

	WKY 4 weeks	**SHR 4 weeks**	**WKY 24 weeks**	**SHR 24 weeks**
Adrenal gland (ng/adrenal gland)				
Dopamine	51 ± 3	72 ± 5 *	326 ± 22 †	164 ± 13 * †
Noradrenaline	635 ± 20	782 ± 39 *	4375 ± 277 †	1654 ± 122 * †
Adrenaline	1734 ± 39	2092 ± 82*	11326 ± 244 †	10522 ± 614 †
Sympathetic ganglion (ng/ganglion)				
Dopamine	1.82 ± 0.09	1.90 ± 0.20	3.47 ± 0.29 †	3.21 ± 0.17 †
Noradrenaline	2.63 ± 0.34	1.94 ± 0.25	14.16 ± 0.08 †	13.41 ± 0.63 †
Plasma (ng/ml)				
Dopamine	0.17 ± 0.03	0.23 ± 0.04	0.07 ± 0.02 †	0.15 ± 0.01 *
Noradrenaline	0.84 ± 0.11	1.34 ± 0.12 *	0.43 ± 0.05 †	0.39 ± 0.03 †
Adrenaline	0.83 ± 0.15	0.70 ± 0.10	0.50 ± 0.14	0.34 ± 0.04 †
Normetadrenaline	0.36 ± 0.03	0.40 ± 0.05	0.44 ± 0.04	0.60 ± 0.05 †
Metadrenaline	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.10 ± 0.01 †

Table 5. Catecholamine content in adrenal gland, sympathetic ganglia and plasma of 4-week and 24-week-old WKY rats and SHR. Values are expressed as mean ± SEM, n = 8 for each group. The effects of strain and age were analyzed by two-way ANOVA, Bonferroni *post-hoc* test was performed. * $p < 0.05$ vs. age-matched WKY; † $p < 0.05$ vs. 4-week-old rats of the same strain. Vavřínová *et al.*, 2019a

4.1.4. *The expression of genes of catecholaminergic system in the sympathetic ganglia*

The best combination of two reference genes (*18S* and *Gapdh*) was used for the normalization of mRNA expression data from sympathetic ganglia of 4-week-old and 24-week-old SHR and WKY rats, as described in Vavřínová *et al*. (2019a), see Attachments.

Lower mRNA and protein expression of *Ddc* and *Dbh* genes were found in the sympathetic ganglia of 4-week-old SHR compared to the aged-matched WKY rats, whereas *Th* expression was similar in both strains (Fig. 3A, B). Moreover, the mRNA expressions of *Qdpr* and *Chga* genes were lower in the sympathetic ganglia of prehypertensive SHR, while the expression of *Vmat1* gene was higher than that in 4-week-old WKY rats (Table 6).

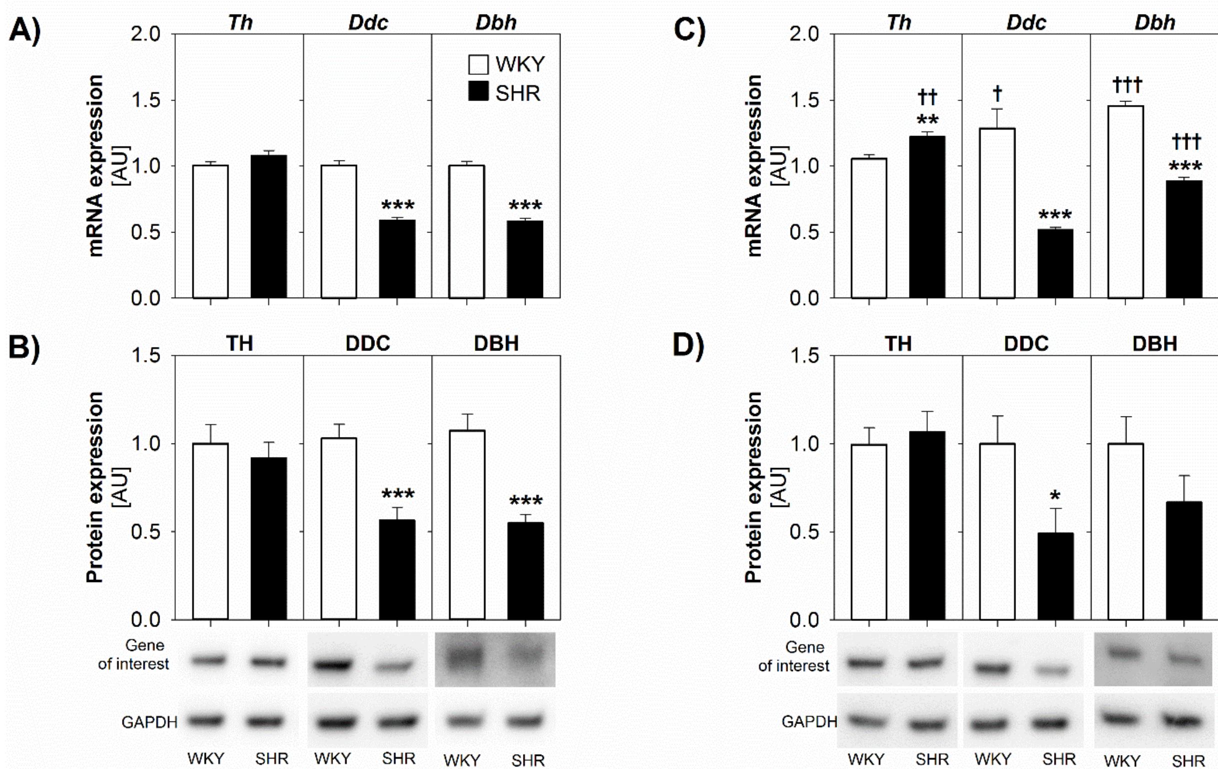

Figure 3. mRNA (A, C) and protein (B, D) expression of genes involved in catecholamine biosynthesis in the sympathetic ganglia of prehypertensive 4-week-old (A, B) or hypertensive 24-week-old (C, D) SHR and age-matched WKY rats. The mRNA expression was standardized to the best combination of reference genes *18S* and *Gapdh*. The protein expression was standardized to GAPDH. Data are plotted relatively to WKY rats as mean ± SEM, n = 8 for each group. The effect of strain and age on mRNA expression was analyzed by two-way ANOVA, Bonferroni *post-hoc* test was performed. * $p<0.05$ vs. age-matched WKY; † $p<0.05$ vs. mRNA expression in 4-week-old rats of the same strain. The statistical significance of Western blot data was determined with Student's t test separately for either age. * $p<0.05$ vs. age-matched WKY. Vavřínová *et al*., 2019a

In the sympathetic ganglia of adult SHR, mRNA expression of *Th* gene was higher, but this change was not observed at the protein level. The mRNA expressions of *Ddc* and *Dbh* genes were lower in sympathetic ganglia of adult SHR compared to age-matched WKY rats. The protein expression of DDC enzyme was also lower, while there was no significant change in protein expression of DBH enzyme (Fig. 3C, D). Furthermore, we observed an increased mRNA expression of *Chgb* and *Npy* genes in the sympathetic ganglia of adult SHR, while *Vmat1* and *Net* genes were underexpressed in comparison to WKY rats.

Taken together, the mRNA and protein expression of genes involved in catecholamine biosynthesis was downregulated in sympathetic ganglia of prehypertensive as well as of adult SHR.

Group of genes	Gene symbol	4 weeks		24 weeks	
		WKY	SHR	WKY	SHR
Catecholamine biosynthesis	*Qdpr*	1.01 ± 0.05	0.81 ± 0.03 *	0.81 ± 0.05 †	0.70 ± 0.03 †
	Gch1	1.01 ± 0.04	1.04 ± 0.04	1.19 ± 0.03 †	1.21 ± 0.05 †
Vesicle-related	*Vmat1*	1.02 ± 0.06	1.33 ± 0.06 *	1.24 ± 0.05 †	0.99 ± 0.05 * †
	Vmat2	1.00 ± 0.03	0.99 ± 0.03	0.77 ± 0.02 †	0.80 ± 0.04 †
	Chga	1.01 ± 0.07	0.83 ± 0.05 *	0.60 ± 0.04 †	0.47 ± 0.02 †
	Chgb	1.01 ± 0.04	1.14 ± 0.07	1.20 ± 0.03 †	1.63 ± 0.05 * †
	Scg2	1.01 ± 0.06	1.01 ± 0.05	1.33 ± 0.05 †	1.27 ± 0.05 †
	Npy	1.01 ± 0.05	0.96 ± 0.04	1.57 ± 0.04 †	1.70 ± 0.05 * †
Catecholamine removal from synaptic cleft	*Net*	1.01 ± 0.07	1.01 ± 0.06	1.29 ± 0.02 †	1.08 ± 0.03 *
	Maoa	1.00 ± 0.04	1.05 ± 0.05	1.20 ± 0.02 †	1.15 ± 0.03
	Maob	1.00 ± 0.04	1.03 ± 0.05	1.09 ± 0.04	1.13 ± 0.04
	Comt	1.00 ± 0.03	0.99 ± 0.04	1.05 ± 0.03	0.93 ± 0.07

Table 6. mRNA expression of genes of catecholaminergic system in sympathetic ganglia of 4-week and 24-week-old WKY rats and SHR. The mRNA expression was standardized to the best combination of reference genes (*18 S* and *Gapdh*). Values are expressed relatively to 4-week-old WKY rats as mean of group ± SEM, n = 8 for each group. The effects of strain and age were analyzed by two-way ANOVA, Bonferroni *post-hoc* test was performed. * $p < 0.05$ vs. age-matched WKY; † $p < 0.05$ vs. 4-week-old rats of the same strain. Vavřínová *et al.*, 2019a

4.1.5. *Catecholamine content in the sympathetic ganglia and sympathetic innervation of femoral artery*

The catecholamine content in the sympathetic ganglia was very low (Table 5) and there was no difference between SHR and WKY rats of either age. Fig. 4 shows the catecholamine content in sympathetic innervation of the femoral arteries visualized by glyoxylic acid staining. The fluorescent signal was approximately twofold higher in SHR of both ages compared to the age-matched WKY rats (2.16 ± 0.08 in prehypertensive SHR, 1.99 ± 0.11 in adult SHR, Student's t test: $p < 0.001$ for both ages).

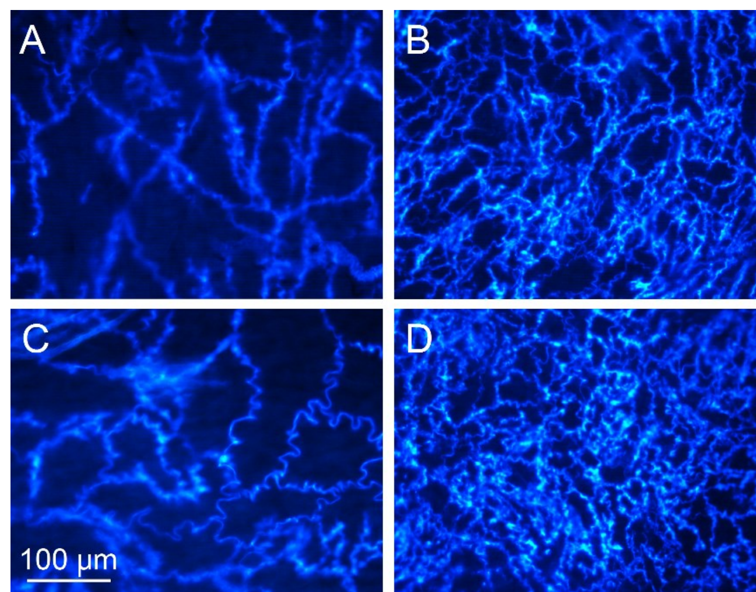

Figure 4. The catecholamine content in the sympathetic innervation of femoral artery visualized by glyoxylic acid staining in: 4-week-old WKY rats (A), 4-week-old SHR (B), 24-week-old WKY rats (C), 24-week-old SHR (D). Vavřínová *et al*., 2019a

4.2. Project 2 – The comparison of sympathoadrenal abnormalities in young prehypertensive and adult spontaneously hypertensive rats

4.2.1. *Body weight and adrenal weight*

Before sympathectomy, the body weights of SHR were lower than those of WKY rats (312±3 vs. 343±7 g; Student's t test: $p<0.001$). Control animals gained a small amount of body weight, whereas 14-day guanethidine treatment prevented such an increase in sympathectomized (SYMPX) SHR and caused even a body weight loss in WKY rats (control SHR, 14±3 g; control WKY rats, 6±3 g; SYMPX SHR, 1±3 g; SYMPX WKY rats, -21±2 g; two-way ANOVA; interaction between strain and treatment: $p<0.01$). The relative weight of the adrenals was significantly greater in control SHR than in WKY rats. Sympathectomy increased the relative adrenal weight and abolished the strain difference (control SHR, 13.8±0.3 mg/100 g; control WKY rats, 11.1±0.2 mg/100 g; SYMPX SHR, 15.5±0.6 mg/100 g; SYMPX WKY rats, 15.2±0.4 mg/100 g; two-way ANOVA; interaction between strain and treatment: $p<0.01$).

4.2.2. *Catecholamine content in the sympathetic innervation of femoral artery*

Fig. 5A shows the catecholamine content in the sympathetic innervation of the femoral arteries visualized by glyoxylic acid staining. The quantification of the fluorescent signal (Fig. 5B) revealed a strain difference, which was abolished by sympathectomy. The fluorescent signal was approximately 2.5-fold higher in control SHR than that observed in WKY rats ($p<0.001$). Guanethidine-induced sympathectomy reduced the fluorescent signal in both strains (-92 % in SYMPX SHR and -88 % in SYMPX WKY rats; $p<0.001$ for SHR and $p<0.01$ for WKY rats).

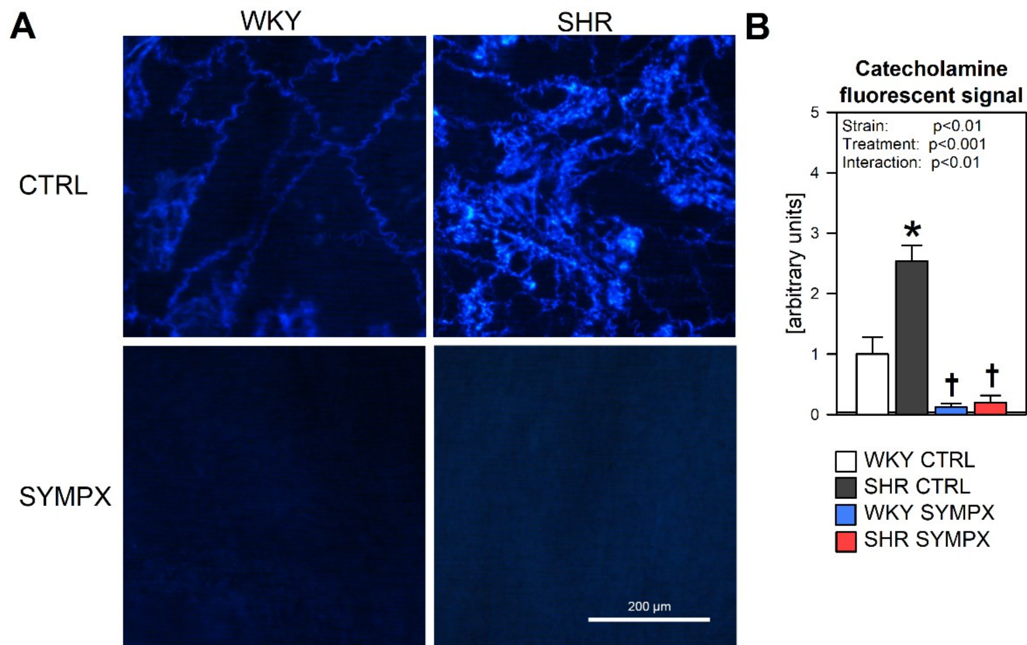

Figure 5. Histochemical visualization of catecholamines in the femoral arteries (SPG method). Representative images of control (CTRL) and sympathectomized (SYMPX) WKY rats and SHR (A) and the quantitative evaluation of the fluorescent signal (B). The values are expressed as the mean ± SEM relative to CTRL WKY rats; n = 8-9 rats in each group. The effects of strain and treatment and their interaction were analyzed by two-way ANOVA, Bonferroni *post-hoc* test was performed. * p<0.05 vs. WKY rats; † p<0.05 vs. CTRL rats. Vavřínová *et al.*, 2019b

4.2.3. *Cardiovascular parameters measured by radiotelemetry*

We used radiotelemetry to compare guanethidine-induced changes in BP, HR and activity over time in adult freely moving SHR and WKY rats (Fig. 6). The average values in the dark and light phase of the day before the start of guanethidine treatment (D0), the third day of guanethidine treatment (D3) and the 14th day of guanethidine treatment are shown in Table 7.

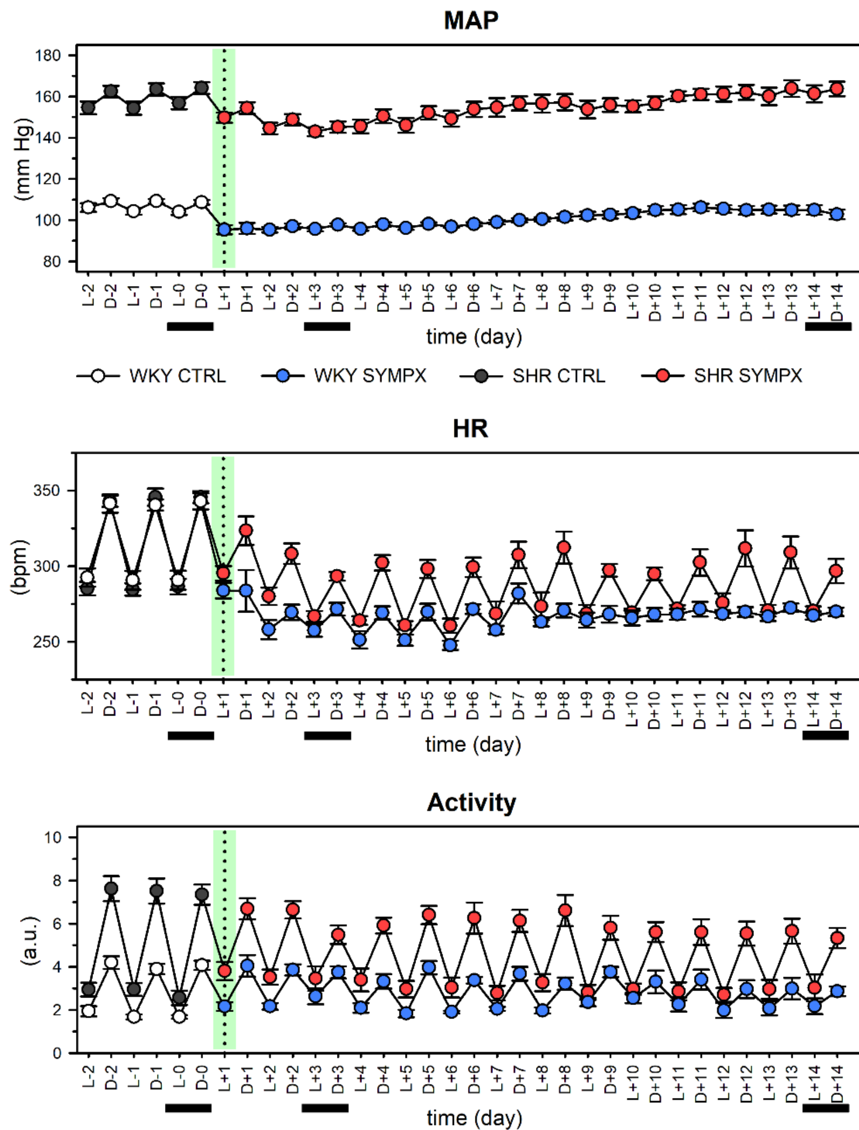

Figure 6. The time-course of mean arterial pressure (MAP), heart rate (HR) and animal activity of WKY rats and SHR measured by telemetry before and during guanethidine administrattion. The first guanethidine injection was given during light phase of the first day (L+1) indicated by vertical dotted line. The black rectangles indicate time-points showed in Table 7.

MAP, HR and animal activity in control rats were higher during the dark phase (active period) than during the light phase (resting period). Untreated SHR had higher MAP than WKY rats during both phases (Table 7). MAP was lowered after three days of guanethidine treatment in both strains, and it returned back to the control level after 14 days. A slight persistent decrease in the MAP was observed only in WKY rats during the dark phase. HR of SHR and WKY rats were similar before guanethidine treatment (Table 7). HR was decreased following 3 days of guanethidine administration in both strains, and the effect persisted until the 14th day of treatment. However, the HR during the dark phase of SYMPX SHR was higher than that of SYMPX WKY rats. SHR were more active than WKY rats during the dark phase, and guanethidine administration reduced the activity of both strains during the dark phase (Table 7).

	MAP (mmHg)		**HR (bpm)**		**Animal activity (AU)**	
	Light	**Dark**	**Light**	**Dark**	**Light**	**Dark**
WKY						
CTRL D0	104±2	109±1	291±6	343±5	1.7±0.1	4.1±0.2
SYMPX D3	96±1 †	98±1 †	258±4 †	272±4 †	2.6±0.4	3.7±0.3
SYMPX D14	105±2	103±2 †	267±3 †	270±3 †	2.2±0.4	2.9±0.2 †
SHR						
CTRL D0	157±3 *	164±3 *	287±5	346±4	2.6±0.3	7.3±0.5 *
SYMPX D3	143±2 *†	145±3 *†	267±4 †	293±3 *†	3.5±0.6	5.5±0.4 *†
SYMPX D14	161±4 *	164±4 *	270±3 †	297±8 *†	3.0±0.6	5.3±0.5 *†
Factor						
Strain	p<0.001	p<0.001	NS	p<0.01	NS	p<0.001
Treatment	p<0.001	p<0.001	p<0.001	p<0.001	p<0.05	p<0.001
Interaction	p<0.05	p<0.001	p=0.056	p<0.01	NS	p<0.001

Table 7. Mean arterial pressure (MAP), heart rate (HR) and animal activity in control (CTRL) and sympathectomized (SYMPX) WKY rats and SHR. Cardiovascular parameters were measured by radiotelemetry in freely-moving animals during the light and dark phases. Data are the means ± SEM of the last day before the start of guanethidine treatment (D0), the third day of guanethidine treatment (D3) and the 14th day of guanethidine treatment (D14). n = 6-7 for each group. Statistical significance was computed by repeated measures two-way ANOVA, in the case of interaction Bonferroni *post-hoc* test was performed; * $p < 0.05$ vs. WKY rats; † $p < 0.05$ vs. CTRL rats (D0) of the same strain. Vavřínová *et al.*, 2019b

The time-courses of low frequency systolic blood pressure variability (LF SBPV; marker of vascular sympathetic activity) and high frequency systolic blood pressure variability (HF SBPV; marker of cardiac sympathetic activity) are shown in Fig. 7 and for their average values see Table 8. The LF SBPV was higher in control SHR than in WKY rats, and the difference was more pronounced during the dark phase. The LF SBPV was markedly attenuated in both strains after 3 days and 14 days of guanethidine treatment, demonstrating the persistent effect of sympathectomy. The HF SBPV was also higher in control SHR and sympathectomy decreased the HF SBPV after 3 days and 14 days of guanethidine administration in both strains.

Spontaneous baroreflex function was determined in freely moving SHR and WKY rats. SHR had a lower baroreflex sensitivity compared to that of WKY rats during both the light and dark phases (Table 8). The number of spontaneous baroreflex sequences was substantially reduced by guanethidine treatment, and baroreflex sensitivity during the dark phase was moderately increased in SYMPX animals of both strains.

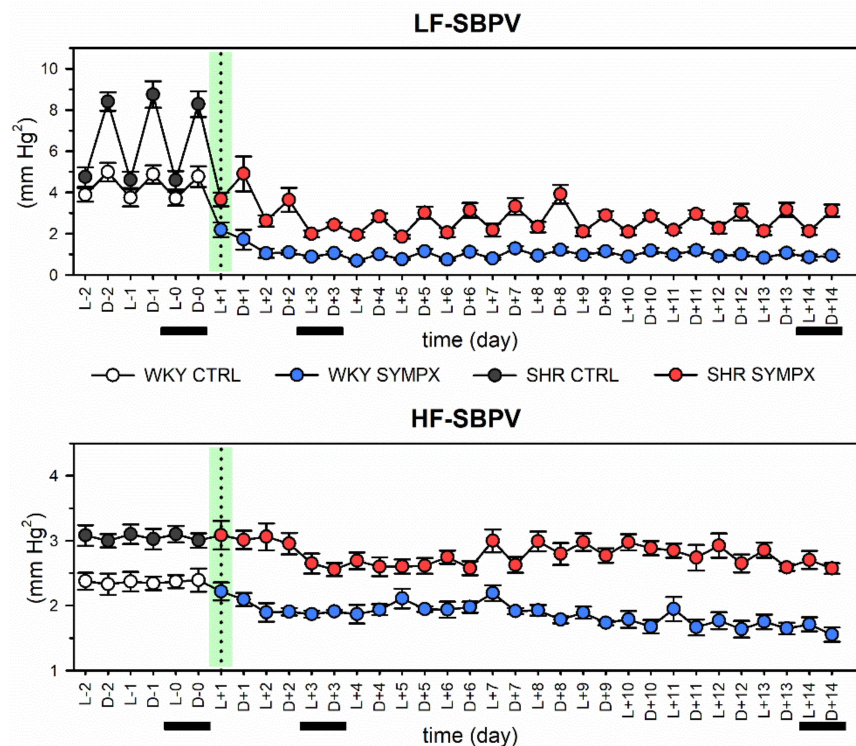

Figure 7. The time-course of low-frequency (LF) and high-frequency (HF) component of systolic blood pressure variability (SBPV) in WKY rats and SHR before and during guanethidine administration. The first guanethidine injection was given during light phase of the first day (L+1) indicated by vertical dotted line. The black rectangles indicate time-points showed in Table 8.

	LF SBPV (mmHg²)		HF SBPV (mmHg²)		sBRS (ms/mmHg)		BRR sequences (n)	
	Light	**Dark**	**Light**	**Dark**	**Light**	**Dark**	**Light**	**Dark**
WKY								
CTRL D0	3.7±0.3	4.8±0.5	2.4±0.1	2.4±0.2	2.2±0.2	2.1±0.1	389±94	480±94
SYMPX D3	0.9±0.1	1.1±0.1 †	1.9±0.1	1.9±0.1	2.3±0.3	2.6±0.3	36±3	96±26 †
SYMPX D14	0.9±0.1	0.9±0.1 †	1.7±0.1	1.6±0.1	2.4±0.3	2.5±0.4	77±16	109±21 †
SHR								
CTRL D0	4.6±0.4	8.3±0.6*	3.1±0.1	3.0±0.1	1.9±0.1	1.6±0.1	272±17	319±30 *
SYMPX D3	2.0±0.2	2.4±0.1 *†	2.6±0.2	2.6±0.1	1.8±0.1	1.8±0.1	72±11	126±14 †
SYMPX D14	2.1±0.2	3.1±0.3 *†	2.7±0.1	2.6±0.1	1.8±0.1	1.8±0.1	115±12	200±27
Factor								
Strain	p<0.01	p<0.001	p<0.001	p<0.001	p<0.05	p<0.001	NS	NS
Treatment	p<0.001	p<0.001	p<0.001	p<0.001	NS	p<0.01	p<0.001	p<0.001
Interaction	NS	p<0.01	NS	NS	NS	NS	NS	p<0.01

Table 8. Low-frequency and high-frequency component of systolic blood pressure variability (LF SBPV, HF SBPV), spontaneous baroreflex sensitivity (sBRS) and number of baroreflex events (BRR) in control (CTRL) and sympathectomized (SYMPX) WKY rats and SHR. Cardiovascular parameters were measured by radiotelemetry in freely-moving animals during the light and dark phase. Data are the means ± SEM of the last day before start of guanethidine treatment (D0), the third day of guanethidine treatment (D3) and the 14th day of guanethidine treatment (D14). n = 6-7 for each group. Statistical significance was computed by repeated measures two-way ANOVA, Bonferroni *post-hoc* test was performed; * $p < 0.05$ vs. WKY rats; † $p < 0.05$ vs. CTRL rats (D0) of the same strain. Vavřínová *et al.*, 2019b

Taken together, sympathetic hyperactivity observed in freely-moving SHR is accompanied with higher blood pressure and locomotor activity as well as with impaired baroreflex function compared to WKY rats. Guanethidine treatment decreased markers of sympathetic activity in freely-moving rats of both strains, but it did not affect the strain differences in BP and locomotor activity.

We examined the stress-induced cardiovascular responses of control and SYMPX animals to the restraint, i.e., under the conditions when the activity of sympathetic nervous system should be enhanced. We observed a higher MAP response to stressor exposure in SHR than in WKY rats, and this response was attenuated by 14-day guanethidine treatment in both strains (Fig.8, Table 9). The maximal stress-induced HR increase was exaggerated in SHR compared to WKY rats, whereas the average HR over 120 min of stress was similar in the control

rats of both strains. Both maximal and average HR response were attenuated by sympathectomy in rats of both strains (Fig.8, Table 9).

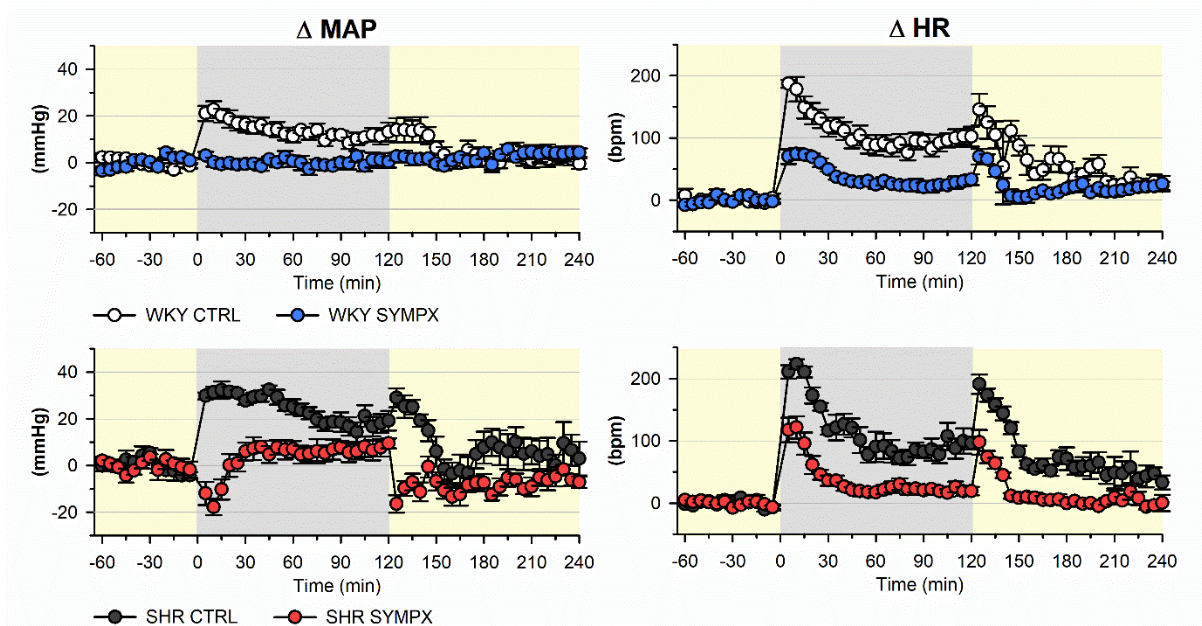

Figure 8. The time-course of changes in mean arterial pressure (MAP) and heart rate (HR) before, during and after restraint stress (0-120 min, marked in grey) in control (CTRL) and sympathectomized (SYMPX) WKY rats and SHR.

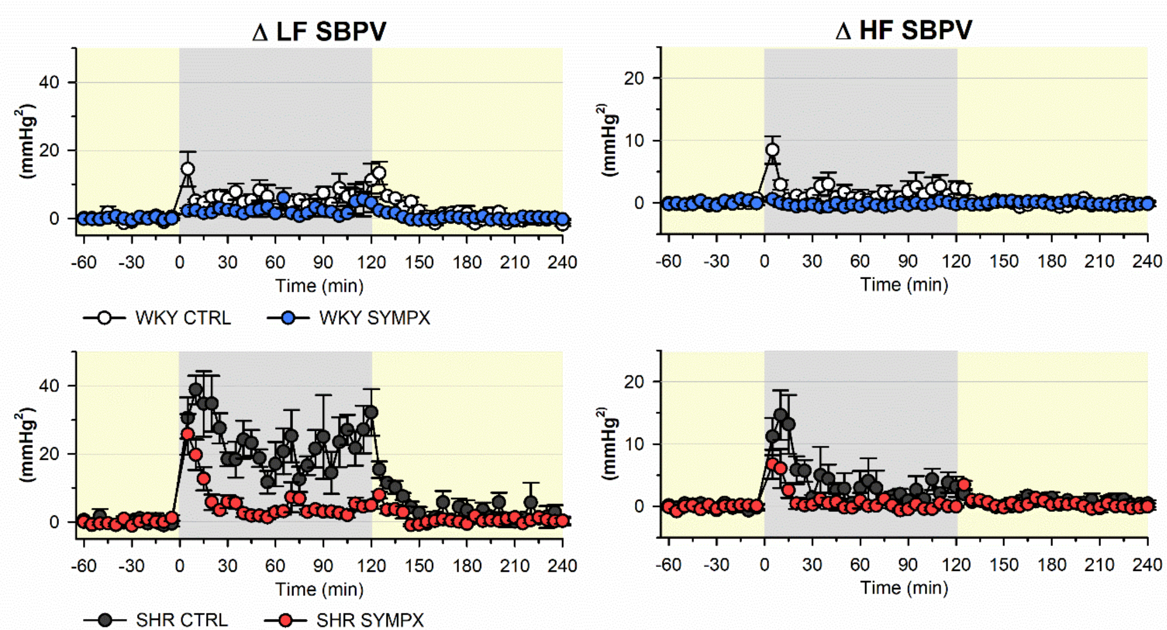

Figure 9. The time-course of changes in low-frequency (LF) and high-frequency (HF) component of systolic blood pressure variability (SBPV) before, during and after restraint stress (0-120 min, marked in grey) in control (CTRL) and sympathectomized (SYMPX) WKY rats and SHR.

SHR responded to the stressor exposure by a greater increase in the LF SBPV compared to WKY rats, and the lowering effect of sympathectomy on LF SBPV was more pronounced in SHR than WKY rats (Fig.9, Table 9). The changes in HF SBPV elicited by stress were higher in SHR compared to WKY rats and guanethidine treatment decreased this parameter in both strains (Fig.9, Table 9). Taken together, after 14 days of guanethidine treatment, sympathectomy-induced cardiovascular changes were more evident under the stressful conditions than in freely moving animals.

	WKY		**SHR**		**two-way ANOVA**		
	CTRL (6-7)	**SYMPX (7)**	**CTRL (7)**	**SYMPX (7)**	**Strain**	**Treatment**	**Interaction**
Basal MAP (mmHg)	107 ± 2	102 ± 2 *	153 ± 2	164 ± 3*†	p<0.001	NS	p<0.01
Basal HR (bpm)	284 ± 8	251 ± 4	271 ± 7 †	264 ± 4	NS	p<0.01	p<0.05
Basal LF SBPV (mm Hg^2)	4.0 ± 0.5	0.8 ± 0.1	3.6 ± 0.3†	1.8 ± 0.2 *†	NS	p<0.001	p<0.05
Basal HF SBPV (mm Hg^2)	2.4 ± 0.2	2.0 ± 0.6	3.1 ± 0.2	2.6 ± 0.2 *†	NS	NS	NS
Max Δ MAP (mm Hg)	23 ± 4	3 ± 2	32 ± 2	10 ± 2	p<0.01	p<0.001	NS
Av. Δ MAP (mm Hg)	14 ± 2	0± 2	24 ± 2	4 ± 3	p<0.01	p<0.001	NS
Max Δ HR (bpm)	187 ± 6	74 ± 10	224 ± 8	122 ± 18	p<0.001	p<0.001	NS
Av. Δ HR (bpm)	109 ± 10	37 ± 5	118 ± 10	37 ± 8	NS	p<0.001	NS
Max Δ LF SBPV (mm Hg^2)	14.5 ± 5.1	6.0 ± 2.9	38.8 ± 4.2	25.7 ± 6.0	p<0.001	p<0.05	NS
Av. Δ LF SBPV (mm Hg^2)	6.4 ± 0.9	2.6 ± 0.9	24.5 ± 4.2 *	5.8 ± 0.8 †	p<0.001	p<0.001	p<0.001
Max Δ HF SBPV (mm Hg^2)	8.5 ± 2.2	0.6 ± 0.5	14.7 ± 4.0	6.8 ± 2.3	p<0.05	p<0.01	NS
Av. Δ HF SBPV (mm Hg^2)	1.8 ± 1.0	- 0.2 ± 0.4	4.2 ± 1.2	0.8 ± 0.4	p=0.05	p<0.001	NS

Table 9. Stress-induced response of mean arterial pressure (MAP), heart rate (HR) low-frequency (LF) and high-frequency (HF) component of systolic blood pressure variability (SBPV) in control (CTRL) and sympathectomized (SYMPX) SHR and WKY rats (analysis of data from Figures 8 and 9). Data are the means ± SEM. Statistical significance was computed by two-way ANOVA, Bonferroni *post-hoc* test was performed; * p < 0.05 vs. WKY rats; † p < 0.05 vs. CTRL rats of the same strain; Basal - average value of the parameter over 60-min period before start of the stress; Av., average value of parameter during stress period; Max, maximal value of parameter during stress period.

4.2.4. *Cardiovascular responses to vasoactive agents*

MAP and HR responses to vasoactive agents were studied in conscious cannulated control and SYMPX SHR and WKY animals (placed in small plastic cages) to obtain a more detailed information about the effects of guanethidine treatment on the cardiovascular system. The basal MAP was higher in SHR than in WKY rats and sympathectomy lowered these basal values in both strains. The basal HR was higher in SHR than in WKY rats, and it was decreased by guanethidine treatment (Table 10).

The ganglionic blocker pentolinium decreased MAP in all experimental groups (Table 10). The effect of pentolinium on MAP was more pronounced in control SHR than in WKY rats ($p<0.05$), and this strain difference was abolished by sympathectomy. The catecholamine releasing agent tyramine increased MAP and HR similarly in SHR and WKY rats, and the effect was almost completely abolished by sympathectomy (Table 10).

	CTRL		**SYMPX**		**two-way ANOVA**		
	WKY	**SHR**	**WKY**	**SHR**	**Strain**	**Treatment**	**Interaction**
Basal MAP (mmHg)	102 ± 2	157 ± 2	91 ± 3	136 ± 2	p<0.001	p<0.001	p=0.053
Basal HR (bpm)	323 ± 8	363 ± 1	282 ± 10	318 ± 6	p<0.001	p<0.001	NS
Pentolinium							
ΔMAP (mmHg)	-38 ± 1	-47 ± 3 *	-15 ± 10 †	-7 ± 4 †	NS	p<0.001	p<0.01
ΔHR (bpm)	-26 ± 6	-16 ± 5	-10 ± 1	26 ± 6	p<0.01	p<0.001	NS
Tyramine							
ΔMAP (mmHg)	49 ± 5	48 ± 4	6 ± 2	4 ± 1	NS	p<0.001	NS
ΔHR (bpm)	94 ± 15	93 ± 13	-3 ± 3	2 ± 2	NS	p<0.001	NS
Captopril							
ΔMAP (mmHg)	-4 ± 1	-15 ± 4	-10 ± 2	-18 ± 3	p<0.01	NS	NS
ΔHR (bpm)	29 ± 7	2 ± 7	32 ± 8	7 ± 5	p<0.01	NS	NS

Table 10. Basal mean arterial pressure (MAP), basal heart rate (HR) and response of MAP and HR to the administration of pentolinium, tyramine and captopril in control (CTRL) and sympathectomized (SYMPX) WKY rats and SHR. Data are the means ± SEM. Statistical significance was computed by two-way ANOVA, Bonferroni *post-hoc* test was performed; * $p < 0.05$ vs. WKY rats; † $p < 0.05$ vs. CTRL rats of the same strain.

The acute administration of the angiotensin-converting enzyme inhibitor captopril decreased MAP more in SHR than in WKY rats, and sympathectomy did not change MAP response to captopril (Table 10). Captopril administration caused HR increase in both control and SYMPX WKY rats but not in SHR with or without sympathectomy (Table 10).

BP changes induced by increasing doses of noradrenaline, adrenaline and angiotensin II (Ang II) were analyzed by three-way ANOVA with the following factors: strain (SHR vs. WKY rats), treatment (control rats vs. SYMPX rats) and the dose of the pressor agent. Regarding the noradrenaline administration, the effect of strain vs. treatment interaction on MAP response depended on the noradrenaline dose (Fig. 10; strain vs. treatment interaction: $p<0.001$ at 0.01 μg/kg dose, NS at 0.1 μg/kg dose, $p<0.001$ at 1 μg/kg dose). There was no strain difference in MAP response after 0.01 μg/kg noradrenaline administration, and sympathectomy augmented this MAP increase in both strains ($p<0.001$ for WKY rats, $p<0.01$ for SHR). As far as the administration of noradrenaline at a dose of 0.1 μg/kg is concerned, MAP increase was more pronounced in SHR than in WKY rats ($p<0.001$), and sympathectomy augmented MAP response in both strains ($p<0.001$). Upon the administration of noradrenaline at a dose of 1 μg/kg, MAP increased similarly in control SHR and WKY rats and sympathectomy augmented the responses in both strains ($p<0.001$). SYMPX SHR responded with a greater MAP increase than SYMPX WKY rats ($p<0.001$).

When MAP response to adrenaline administration was analyzed by three-way ANOVA, there was no significant interaction between strain, treatment and dose. MAP response to adrenaline was exaggerated in SHR regardless of the adrenaline dose or guanethidine treatment (Fig. 10). The effect of sympathectomy on MAP response depended on the adrenaline dose. MAP response upon the administration of 0.01 μg/kg adrenaline was similar in control and SYMPX rats, but MAP responses were augmented in SYMPX rats to the administration of 0.1 and 1 μg/kg adrenaline ($p<0.001$ for both doses). Regarding the Ang II administration, there was

no significant interaction between strain, treatment and dose (Fig. 10). The effect of both strain and treatment on the MAP response to Ang II depended on the dose used. MAP responses were more pronounced in SHR than in WKY rats upon the administration of 5, 10, 20, 50 and 100 ng/kg Ang II ($p<0.01$). Sympathectomy augmented MAP responses to the administration of 5, 10 and 20 μg/kg Ang II ($p<0.01$).

Taken together, the experiments with pentolinium and tyramine confirmed the persistence of effective sympathectomy after 14 days of guanethidine administration. The involvement of renin-angiotensin system in BP maintenance (indicated by cardiovascular response to captopril) has not been affected by sympathectomy. The sensitivity to catecholamines was markedly augmented by sympathectomy in both strains.

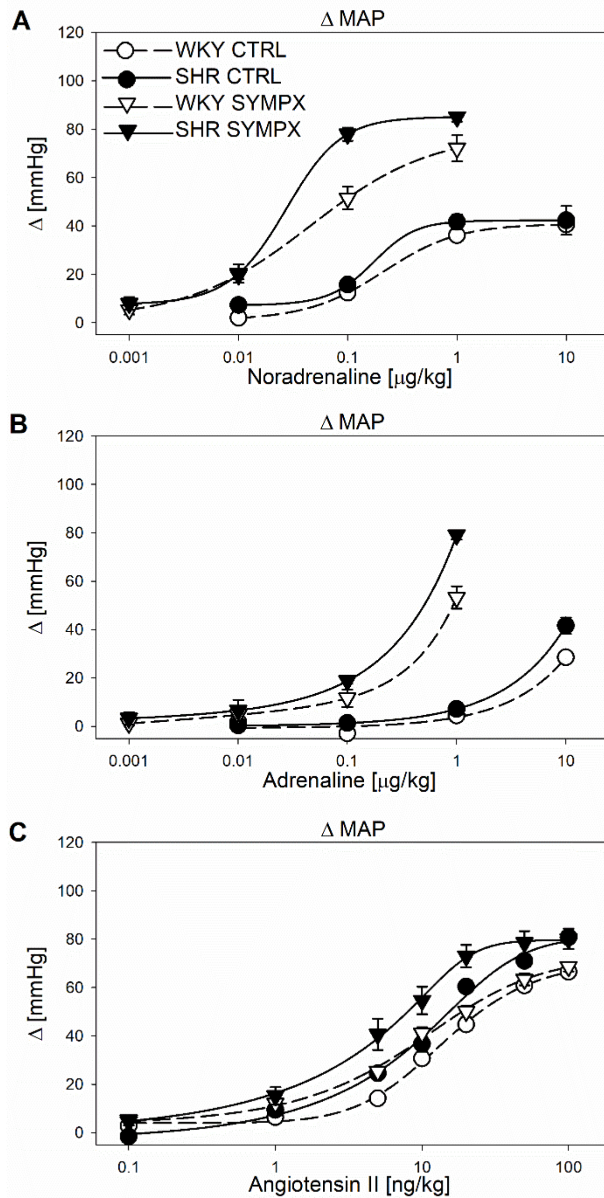

Figure 10. Mean arterial pressure (MAP) response to the intravenous administration of noncumulative doses of noradrenaline (A), adrenaline (B) or angiotensin II (C) in conscious control (CTRL) and sympathectomized (SYMPX) WKY rats and SHR. The values are expressed as the mean ± SEM; n = 6-8 rats for noradrenaline and adrenaline, n = 9-11 rats for angiotensin II. The effects of strain, treatment, and dose and their interactions were analyzed by three-way ANOVA (the results are listed in Attachments), Bonferroni *post-hoc* test was performed. Vavřínová *et al.*, 2019b

4.2.5. *Plasma catecholamines and corticosteroids*

There were no differences in the plasma levels of noradrenaline or normetadrenaline between control SHR and WKY rats. Sympathectomy decreased plasma levels of noradrenaline and normetadrenaline in both strains (Fig. 11A, 11B). Control SHR exhibited similar plasma adrenaline levels but higher plasma metadrenaline levels compared to those in control WKY rats (Fig. 11C, 11D; $p<0.05$). Guanethidine treatment elevated plasma levels of adrenaline and metadrenaline, and this effect was more pronounced in WKY rats (4-fold increase in SYMPX WKY rats, $p<0.001$ vs. 1.5-fold increase in SYMPX SHR, $p<0.05$). The evaluation of plasma corticosteroid levels revealed that control SHR exhibited plasma levels of corticosterone similar to those of WKY rats (Fig. 11E) and plasma aldosterone levels lower than those of WKY rats (Fig. 11F, $p<0.01$). Sympathectomy increased plasma levels of both corticosterone and aldosterone in WKY rats ($p<0.05$ for corticosterone and $p<0.001$ for aldosterone) but not in SHR.

4.2.6. *Catecholamine content in the adrenal gland*

There was a lower noradrenaline content in the adrenal glands of control SHR than in those of WKY rats, and sympathectomy increased the noradrenaline content in the adrenal glands of WKY rats but not in those of SHR (Table 11). Moreover, SHR exhibited a lower adrenal content of adrenaline than WKY rats, and sympathectomy increased the adrenaline content in the adrenal glands irrespective of the strain.

	CTRL		**SYMPX**		**two-way ANOVA**		
	WKY	**SHR**	**WKY**	**SHR**	**Strain**	**Treatment**	**Interaction**
Noradrenaline (ng/adrenal gland)	4840 ± 282	2388 ± 280 *	7320 ± 847 †	2642 ± 166 *	p<0.001	p<0.05	p<0.05
Adrenaline (ng/adrenal gland)	13990 ± 480	12770 ± 370	16060 ± 640	13390 ± 460	p<0.001	p<0.05	NS

Table 11. Catecholamine content in adrenal gland of control (CTRL) and sympathectomized (SYMPX) WKY rats and SHR. Values are expressed as mean ± SEM, n = 6-7 for each group. The effects of strain and treatment and their interaction were analyzed by two-way ANOVA, Bonferroni *post-hoc* test was performed. * $p < 0.05$ vs. age-matched WKY; † $p < 0.05$ vs. 4-week-old rats of the same strain. Vavřínová *et al.*, 2019b

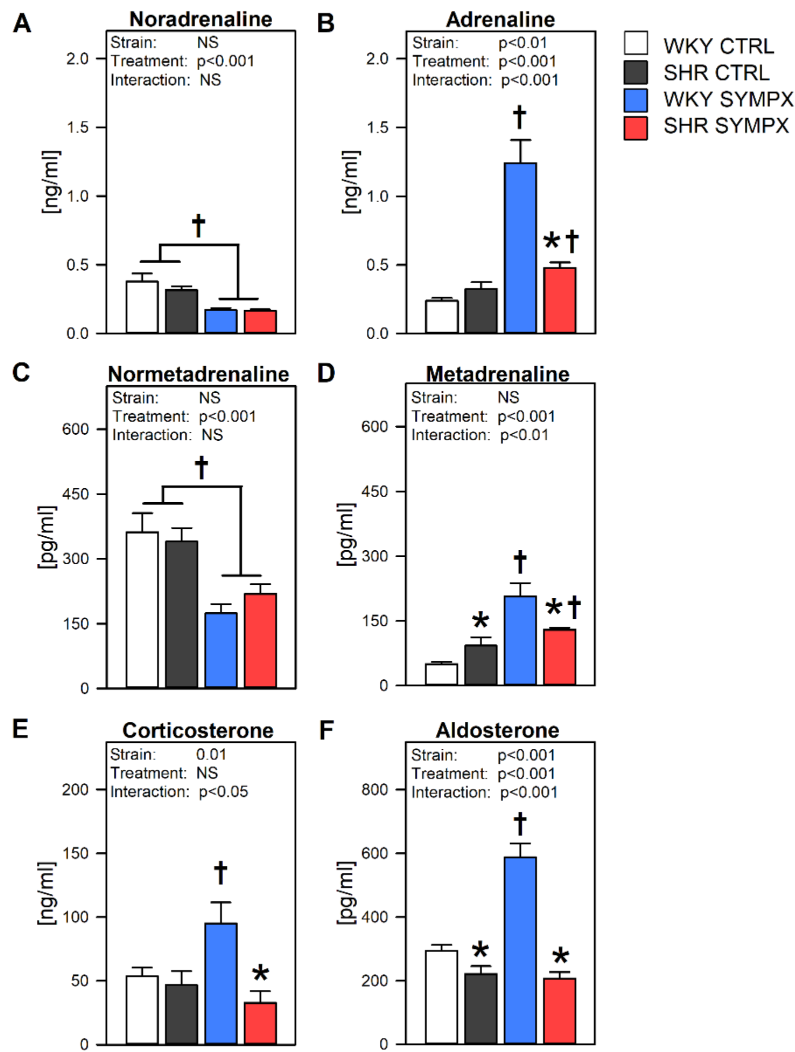

Figure 11. Plasma noradrenaline (A), normetadrenaline (B), adrenaline (C) metadrenaline (D), corticosterone (E) and aldosterone (F) levels in control (CTRL) and sympathectomized (SYMPX) WKY rats and SHR. The values are expressed as the mean ± SEM; n = 8 rats for each group. The effects of strain and treatment and their interaction were analyzed by two-way ANOVA, Bonferroni *post-hoc* test was performed. * p<0.05 vs. WKY rats; † p<0.05 vs. CTRL rats.

4.2.7. *Gene expression in vascular smooth muscle cells and in the adrenal medulla*

Reference genes (*Hmbs* and *Ywhaz* in the aortic smooth muscles and *Gapdh* and *Hprt1* in the adrenal medulla) were selected as described in our previous study (Vavřínová *et al*., 2016). The most abundant mRNA for adrenergic receptor in aortic vascular smooth muscle was *Adra1d* subtype. The *Adra1b* mRNA was five times less abundant compared to *Adra1d*, whereas the mRNA expression of *Adra1a, Adra2a*, *Adra2b*, *Adra2c*, *Adrb1* or *Adrb2* genes was too low for

reliable quantification. The mRNA expression levels of *Adra1d* and *Adra1b* genes were lower in vascular smooth muscle of SHR than in that of WKY rats and these expressions were not affected by sympathectomy (Table 12). The G-proteins participating in α_1-adrenergic contraction are the G_q and G_{11} subtypes, encoded by the *Gnaq* and *Gna11* genes. The mRNA expression of *Gnaq* was lower in the vascular smooth muscle of SHR compared to WKY rats, while the expression of *Gna11* was similar in both strains. There was no effect of guanethidine treatment on mRNA expression of *Gnaq* or *Gna11* (Table 12).

Gene symbol	Ratio mean SHR/ mean WKY (arbitrary units)				two-way ANOVA		
	CTRL		SYMPX				
	WKY	SHR	WKY	SHR	Strain	Treatment	Interaction
Vascular smooth muscle cells							
Adra1d	1.01 ± 0.05	0.72 ± 0.05	1.05 ± 0.08	0.75 ± 0.03	p<0.001	NS	NS
Adra1b	1.04 ± 0.10	0.87 ± 0.06	1.08 ± 0.12	0.93 ± 0.04	p=0.051	NS	NS
Gnaq	1.03 ± 0.1	0.92 ± 0.05	1.08 ± 0.06	0.95 ± 0.04	p<0.05	NS	NS
Gna11	1.02 ± 0.07	0.95 ± 0.05	0.92 ± 0.07	1.01 ± 0.03	NS	NS	NS

Table 12. The mRNA expression of genes for adrenergic receptors and G-proteins in vascular smooth muscle cells of aorta. The mRNA expression was standardized to the best combination of reference genes *Hmbs* and *Ywhaz*. The values are expressed relatively to control (CTRL) WKY rats as the mean ± SEM, n = 7-8 for each group. Statistical significance was computed by two-way ANOVA. Bonferroni *post-hoc* test was performed. Vavřínová *et al*., 2019b

The mRNA expression of genes involved in catecholamine biosynthesis (*Th*, *Ddc*, *Dbh* and *Pnmt)* was lower in the adrenal medulla of SHR than that of WKY rats. Guanethidine treatment increased the mRNA expression of *Th*, *Dbh* and *Pnmt* genes in the adrenal medulla of both strains (Figure 12).

Taken together, the mRNA expression of adrenergic receptors and G-proteins in vascular smooth muscles nas not been affected by guanethidine treatment, whereas the expression of genes involved in catecholamine biosynthesis was induced by sympathectomy in both SHR and WKY rats.

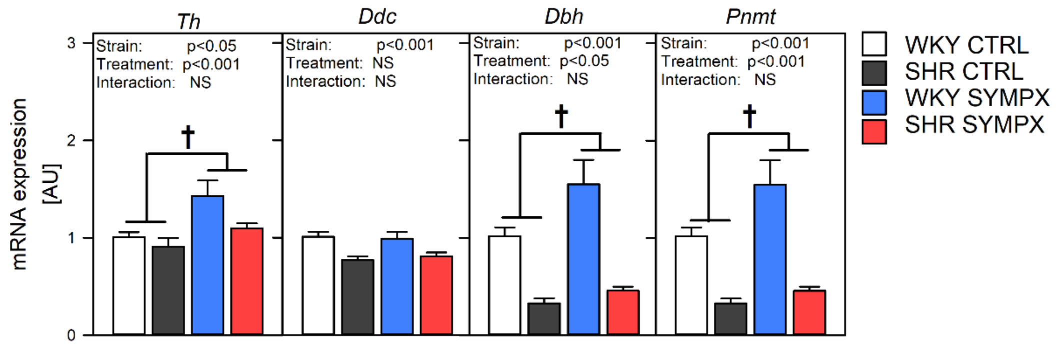

Figure 12. mRNA expression of genes involved in catecholamine biosynthesis in the adrenal medulla of adult control (CTRL) and sympathetcomized (SYMPX) SHR and WKY rats. The mRNA expression was standardized to the best combination of reference genes Gapdh and Hprt1. Data are plotted relatively to CTRL WKY rats as mean ± SEM, n = 7-8 for each group. The effects of strain and treatment and their interaction were analyzed by two-way ANOVA, Bonferroni *post-hoc* test was performed. * $p<0.05$ vs. WKY rats; † $p<0.05$ vs. CTRL rats.

4.3. *Project 3 – The comparison of stress-induced cardiovascular and hormonal responses in adult normotensive and spontaneously hypertensive rats*

Stress-induced changes of BP, HR, low-frequency and high-frequency component of systolic blood pressure variability are shown in Fig. 8 and Fig. 9.

4.3.1. *Plasma hormones*

Figures 13 and 14 show plasma levels of noradrenaline, normetadrenaline, adrenaline, metadrenaline, ACTH, corticosterone, plasma renin activity and aldosterone in stress-naive as well as restrained SHR and WKY rats.

Stress-naive SHR showed higher plasma noradrenaline levels than WKY rats ($p<0.001$; Fig. 13A). Plasma noradrenaline levels were increased after 10 min of restraint in both strains ($p<0.001$ for both strains) and stress-induced noradrenaline level was higher in SHR compared to WKY rats. After 120 min of restraint, noradrenaline plasma levels were still increased in WKY rats (WKY rats basal vs. 120 min: $p<0.05$), whereas noradrenaline plasma levels in SHR were even lower than those observed in stress-naive SHR (SHR basal vs. 120 min: $p<0.001$) and the strain difference disappeared. Plasma normetadrenaline levels were similar in stress-naive SHR and WKY rats (Fig. 13B). Restraint increased normetadrenaline levels after 10 min only in SHR but not in WKY rats (SHR basal vs. 10 min: $p<0.001$; SHR vs. WKY rats: $p<0.001$). Plasma adrenaline levels were similar in stress-naive SHR and WKY rats (Fig. 13C). Restraint increased adrenaline levels after 10 min only in SHR but not in WKY rats (SHR basal vs. 10 min: $p<0.001$; SHR vs. WKY rats: $p<0.01$). After 120 min of restraint, adrenaline levels returned back to basal levels in SHR, but they were increased in WKY rats (WKY rats basal vs. 120 min: $p<0.01$; SHR vs. WKY rats: $p<0.01$). Plasma metadrenaline levels were similar in stress-naive SHR and WKY rats (Fig. 13D). Restraint increased metadrenaline levels after 10 min only in SHR but not in

WKY rats (SHR basal vs. 10 min: p<0.001; SHR vs. WKY rats: p<0.001). No strain differences were seen after 120 min of stress and after recovery.

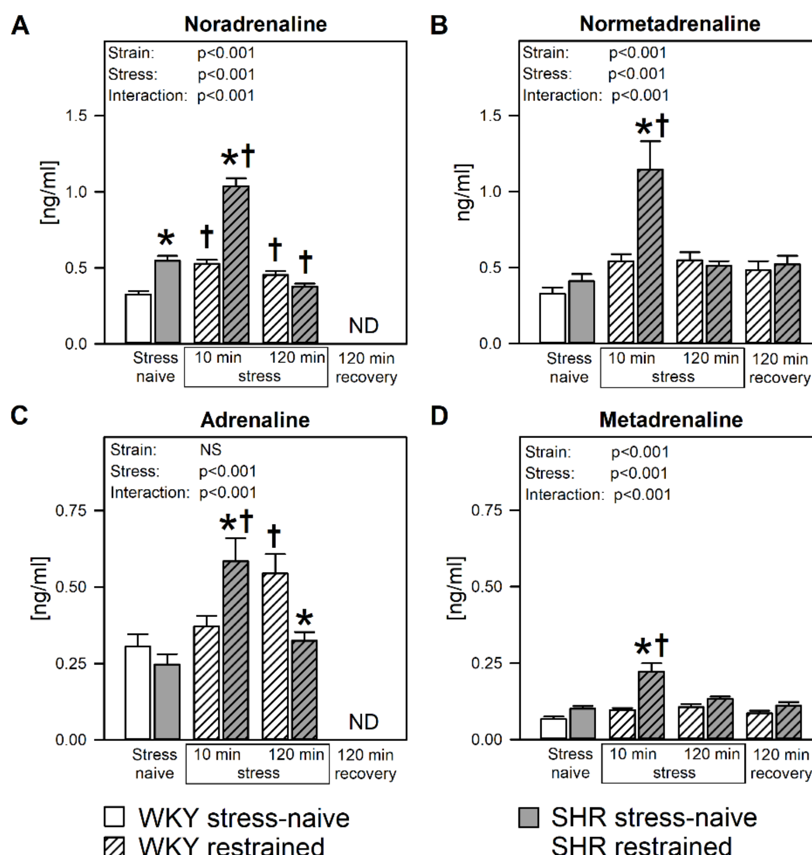

Figure 13. Plasma levels of noradrenaline (A), normetadrenaline (B), adrenaline (C) and metadrenaline in stress-naive and restrained WKY rats and SHR. The values are expressed as the mean ± SEM; n = 8 rats for each group. The effects of strain and stress and their interaction were analyzed by two-way ANOVA, Bonferroni *post-hoc* test was performed. * p<0.05 vs. WKY rats; † p<0.05 vs. CTRL rats. ND, not determined.

Plasma ACTH levels were similar in stress-naive SHR and WKY rats (Fig. 14A). Restraint increased ACTH levels in both SHR and WKY rats after 10 and 120 min while ACTH increase after 10 min was more pronounced in SHR (p<0.001). After 120 min recovery, ACTH levels returned back to the basal level in WKY rats but remained increased in SHR (SHR vs. WKY rats: p<0.05). In stress-naive animals, there was a similar plasma level of corticosterone in SHR and WKY rats (Fig. 14B). Restraint induced corticosterone increase, which was more pronounced in SHR than in WKY rats (p<0.01 after 10 min, p<0.001 after 120 min). Plasma

levels of corticosterone were further increased after 120 min stress in both strains, while after 120 min of recovery they were similar to those in stress-naive rats.

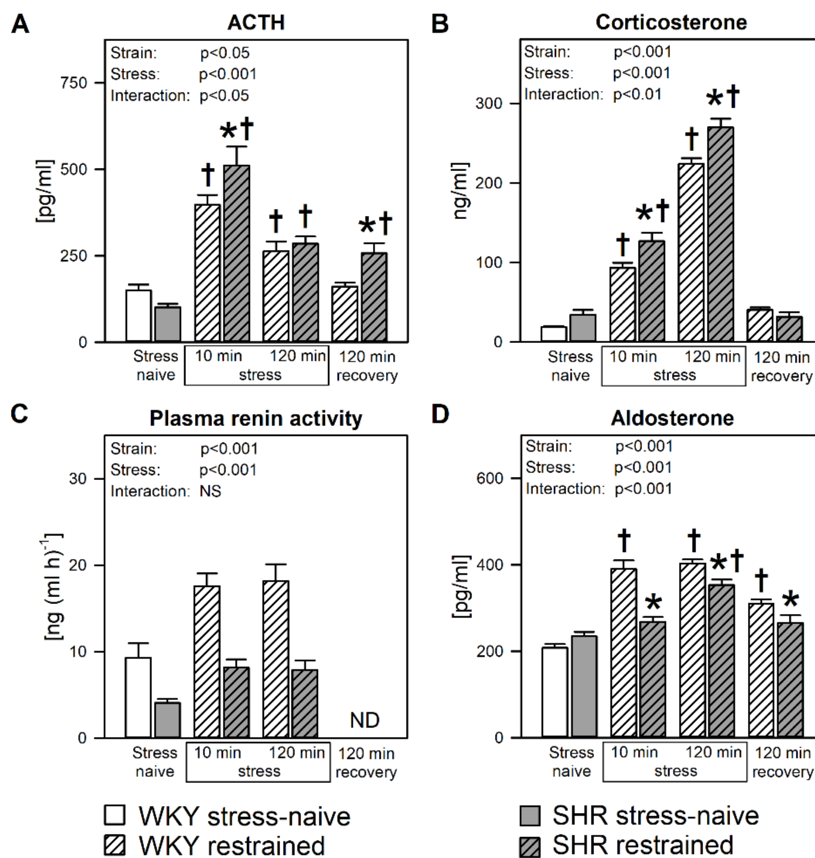

Figure 14. Plasma levels of adrenocorticotropic hormone (ACTH; A), corticosterone (B), plasma renin activity (C) and aldosterone plasma levels in stress-naive and restrained WKY rats and SHR. The values are expressed as the mean ± SEM; n = 8 rats for each group (with the exception of plasma renin activity, where n=9/6/10 for stress-naive rats, 10 min restraint and 120 min restraint, respectively). The effects of strain and stress and their interaction were analyzed by two-way ANOVA, Bonferroni *post-hoc* test was performed. * $p<0.05$ vs. WKY rats; † $p<0.05$ vs. CTRL rats. ND, not determined.

Plasma renin activity was lower in SHR than in WKY rats and it was increased in rats of both strains after 10 and 120 min of restraint stress ($p<0.001$ for both time-points; Fig. 14C). In stress-naive animals, there was a similar plasma level of aldosterone in SHR and WKY rats (Fig. 14D). Plasma aldosterone was increased in WKY rats after 10 and 120 min of restraint and also after 120 min recovery ($p<0.001$ for all time-points). In SHR, restraint caused aldosterone to increase only after 120 min ($p<0.001$). Stress-induced aldosterone levels were lower in SHR than those observed in WKY rats ($p<0.001$ after 10 min, $p<0.01$ after 120 min and $p<0.05$ after recovery).

Taken together, we observed more pronounced stress-induced increase of stress-related hormones (catecholamines, ACTH, corticosterone) in SHR compared to WKY rats. On the other hand, plasma renin activity and aldosterone levels were lower in SHR.

4.3.2. *Gene expression in the adrenal medulla*

Reference genes *Gapdh* and *Sdha* were selected as described in our previous study (Vavřínová *et al*., 2016). The mRNA expression of *Th* gene was similar in adrenal medulla of stress-naive SHR and WKY rats (Figure 15). The restraint stress increased mRNA expression of *Th* gene in both SHR and WKY rats (3-fold in WKY, 4.5-fold in SHR; $p<0.001$ for both strains) and *Th* expression was higher in restrained SHR than in restrained WKY rats ($p<0.001$). The mRNA expression of *Ddc* gene was lower in adrenal medulla of SHR compared to WKY rats and it was not affected by restraint stress (Figure 15). The mRNA expression of *Dbh* gene was lower in adrenal medulla of stress-naive SHR in comparison to that of WKY rats ($p<0.001$), and similarly increased (2-fold) after restraint stress ($p<0.01$ for SHR; $p<0.001$ for WKY rats), the strain difference being preserved. There was lower mRNA expression of *Pnmt* gene in stress-naive SHR compared to WKY rats (Student's t test: $p<0.05$). Restraint stress increased *Pnmt* expression 4-fold in WKY rats and 3-fold in SHR (basal vs. restraint for both strains: $p<0.001$; restrained SHR vs. WKY rats: $p<0.001$).

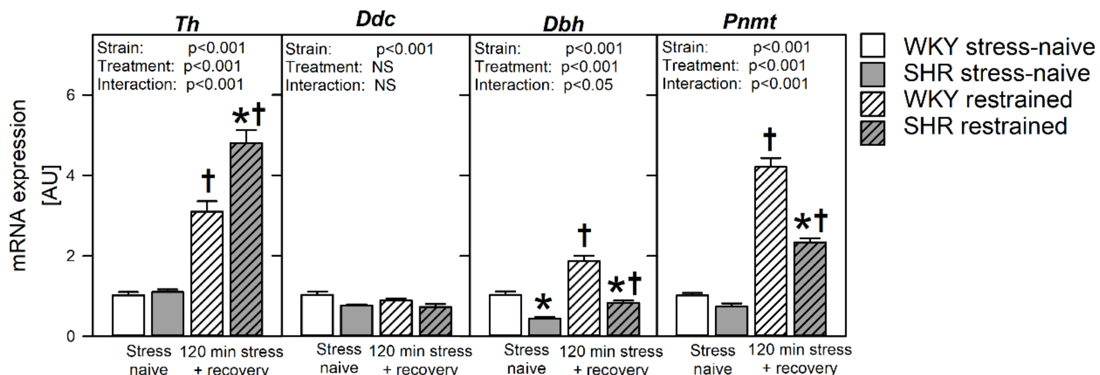

Figure 15. mRNA expression of genes involved in catecholamine biosynthesis in adrenal medulla of stress-naive and restrained (120 min of stress + 120 min recovery) WKY rats and SHR. The mRNA expression was standardized to the best combination of reference genes (*Gapdh* and *Sdha*). Data are plotted relatively to stress-naive WKY rats as mean ± SEM, n = 7-8 for each group. Statistical significance was computed by two-way ANOVA. Bonferroni post-hoc test was performed.

The mRNA expression of *Nr3c1* gene (encoding glucocorticoid receptor) was similar in adrenal medulla of stress-naive SHR and WKY rats (Table 13). We observed a higher expression of *Nr3c1* in restrained SHR compared to restrained WKY rats ($p<0.001$). The mRNA expression of *Nr3c2* gene (encoding mineralocorticoid receptor) was 2-fold higher in adrenal medulla of stress-naive SHR compared to WKY rats ($p<0.001$) and restraint stress decreased the *Nr3c2* expression in SHR ($p<0.001$), whereas there was no stress-induced change in the expression of *Nr3c2* in WKY rats. The mRNA expression of *Fkbp5* gene (encoding Fkbp prolyl isomerase 5) was higher in the adrenal medulla of SHR (Table 13). Restraint stress induced *Fkbp5* mRNA expression increase in adrenal medulla of both SHR and WKY rats. The expression of *Hsd11b1* gene (encoding enzyme 11β-hydroxysteroid dehydrogenase type 1, which converts 11-dehydrocorticosterone to active corticosterone), was similar in the adrenal medulla of SHR and WKY rats and it was not affected by restraint stress. The mRNA expression of *H6pd* gene (encoding glucose-6-phosphate dehydrogenase, which generates NADPH required for oxo-reductase activity of *Hsd11b1*), was similar in adrenal medulla of SHR and WKY rats and it was similarly increased by restraint stress in both strains. The expression of *Hsd11b2* gene (encoding enzyme 11β-hydroxysteroid dehydrogenase type 2, which deactivates corticosterone), in the adrenal medulla was too low for a reliable quantification.

The most abundant mRNA for Ang II receptor in the adrenal medulla was subtype 2 (*Agtr2* gene), which was similarly expressed in SHR and WKY rats (Table 13). The mRNA expression of *Agtr2* gene substantially decreased after the restraint stress in both strains (Table 14). The mRNA for Ang II receptor subtypes 1a (gene *Agtr1a*) and 1b (gene *Agtr1b*) was 10-fold and 40-fold less abundant in adrenal medulla than *Agtr2* subtype, respectively. The mRNA expression of *Agtr1a* gene was lower in SHR and it was not affected by stress. *Agtr1b* gene was also underexpressed in SHR and its mRNA expression decreased after the restraint stress in rats of both strains.

Gene symbol	Ratio mean SHR/ mean WKY (arbitrary units)				two-way ANOVA		
	Stress-naive		After 120 min restraint and 120 min recovery				
	WKY	SHR	WKY	SHR	Strain	Restraint	Interaction
Nr3c1	1.03 ± 0.09	1.18 ± 0.03	0.77 ± 0.04	1.41 ± 0.12*	p<0.001	NS	p<0.05
Nr3c2	1.05 ± 0.12	1.92 ± 0.12*	0.91 ± 0.07	1.09 ± 0.12†	p<0.001	p<0.001	p<0.01
Fkbp5	1.06 ± 0.12	1.29 ± 0.12	1.88 ± 0.14	2.59 ± 0.36	p<0.05	p<0.001	NS
Hsd11b1	1.04 ± 0.11	0.96 ± 0.05	1.06 ± 0.12	1.03 ± 0.12	NS	NS	NS
H6pd	1.06 ± 0.12	1.25± 0.21	1.88 ± 0.26	2.00 ± 0.26	NS	p<0.001	NS
Agtr1a	1.02 ± 0.07	0.69± 0.07	1.16 ± 0.07	0.84 ± 0.09	p<0.001	NS	NS
Agtr1b	1.10 ± 0.17	0.57± 0.07	0.41 ± 0.04	0.29 ± 0.08	p<0.01	p<0.001	NS
Agtr2	1.06 ± 0.13	0.95± 0.09	0.25 ± 0.03	0.42 ± 0.16	NS	p<0.001	NS

Table 13. The mRNA expression of genes of corticosteroid signaling and angiotensin II receptors in adrenal medulla of stress-naive and restrained (120 min of restraint + 120 min recovery) WKY rats and SHR. The mRNA expression was standardized to the best combination of reference genes (*Gapdh* and *Sdha*). The values are expressed relatively to stress-naive WKY rats as the mean ± SEM, n = 8 for each group. Statistical significance was computed by two-way ANOVA. Bonferroni *post-hoc* test was performed. * $p < 0.05$ vs. WKY; † $p < 0.05$ vs. stress-naive rats of the same strain.

Taken together, we observed the different pattern of stress-induced mRNAexpression of particular catecholamine biosynthetic enzymes in adrenal medulla of SHR and WKY rats. Concerning genes involved in regulation of expression of catecholamine biosynthetic enzymes, the mRNA expression of genes for glucocorticoid receptors and mineralocorticoid receptors was higher in SHR, whereas the expression of genes for Ang II type 1 receptors was lower in SHR compared to WKY rats.

5. DISCUSSION

The aim of the Thesis was to study the role of sympathoadrenal and sympathoneural systems in the development and maintenance of high blood pressure in rats with genetic hypertension. In the Project 1, the ontogenetic changes in the sympathoneural and sympathoadrenal systems were studied in SHR and WKY rats. The increased adrenal catecholamine content and increased density of vascular sympathetic innervation were observed in prehypertensive SHR. In contrast, the expression of enzymes of catecholamine biosynthesis was downregulated in both the sympathetic ganglia and adrenal medulla of adult as well as prehypertensive SHR compared to aged-matched WKY rats. It resulted in the lower catecholamine content in the adrenal glands. It results in the lower catecholamine content in the adrenal glands but not in the lower vascular sympathetic innervation of adult SHR. In contrast, vascular sympathetic innervation was not reduced in adult SHR. In the Project 2, the effects of chemical sympathectomy induced by guanethidine on cardiovascular parameters were evaluated in adult SHR and WKY rats. Guanethidine treatment decreased the BP, HR, low-frequency and high-frequency components of systolic blood pressure variability (LF SBPV and HF SBPV, markers of sympathetic vascular and cardiac activity, respectively) in both SHR and WKY rats. However, BP decrease was only temporary despite the persistent effect of sympathectomy, which might be explained by a major increase in sensitivity of cardiovascular system to catecholamines and the increased plasma levels of adrenaline. In Project 3, stress-induced cardiovascular response of adult SHR and WKY rats elicited by restraint was compared. Acutely stressed SHR showed exaggerated BP, HR, LF SBPV, and HF SBPV responses, which were accompanied by higher plasma levels of catecholamines, ACTH and corticosterone. On the other hand, the plasma renin activity and plasma aldosterone levels were lower in stressed SHR compared to WKY rats. Restraint stress-induced increase in mRNA expression of *Th* gene, involved in catecholamine biosynthesis, was more pronounced in adrenal medulla of SHR,

whereas stress-induced mRNA expression of *Dbh* and *Pnmt* genes was lower in SHR compared to WKY rats.

Project 1 – The comparison of sympathoneural and sympathoadrenal abnormalities in young prehypertensive and adult spontaneously hypertensive rats

The mRNA and protein expression of genes involved in catecholamine biosynthesis *Ddc* and *Dbh* was lower in the sympathetic ganglia of prehypertensive 4-week-old SHR, whereas the expression of *Th* gene was unchanged compared to the aged-matched WKY rats. The dopamine and noradrenaline content was similar in the sympathetic ganglia of 4-week-old SHR and WKY rats. On the other hand, the histochemical visualization of catecholamines showed a higher density of sympathetic innervation in the femoral arteries of prehypertensive SHR compared to aged-matched WKY rats which is consistent with the results reported earlier (Mangiarua and Lee, 1990; Scott and Pang, 1983). In line with the increased density of sympathetic innervation, we observed a higher plasma level of noradrenaline in prehypertensive SHR compared WKY rats which is consistent with previous reports (Grobecker *et al*., 1976; Szemeredi *et al*., 1988). On the other hand, Cabassi *et al*. (1998) found similar plasma levels of noradrenaline in young SHR and WKY rats. However, the plasma noradrenaline level is a very unstable parameter which can change within seconds and might be influenced by many factors including animal handling, anesthesia or blood sampling. The plasma levels of normetadrenaline, which is a metabolite of noradrenaline, were similar in 4-week-old SHR and WKY rats suggesting that the function of sympathetic nervous system under the rest conditions might be similar in prehypertensive SHR and WKY rats. In the adrenal glands of prehypertensive SHR, we found a lower mRNA expression of *Dbh* and *Pnmt* genes but an unchanged mRNA expression of *Th* and *Ddc* compared to 4-week-old WKY rats which is consistent with previous results (Friese *et al*., 2005). In line with mRNA data, the protein expression of *Th* gene was unchanged while protein expression of *Ddc* and *Dbh* genes was lower in the adrenal glands of 4-week-old SHR in comparison with the

aged-matched WKY rats. The protein expression of *Pnmt* gene was similar in both strains despite the decreased mRNA expression observed in the adrenal gland of prehypertensive SHR. However, activity of PNMT enzyme is regulated (e.g. after the exposure to glucocorticoids) not only transcriptionally but also through the control of translation and enzyme degradation (Berenbeim *et al*., 1979; Wong *et al*., 1995). In adrenal gland of prehypertensive SHR, activity of TH enzyme was reported to be either decreased (Grobecker *et al*., 1976; Moura *et al*., 2005) or increased (Teitelman *et al*., 1981). The catecholamine content in the adrenal glands of young SHR was described as unchanged or decreased (Grobecker *et al*., 1976; Moura *et al*., 2005). We did not measure the activity of enzymes involved in catecholamine biosynthesis but we found a greater amount of dopamine, noradrenaline and adrenaline in the adrenal glands of 4-week-old SHR compared to WKY rats. Moreover, we observed similar plasma adrenaline and metadrenaline levels in 4-week-old SHR and WKY rats. Furthermore, the mRNA expression of genes involved in catecholamine reuptake and degradation was similar in adrenal medulla of both strains. The higher catecholamine content might be associated with some post-translational mechanisms increasing the activity of TH enzyme in adrenal gland of prehypertensive SHR. In general, despite of the decreased expression of genes involved in catecholamine biosynthesis (in both sympathoneural and sympathoadrenal system), there is the elevated density of vascular sympathetic innervation as well as increased adrenal content of catecholamines in prehypertensive SHR. The abnormalities observed in sympathoneural and sympathoadrenal system of SHR might cause exaggerated responsiveness of the system to various stimuli (including stress) and contribute to hypertension development in this strain.

In sympathetic ganglia of adult SHR with established hypertension, the pattern of mRNA and protein expression of genes involved in catecholamine biosynthesis was similar to that observed in young SHR (decreased expression of *Ddc* and *Dbh* genes) with the exception of slightly increased mRNA expression of *Th* in adult SHR compared to WKY rats. The unchanged

catecholamine content in the sympathetic ganglia and a denser vascular sympathetic innervation in adult SHR were also analogous to the findings in prehypertensive animals and are in accordance with our previously published results (Bencze *et al*., 2016) as well as with the findings of other laboratories (Mangiarua and Lee, 1990; Mano *et al*., 1992; Scott and Pang, 1983). In the adrenal glands of adult SHR, mRNA expression of all genes encoding enzymes of catecholamine biosynthesis (*Th*, *Ddc*, *Dbh* and *Pnmt*) was decreased in comparison to the adult WKY rats. In accordance, we found a lower protein expression of *Th*, *Ddc* and *Dbh* genes in the adrenal glands of adult SHR compared to WKY rats. Similarly to young animals, we observed similar protein expression of *Pnmt* gene in adrenal gland of adult SHR and WKY rats. The decreased mRNA and protein expression of *Th* gene in adrenal gland of adult SHR were reported previously (Grundt *et al*.,2009; Moura *et al*., 2005). On the contrary, our results are at a variance with the studies describing a higher mRNA expression of *Th* and *Pnmt* genes in adrenals of SHR (Nguyen *et al*., 2009; Reja *et al*., 2002a). Our observation of decreased adrenal content of dopamine and noradrenaline but an unchanged amount of adrenaline in the adrenal glands of adult SHR is in line with the protein expression of the respective biosynthetic enzymes. This is also consistent with reduced noradrenaline (Korner *et al*., 1993; Moura *et al*. 2005) and unchanged adrenaline (Lee *et al*., 1991a; O'Connor et al., 1999) reported in the adrenal glands of adult SHR. We found similar plasma levels of noradrenaline, adrenaline, normetadrenaline and metadrenaline in adult SHR and WKY rats which is in agreement with some earlier reports (Kvetnansky *et al*., 1979a; Szemeredi *et al*., 1988), but the other groups reported increased plasma levels of noradrenaline (Moura *et al*. 2005) or adrenaline (Vlachakis *et al*., 1980) in SHR with established hypertension. The discrepant results concerning the expression of enzymes involved in catecholamine biosynthesis or plasma levels of catecholamines might be explained by the influence of stress since the catecholaminergic system is extremely susceptible to the stressful conditions. Kvetnansky *et al*. (2004) demonstrated that a single or repeated

immobilization changed mRNA and protein expression of *Th*, *Dbh* and *Pnmt* genes in both adrenal glands and sympathetic ganglia. It was reported that the stress-induced hormonal and cardiovascular response as well as stress-induced changes in adrenal expression differ between SHR and WKY rats (Behuliak *et al*., 2018; Grundt *et al*., 2009; McCarty *et al*., 1978). This will be discussed together with the data obtained in the Project 3.

In conclusion, the pathway of catecholamine biosynthesis in the adrenal glands of SHR with established hypertension is downregulated at the different levels, i.e. mRNA expression, protein expression and the catecholamine content. Such downregulated expression is also present in the sympathetic ganglia of adult SHR, but it is not associated with a decrease in catecholamine content in ganglia or vascular wall. The partial downregulation of the expression of genes of the catecholaminergic system is present already in 4-week-old SHR. At this developmental stage, SHR are still normotensive or their BP is only slightly increased but they already show higher sympathetic activity and increased heart rate (Behuliak *et al*., 2015; Judy *et al*., 1979). Thus, downregulation of the expression of genes of the catecholaminergic system might be a compensatory mechanism counteracting the hyperfunction of the sympathoneural system which develops concurrently with the progression of hypertension in SHR.

Project 2 – The comparison of sympathoadrenal abnormalities in young prehypertensive and adult spontaneously hypertensive rats

More important role of the sympathetic nervous system in regulation of cardiovascular system in adult SHR compared to WKY rats was documented by a higher fluorescent signal of catecholamines in the femoral arteries, a more pronounced BP decrease after the treatment with ganglionic blocker pentolinium and a higher LF SBPV and HF SBPV (markers of sympathetic vascular and cardiac activity, respectively), which is in accordance with previous reports (Behuliak *et al*., 2018; Chiu and McNeill, 1992; Head *et al*., 1985; Zicha *et al*., 2014). Sympathectomy by chronic guanethidine administration reduced the MAP and HR as well as the

LF SBPV and HF SBPV in both SHR and WKY rats after three days of treatment. HR, LF and HF SBPV were still decreased after 14 days of guanethidine treatment, but the MAP in sympathectomized animals returned back to the level observed in untreated animals. Similarly to our finding, Johnson and O'Brien (1976) reported that chronic guanethidine treatment of adult Sprague-Dawley rats did not significantly change the resting BP. In contrast, Benarroch *et al.* (1990) observed the lowering of basal BP in adult Sprague-Dawley rats treated with guanethidine. Nevertheless, we also found BP reduction in rats treated with guanethidine for 14 days but this was observed only under the conditions of acute restraint stress (Behuliak *et al.*, 2018). BP recovery to the control values in freely moving animals cannot be ascribed to an insufficient degree of sympathectomy in either rat strain because the LF SBPV (marker of sympathetic vascular activity) was still decreased after 14 days of guanethidine treatment. Moreover, guanethidine treatment decreased the catecholamine fluorescent signal to less than 15 % in both SHR and WKY rats compared to non-sympathectomized controls. In line with this finding, MAP responses to ganglionic blocker pentolinium and catecholamine releasing agent tyramine were also attenuated by sympathectomy.

It was reported that after peripheral sympathectomy, vascular tone can be maintained by the augmented postjunctional sensitivity of vascular smooth muscle cells to vasoconstrictors (Fleming, 1981). We observed similar MAP sensitivity to noradrenaline and adrenaline in control non-sympathectomized SHR and WKY rats. This is in agreement with our previous results concerning isolated arteries (Bencze *et al.*, 2016). We demonstrated the enhancement of MAP sensitivity to noradrenaline (up to 14-fold) and adrenaline (up to 10-fold) following sympathectomy in both SHR and WKY rats which is in accordance with the increased BP response to the α_1-adrenergic agonist phenylephrine observed in adult sympathectomized Wistar (Kamikihara *et al.*, 2007) and WKY rats (Rizzoni *et al.*, 2000). To evaluate whether the sympathectomy-induced augmentation of the MAP response is specific for catecholamines or it

is generalized for various vasoconstrictors, MAP response to Ang II was also determined. Guanethidine treatment increased the sensitivity of MAP to Ang II only twofold in both SHR and WKY rats. A smaller effect of sympathectomy on cardiovascular sensitivity to Ang II compared to catecholamines was also described by Rizzoni *et al*. (2000). Kamikihara *et al*. (2007) reported that the increased vascular sensitivity to phenylephrine in rats sympathectomized by reserpine is accompanied by the mRNA overexpression of α_{1D}-adrenergic receptor in the rat tail artery. On the contrary, we found no effect of sympathectomy on the mRNA expression of adrenergic receptors as well as G-protein subtypes that mediate adrenergic vasoconstriction in aortic smooth muscle. The discrepant results might be caused by the use of different sympatholytic agents or the examination of various vascular beds. However, the enhanced sensitivity of the cardiovascular system to catecholamines can be reached by mechanisms other than the *de novo* synthesis of receptors, e.g., by their increased affinity for noradrenaline (Colucci *et al*., 1982).

Baroreflex function was evaluated by the spontaneous sequence technique in freely moving SHR and WKY rats. SHR showed a lower baroreflex sensitivity compared to WKY rats which is in agreement with the reports that impaired baroreflex sensitivity is associated with sympathetic hyperactivity in SHR as well as with human essential hypertension (Behuliak *et al*., 2018; Ferrari *et al*., 1991; Head, 1995). Impaired baroreflex function in SHR is consistent with the greater MAP decrease but the reduced HR increase to the acute administration of captopril (angiotensin-converting enzyme inhibitor) in SHR when compared to WKY rats. Sympathectomy slightly improved the baroreflex sensitivity in both strains which is in accordance with previous reports concerning SHR, WKY and Sprague-Dawley rats (Ferrari *et al*., 1991; Mircoli *et al*., 2002). Nevertheless, the strain differences in baroreflex sensitivity persisted in guanethidine-treated SHR and WKY rats.

In our study, sympathectomy lowered plasma levels of noradrenaline and normetadrenaline by 50 % in both SHR and WKY rats, which is in line with the data obtained in neonatally sympathectomized SHR (Tipton *et al*., 1984). On the other hand, guanethidine treatment was accompanied with the elevation of plasma adrenaline and metadrenaline levels in both strains, the effect being three times stronger in WKY rats. Plasma noradrenaline predominantly originates from the peripheral sympathetic nerve endings, whereas the chromaffin cells of adrenal medulla are considered to be the source of less than 10 % of this hormone under the normal conditions. The contribution of adrenal medulla to plasma noradrenaline levels can increase up to 30-45 % under the stress condition (Goldstein, 1983), suggesting that the adrenal gland has a capacity to partially compensate for the lack of noradrenaline in sympathectomized animals. Adrenal hypertrophy, greater adrenal catecholamine content and a higher activity of TH enzyme in adrenals have already been described in Sprague-Dawley rats subjected to sympathectomy (Kvetnansky *et al*., 1979b; Qiu *et al*., 1999). We observed a more pronounced adrenal enlargement in guanethidine-treated WKY rats than in SHR. We also found greater noradrenaline content in adrenals of sympatectomized WKY rats, while the amount of adrenaline was increased by sympathectomy in both strains. We have observed the substantially decreased mRNA expression of all genes involved in catecholamine biosynthesis (*Th, Ddc, Dbh, Pnmt*) in adrenal glands of adult SHR in the Project 1. The mRNA expression of *Ddc, Dbh* and *Pnmt* genes was also lower in adrenal medulla of control SHR than in WKY rats in the Project 2, but the mRNA expression of *Th* gene was similar in both strains. The possible explanation for these findings might be that daily intraperitoneal administration of saline in the Project 2 induced the expression of *Th* gene. Moreover, the mRNA expression in the Project 1 was measured in macrosamples of adrenal medulla and might be partially affected by the size of adrenal medulla in either strain, whereas the expression in the Project 2 was evaluated in microdissected samples of the same size in both strains. Sympathectomy of adult rats elevated the mRNA expression of

the *Th, Dbh* and *Pnmt* genes similarly in the adrenal medulla of SHR and WKY rats and the strain difference still persisted. This finding suggests that downregulation of catecholamine biosynthetic pathway in adrenal gland of adult SHR is not caused by a direct negative feedback compensating sympathetic hyperactivity in this strain.

In addition to the elevated plasma adrenaline levels, the sympatectomy also caused two-fold increase in plasma levels of corticosterone and aldosterone in WKY rats but not in SHR. Aldosterone secretion is regulated by Ang II; thus, a greater role of the renin-angiotensin system (RAS) in sympathectomized animals might explain the increased plasma aldosterone levels. It was reported that guanethidine treatment lowers plasma renin activity while it increases the density of Ang II receptors in the adrenal gland (Qiu *et al*., 1999). However, the sympathectomy in our settings did not change the acute BP response to angiotensin-converting enzyme inhibitor captopril in either strain. Thus, the contribution of the kidney and RAS to BP maintenance in sympathectomized animals cannot be established without further experiments. On the other hand, both corticosterone and aldosterone are released as a result of exposure to psychological stressor (Kubzansky and Adler, 2010). A more pronounced body weight loss and enhanced adrenal growth observed in sympathectomized WKY rats indicates that sympathectomy by guanethidine might be a more stressful intervention for adult WKY rats than for SHR. Altogether, guanethidine-induced sympathectomy decreased HR and improved baroreflex sensitivity in adult hypertensive SHR and normotensive WKY rats. Basal BP in freely-moving animals was lowered by sympathectomy only temporarily but the effect of treatment on BP was revealed under the stressful conditions. BP recovery might be explained by the involvement of compensatory mechanisms, such as the more than tenfold increase in BP sensitivity to catecholamines and/or elevation of plasma levels of adrenaline.

In the project 2, SHR showed more pronounced pressor response during the acute restraint compared to WKY rats which is in accordance with previous reports concerning

restraint (McDougall *et al*., 2000) as well as the other forms of stress such as handling, open-field or air-jet (McDougall *et al*., 2005; van den Buuse *et al*., 2001). This finding is in line with the increased vascular sympathetic activity documented by indirect parameter LF SBPV which was markedly elevated in SHR during the restraint stress. Furthermore, we observed an exaggerated maximal heart rate increase in adult SHR compared to WKY rats, which is in accordance with a more pronounced increase in cardiac sympathetic activity (documented by indirect parameter HF SBPV) in stressed SHR compared to WKY rats. In contrast, McDougall *et al*. (2000) showed the similar maximal heart rate increase evoked by the restraint in SHR and WKY rats. However, the other research groups showed a more pronounced heart rate increase in SHR during stress elicited by either air-jet or by open-field exposure (Ely *et al*., 1985; van den Buuse *et al*., 2001). The excessive activation of sympathoneural and sympathoadrenal systems in SHR was also documented in the Project 3 by higher stress-induced plasma levels of noradrenaline and adrenaline as well as of their metabolites normetadrenaline and metadrenaline. These findings are in a good agreement with previous observations concerning immobilization stress in SHR (Kvetnansky *et al*., 1979a; McCarty *et al*., 1978). The exaggerated noradrenaline release during stress probably results from a denser sympathetic innervation and higher catecholamine content in SHR arteries. Augmented stress-induced adrenaline release seems to be at variance with the lower expression of catecholamine biosynthetic enzymes and lower catecholamine content in the adrenal gland of adult stress-naive SHR when compared to WKY rats. However, the catecholamine content in adrenal gland is three orders of magnitude greater than the amount of plasma catecholamines and thus a lower catecholamine store may not lead to the attenuation of catecholamine efflux.

In conclusion, peripheral sympathectomy of adult hypertensive SHR by guanethidine administration decreased heart rate and improved baroreflex sensitivity in freely moving rats. In contrast, basal blood pressure was lowered by guanethidine treatment only in the first five days,

although sympathectomy prevented stress-induced blood pressure increase in rats studied after two-week guanethidine treatment. BP recovery in sympathectomized rats might be explained by compensatory mechanisms, such as the more than tenfold increase in BP sensitivity to catecholamines and the increased plasma levels of adrenaline.

Project 3 – The comparison of stress-induced cardiovascular and hormonal responses in adult normotensive and spontaneously hypertensive rats

Stress-induced elevation in the expression of genes involved in catecholamine biosynthesis is considered to be a mechanism aiming to replenish catecholamine stores (Wong, 2006). The catecholamine secretion from the adrenal gland as well as the expression and activity of catecholamine biosynthetic enzymes in chromaffin cells is regulated by several mechanisms including acetylcholine from sympathetic innervation, glucocorticoids and Ang II (Livett and Marley, 1993; Stachowiak *et al*., 1990; Wong, 2006). Other experiments combining stress with hypophysectomy, preganglionic denervation of sympathetic nerves or the adrenal medulla and treatment with various hormones or neural agents (e.g. ACTH, glucocorticoid, acetylcholine etc.) further suggest that the induction of *Th* gene may be primarily mediated by neural activity, whereas regulation of *Pnmt* gene is dependent mainly on hormonal influence (Axelrod and Reisine, 1984; Viskupic *et al*., 1994). It was reported that the sympathetic activity is increased in splanchnic and renal nerves of SHR (Judy et al., 1979; Judy and Farrell, 1979; Okamoto *et al.*, 1967) and we also observed markedly elevated sympathetic activity (documented by indirect parameters LF and HF SBPV) in SHR under the stress conditions. Moreover, it was found that the secretion of catecholamines evoked by the stimulation of nicotinic and muscarinic receptors as well as membrane depolarization is enhanced in the perfused adrenal glands of SHR compared to WKY (Lim *et al*., 2002; Miranda-Ferreira *et al*., 2009). Accordingly, in the Project 3 we observed a greater elevation of mRNA expression of *Th* gene in adrenal medulla of stressed SHR compared to WKY rats. This finding is consistent with the greater increase in mRNA expression

of *Th* gene in the adrenal glands of adult SHR after 25 min of mild stress caused by tail cuff measurement of blood pressure (Grundt *et al*., 2009).

In the Project 3, plasma corticosterone levels were found higher in restraint SHR than in WKY rats which is in accordance with previous papers concerning higher plasma corticosterone levels in SHR subjected to ether exposure or immobilization (Häusler *et al*., 1983; Kvetnansky *et al*., 1979a). Corticosterone production is triggered by ACTH (Chrousos, 2009). We observed higher ACTH levels in SHR subjected to restraint. Accordingly, exaggerated ACTH response after ether exposure, cold exposure or immobilization was previously reported in SHR (Djordjevic *et al*., 2007; Häusler *et al*., 1983). ACTH secretion from pituitary is controlled by CRH (Chrousos, 2009). The decreased CRH content in hypothalamus and impaired ACTH response to CRH administration were described in young SHR (Hashimoto *et al*., 1989; Hattori *et al*., 1986a; Hattori *et al*., 1986b). This suggests that another mechanism underlie the exaggerated stress-induced ACTH release in hypertensive rats. It was documented that peripheral administration of adrenergic agonists induces release of ACTH and corticosterone (Berkenbosch *et al*., 1981; Tilders *et al*., 1982). Moreover, Lowrance *et al*. (2016) showed that sympathetic nervous system directly contributes to glucocorticoid production during stress. Thus, the hyperactivity of sympathoneural and sympathoadrenal systems might be involved in the overactivation of HPA axis in SHR. Surprisingly, the mRNA expression of *Pnmt* gene which is regulated by glucocorticoids is decreased in adrenal medulla of stress-naive as well as stressed SHR compared to WKY rats. The corticosterone influence on the expression of targeted genes is exxerted through high affinity mineralocorticoid receptors (encoded by *Nr3c2* gene) and low affinity glucocorticoid receptors (encoded by *Nr3c1* gene (Hadoke et al., 2009; Hodel, 2001; Sapolsky *et al*., 2000). Mineralocorticoid receptor in rat extra-renal tissues bind aldosterone and corticosterone with similar affinities. Plasma corticosterone levels are 100-1000 times higher than plasma aldosterone levels. Therefore, the selectivity of mineralocorticoid receptors for

aldosterone is dependent on pre-receptor metabolism of glucocorticoids (Hadoke et al., 2009), e.g. presence of 11β-hydroxysteroid dehydrogenase 2 (encoded by *Hsd11b2* gene) which inactivates corticosterone by oxidizing its 11β-hydroxy group to 11-keto form (Sapolsky *et al*., 2000). The induction of expression of *Fkbp5* gene (Fkbp prolyl isomerase 5) is considered to be a marker of activation of glucocorticoid receptors. Moreover, *Fkbp5* decreases the affinity of glucocorticoid receptor, its translocation to the nucleus and glucocorticoid-dependent transcriptional activity (Binder, 2009; Zannas *et al*., 2016). The mRNA expression of both glucocorticoid and mineralocorticoid receptors was higher in adrenal medulla of SHR compared to WKY rats. The mRNA expression of *Hsd11b2* gene in the adrenal medulla was under the limit of detection. This is in accordance with important role of glucocorticoids in regulation of catecholamine synthesis and release (Livett and Marley, 1993; Wong, 2006). The mRNA expression of *Fkbp5* gene was higher in adrenal medulla of stress-naive as well as stressed SHR which is in line with higher corticosterone levels in this strain and might partially protect adrenal chromaffin cells from the excessive glucocorticoids. It would require further experiments to determine the role of glucocorticoid signaling in the altered expression of catecholamine biosynthetic enzymes observed in SHR. However, we have recently demonstrated an important permissive and/or stimulating role of glucocorticoids in the maintenance of sympathetically mediated peripheral vascular resistance and in the adequate response of cardiovascular system to stressor exposure (Bencze *et al*., 2020). Glucocorticoids prolong catecholamine stability in the sympathetic synapse, increase the efficacy of catecholamines at adrenergic receptors and influence receptor-G protein coupling and catecholamine-induced cAMP synthesis (Dailey and Westfall, 1978; Haigh and Jones, 1990; Sapolsky *et al*., 2000). Furthermore, corticosteroids play an important role in the control of vascular smooth muscle tone by their permissive effects, potentiating the responses to vasoconstrictors (Ullian, 1999; Yang and Zhang, 2004). Thus, the response of cardiovascular system to the enhanced activation of sympathoneural and

sympathoadrenal systems in SHR might be even intensified by glucocorticoid excess found in this strain. Some studies have indicated that adrenalectomy prevents hypertension development in prehypertensive SHR and decreases BP in SHR with established hypertension. The effect of adrenalectomy was reversed by glucocorticoid but not mineralocorticoid replacement (Ruch *et al.*, 1984; Stern *et al.*, 1983; Yagil *et al.*, 1989).

In addition to abovementioned regulatory mechanisms, catecholamine release as well as the expression of enzymes involved in catecholamine biosynthesis are also regulated by Ang II (Sabban, 1997; Stachowiak *et al.*, 1990). In the Project 3, we observed an attenuated plasma renin activity in adult stress-naive and stressed SHR (renin catalyzes the first step in a RAS cascade by converting angiotensinogen to angiotensin I) which is consistent with the previous reports (Freeman et al., 1975; Herlitz *et al.*, 1982; Sen *et al.*, 1972). Lower plasma levels of aldosterone in SHR (whose production is stimulated by Ang II) observed in our study and by others laboratories (Freeman et al., 1975) also provide an indirect evidence that SHR might have reduced peripheral Ang II levels. It was proposed that the suppression of RAS system in SHR might be a compensatory response against high blood pressure (Shiono and Sokabe, 1976) or a result of high blood pressure acting on kidney, e.g. through increased pressure in the afferent arterioles (Herlitz *et al.*, 1982; Sen *et al.*, 1972; Watanabe *et al.*, 1983). The predominant Ang II receptors in the adrenal medulla are AT_2 subtypes (Jezova *et al.*, 2003) which is in accordance with our mRNA expression data. However, a synergistic effect of AT_1 and AT_2 receptors in the transcriptional regulation of NE synthesis in adrenal medulla was revealed (Jezova *et al.*, 2003). We found a lower mRNA expression of AT_1 receptors in adrenal medulla of stress-naive as well as stressed SHR compared to WKY rats, whereas the mRNA expression of AT_2 receptors was similar in both strains. We observed similar mRNA expression pattern of AT_1 and AT_2 receptors also in adrenal medulla of 4-week-old SHR (data not shown). These findings suggest that the

decreased stimulation of adrenal chromaffin cells by Ang II in SHR might underlie the decreased expression of enzymes involved in catecholamine biosynthesis in this hypertensive strain.

In conclusion, SHR exhibit the exaggerated cardiovascular response to restraint which is accompanied by the excessive activity of sympathetic nervous system and elevated plasma levels of catecholamines compared to normotensive WKY rats. We also observed the over-activation of HPA axis in SHR which can further potentiate the involvement of sympathetic nervous system in cardiovascular stress response. On the other hand, plasma renin activity and aldosterone levels were suppressed in adult SHR. Moreover, SHR subjected to stressor exposure showed more pronounced induction of mRNA expression of *Th* gene in adrenal medulla compared to stressed WKY rats. On the other hand, the expression of other genes involved in catecholamine biosynthesis (*Ddc*, *Dbh*, *Pnmt*) remained lower in adrenal medulla of stressed SHR compared to WKY rats. This finding supports the idea that *Th* is primarily regulated by the activity of sympathetic nervous system, whereas the other enzymes of catecholamine biosynthesis are rather regulated by other mechanisms. The possible cause of the downregulated expression of genes involved in catecholamine biosynthesis in SHR might be the lower stimulation of adrenal chromaffin cells by Ang II.

6. SUMMARY

In my PhD Thesis, I studied the role of sympathoneural and sympathoadrenal systems in the development and maintenance of high blood pressure of the spontaneously hypertensive rats (SHR). We found increased adrenal catecholamine content and increased density of sympathetic innervation in prehypertensive SHR compared to age-matched WKY rats. These changes could be involved in the pathogenesis of high blood pressure. The downregulation of the mRNA and protein expression of genes involved in catecholamine biosynthesis (*Th*, *Ddc*, *Dbh*, *Pnmt*) is probably a compensatory mechanism counteracting the hyperfunction of the sympathoneural system. The suppression of catecholamine biosynthesis develops concurrently with the progression of hypertension in SHR and leads to the lower catecholamine content in the adrenal glands but not in the lower vascular sympathetic innervation of adult SHR. We documented a greater role of sympathetic nervous system in blood pressure maintenance in adult SHR compared to WKY rats. However, chemical sympathectomy by guanethidine is not an effective method for pernament blood pressure lowering in adult SHR. This might be explained by the involvement of compensatory mechanisms in sympathectomized rats, such as the more than tenfold increase in BP sensitivity to catecholamines and the increased plasma levels of adrenaline. Nevertheless, we observed the improvement of some cardiovascular parameters in sympathectomized SHR including reduction of heart rate, attenuated blood pressure response to stress and enhancement of baroreflex sensitivity. Finally, we demonstrated exaggerated cardiovascular response and excessive activation of sympathoneural and sympathoadrenal systems during the acute restraint in adult SHR compared to WKY rats. Furthermore, SHR subjected to restraint showed the overactivation of hypothalamic-pituitary-adrenal axis which might intensify sympathetically mediated rise in peripheral vascular resistance and enhanced cardiovascular response to stressor exposure. In line with sympathetic hyperactivity, we observed a greater elevation of mRNA expression of *Th* gene in the adrenal medulla of stressed

SHR compared to WKY rats. In contrast, the mRNA expression of other genes involved in catecholamine biosynthesis (*Ddc*, *Dbh*, *Pnmt*) remained lower in adrenal medulla of stressed SHR, suggesting the involvement of other mechanisms in the regulation of these enzymes. The possible explanation might be a lower stimulation of adrenal chromaffin cells by angiotensin II in SHR resulting from the attenuated plasma renin activity and the decreased mRNA expression of adrenal angiotensin II receptors observed in this strain.

In conclusion, the data presented in my PhD Thesis confirmed that the sympathetic nervous system contributes to the development and maintenance of high blood pressure in SHR. Its effects on cardiovascular system might be potentiated by the excessive activation of hypothalamic-pituitary-adrenal axis observed in this rat strain. Similar mechanisms are involved in the development and maintenance of high blood pressure in humans. Therefore, the investigation of abovementioned phenomena in SHR can contribute to a better understanding and treatment of human essential hypertension. The resistance of adult SHR to the treatment targeting the peripheral sympathetic nervous system can provide an insight into the compensatory mechanisms which counteract the effective treatment of high blood pressure. Therefore, the drugs affecting central regulation of cardiovascular system (e.g. ACE inhibitors or angiotensin receptor blockers) might be better for the effective lowering of blood pressure in hypertension.

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8. LIST OF PUBLICATIONS

8.1. Publications enclosed in full length

Vavřínová A, Behuliak M, Zicha J. **The importance of the selection of appropriate reference genes for gene expression profiling in adrenal medulla or sympathetic ganglia of spontaneously hypertensive rat.** Physiol Res. 2016; 65(3):401-411. (IF = 1.646)

Vavřínová A, Behuliak M, Bencze M, Vaněčková I, Zicha J. **Which sympathoadrenal abnormalities of adult spontaneously hypertensive rats can be traced to a prehypertensive stage?** Hypertens Res. 2019; 42(7):949-959. (IF=3.217)

Vavřínová A, Behuliak M, Bencze M, Vodička M, Ergang P, Vaněčková I, Zicha J. **Sympathectomy-induced blood pressure reduction in adult normotensive and hypertensive rats is counteracted by enhanced cardiovascular sensitivity to vasoconstrictors.** Hypertens Res. 2019; 42(12):1872-1882. (IF=3.217)

Bencze M, Vavřínová A, Zicha J, Behuliak M. **Pharmacological suppression of endogenous glucocorticoid synthesis attenuated blood pressure and heart rate response to acute restraint in Wistar rats.** Physiol Res. 2020; 69(3): 415-426. (IF = 1.646)

8.2. Other publications

Bencze M, Behuliak M, Vavřínová A, Zicha J. **Broad-range TRP channel inhibitors (2-APB, flufenamic acid, SKF-96365) affect differently contraction of resistance and conduit femoral arteries of rat.** Eur J Pharmacol. 2015; 765:533-540. (IF = 2.730)

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Bencze M, Behuliak M, Vavřínová A, Zicha J. **Altered contractile responses of arteries from spontaneously hypertensive rat: The role of endogenous mediators and membrane depolarization.** Life Sci. 2016; 166:46-53. (IF = 2.685)

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9. ATTACHEMENTS