The Abnormalities of Adrenomedullary Hormonal System in Genetic Hypertension: Their Contribution to Altered Regulation of Blood Pressure

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Summary
It is widely accepted that sympathetic nervous system plays a crucial role in the development of hypertension. On the other hand, the role of adrenal medulla (the adrenomedullary component of the sympathoadrenal system) in the development and maintenance of high blood pressure in man as well as in experimental models of hypertension is still controversial. Spontaneously hypertensive rats (SHR) are the most widely used animal model of human essential hypertension characterized by sympathetic hyperactivity. However, the persistence of moderately elevated blood pressure in SHR subjected to sympathectomy neonatally as well as the resistance of adult SHR to the treatment by sympatholytic drugs suggests that other factors (including enhanced activity of the adrenomedullary hormonal system) are involved in the pathogenesis of hypertension of SHR. This review describes abnormalities in adrenomedullary hormonal system of SHR rats starting with the hyperactivity of brain centers regulating sympathetic outflow, through the exaggerated activation of sympathoadrenal preganglionic neurons, to the local changes in chromaffin cells of adrenal medulla. All the above alterations might contribute to the enhanced release of epinephrine and/or norepinephrine from adrenal medulla. Special attention is paid to the alterations in the expression of genes involved in catecholamine biosynthesis, storage, release, reuptake, degradation and adrenergic receptors in chromaffin cells of SHR. The contribution of the adrenomedullary hormonal system to the development and maintenance of hypertension as well as its importance during stressful conditions is also discussed.

Key words
Adrenal gland • Chromaffin cells • Epinephrine • Blood pressure • Cardiovascular system • Stress

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Introduction
The sympathetic nervous system (SNS) in cooperation with other humoral and local factors is involved in the regulation of arterial blood pressure (BP) through the changes of regional vascular resistance and/or cardiac output. SNS contribution varies under particular circumstances, such as postural changes, physical exercise, stress, etc. It is widely accepted that SNS plays a crucial role in the development of human hypertension (Fisher and Paton 2012) and various forms of experimental hypertension (Mancia and Grassi 2014). On the other hand, the role of adrenal medulla (the adrenomedullary component of the sympathoadrenal system) in the development and maintenance of high blood pressure in man as well as in experimental models is still controversial (Floras 1992, Elam and Grassi 2000). Spontaneously hypertensive rats (SHR) are the mostly used animal model of human essential hypertension (Yagil and Yagil 2001). They develop hypertension...
The functional organization of the adrenomedullary system

In general, the autonomic nervous system (sympathoadrenal and parasympathetic branch) is regulated by a complex central neural network (e.g. nucleus of the solitary tract, NTS; paraventricular nucleus of hypothalamus, PVN; rostral ventrolateral medulla, RVLM; nucleus ambiguous, NA etc.), which control the activity of particular efferent preganglionic neurons innervating either sympathetic postganglionic neurons, adrenal medulla or parasympathetic postganglionic neurons. The sympathetic and parasympathetic postganglionic neurons form synapses with target tissues (e.g. vascular smooth muscle cells, cardiac conduction system, etc.), whereas the chromaffin cells of adrenal medulla release catecholamines into the blood stream thus influencing the distant tissues in the organism. The regulation of sympathoadrenal and parasympathetic nervous system is complex and exerts reciprocal interactions of both systems at the level of PVN, RVLM, preganglionic neurons as well as at the level of postganglionic neurons and target tissues allowing the precise regulation of organ and tissue functions (Ondicova and Mravec 2010). Considering the adrenomedullary system, the evidence indicates that there are two separate populations of chromaffin cells releasing either epinephrine or norepinephrine, which are regulated by distinct neural pathways allowing the differential secretion according to the physiological demands of the organism (Flatmark 2000, de Diego et al. 2008). Thus, there is a high norepinephrine release by adrenal medulla during cold exposure (although the major part of norepinephrine during cold exposure is released by sympathetic nerve endings), whereas high epinephrine release was found during hypoglycemia (Khalil et al. 1986, Vollmer et al. 1992).

Transneuronal retrograde cell-body labeling technique demonstrated that there are at least 5 brain areas directly involved in the regulation of efferent sympathoadrenal preganglionic neurons: caudal raphe nuclei, ventromedial medulla, rostral ventrolateral medulla (RVLM), A5 cell group, and paraventricular nucleus of hypothalamus (PVN) (Strack et al. 1989). RVLM is one of the most important central regions involved in cardiovascular regulation, which integrates the information coming from various peripheral receptors (vestibular receptors, skeletal muscle receptors, nociceptors etc.), nucleus of the solitary tract (mediating...
baroreceptor and chemoreceptor reflexes), PVN (involved in the regulation of body fluids, metabolism and temperature) and higher brain regions. RVLM is also a crucial structure for the baroreflex regulation which determines both sympathoadrenal and sympathoneural outflow (Guyenet 2006, Dampney 2016). Anterograde and retrograde tracing method demonstrated that neurons projecting to sympathoadrenal preganglionic neurons are concentrated in the more rostral part of the rat RVLM and less at the caudal level of the nucleus (Zagon and Smith 1993, Pyner and Coote 1998). In contrast, the neurons projecting to cervical preganglionic neurons are more dispersed from rostral to caudal levels of the RVLM nucleus (Pyner and Coote 1998). However, a double-virus transneuronal labeling technique revealed that some RVLM neurons are involved in the regulation of both sympathoneural and sympathoadrenal outflow, which probably work under certain circumstances where parallel sympathetic activation is desirable, such as during the fight-or-flight response (Jansen et al. 1995). Physiological studies in cats revealed the existence of the RVLM territories the stimulation of which could preferably activate particular sympathetic outflows regulating different functions of the organism, e.g. muscle vasoconstriction, visceral vasoconstriction and kidney function (McAllen and May 1994). However, no functional experiments differentiating RVLM areas involved in the regulation of either sympathoneural or sympathoadrenal outflows in the rat were published so far. In RVLM of SHR, immunohistochemical staining demonstrated increased basal number of Fos-positive immunoreactive neurons compared to WKY rats (Minson et al. 1996, Palmer and Printz 1999). On the other hand, the BP reduction after the administration of NO donor sodium nitroprusside increased a number of Fos-positive immunoreactive RVLM neurons in WKY rats but not in SHR (Minson et al. 1996). Similarly, a psychological stimulus (airpuff startle) caused lower activation of RVLM neurons in SHR than in WKY rats (2-fold or 4-fold increase, respectively) (Palmer and Printz 1999). A faster firing rate was described in RVLM and PVN neurons of SHR compared to WKY rats (Matsuura et al. 2002, Li et al. 2008, Stern et al. 2012) which is in line with the above mentioned increased basal Fos immunoreactivity. Moreover, the hyperpolarization of RVLM and PVN neurons (induced by the microinjection of a viral vector coding for human inward-rectifier Kir2.1-potassium channel) resulted in the reduction of sympathetic outflow and BP decrease in SHR (Gerald et al. 2014, Gerald et al. 2016). Thus, it is clear that the hyperactivity of the brain centers regulating sympathetic outflow contribute to high BP in SHR but a more detailed information concerning the regulation of sympathoneural and sympathoadrenal outflows would be desirable.

The cell bodies of sympathoadrenal preganglionic neurons are located in the intermediolateral cell column between the first and the thirteenth thoracic segments (Zagon et al. 1993, Pyner and Coote 1994, Pyner and Coote 1998, Mueller et al. 2011). Sympathoadrenal preganglionic neurons occupy the lateral aspect of the intermediolateral cell column, whereas preganglionic neurons projecting to superior cervical ganglion and stellate ganglion are located more medially or centrally, respectively (Pyner and Coote 1994). The axons of the sympathoadrenal preganglionic neurons arise from the sympathetic trunk as the splanchnic nerves and form the synapses with chromaffin cells of adrenal medulla (Mueller et al. 2011). It was proposed that there are two distinct populations of sympathoadrenal preganglionic neurons differing in their sensitivity to various stimuli and also in their conduction velocity. Morrison et al. (2000) demonstrated that the first group comprised slowly conducting preganglionic neurons. These neurons were markedly excited during the pseudo-hypoglycemia induced by 2-deoxy-D-glucose, but they exhibited little or no sensitivity to the baroreceptor reflex activation. They probably innervate epinephrine-producing chromaffin cells. By contrast, the second group of sympathoadrenal preganglionic neurons showed rapid conduction velocity similar to barosensitive vasoconstrictor sympathetic preganglionic neurons. These neurons were unaffected by pseudo-hypoglycemia, but they were highly sensitive to baroreceptor reflex activation. They likely regulate norepinephrine release from the adrenal medulla (Morrison et al. 2000). This suggests that norepinephrine-producing chromaffin cells of adrenal medulla might be regulated in concert with sympathetic neurons innervating blood vessels. In the spinal cord of SHR, there is a greater basal incidence of Fos-positive sympathoadrenal preganglionic neurons compared to WKY rats (Minson et al. 1996), which is in accordance with the increased constitutive activity of the splanchnic nerves in this hypertensive strain (Morrison and Whitehorn 1984, Ricksten et al. 1984). Moreover, a greater number of sympathoadrenal preganglionic neurons was activated by nitroprusside administration in SHR (Minson et al. 1996) and the enhanced response to hypothalamic stimulation was described in splanchnic

Adrenomedullary System in Experimental Hypertension 309
nerves of SHR compared to WKY rats (Takeda and Buñag 1978, Morrison and Whitehorn 1984). Furthermore, SHR exhibited more pronounced activation of sympathoadrenal preganglionic fibers to various stimuli, including ganglionic blockade by trimethaphan, mental stress caused by air-jet and pseudo-hypoglycemia induced by 2-deoxy-D-glucose when compared to normotensive WKY rats (Zhang and Thorén 1998). The enhanced activation of preganglionic neurons observed after various stimuli in SHR is not associated with the augmented activation of RVLM neurons (see above) and the contribution of other brain areas remains to be elucidated. However, excitatory stimulation elicited greater adrenal nerve responses in anesthetized spinaly transected SHR compared to WKY rats (Schramm and Chornoboy 1982). Taken together, the hyperexcitability of sympathoadrenal preganglionic neurons in SHR seems to be at least partially independent of the central influence. This abnormality contributes to the greater stress-induced catecholamine release by adrenal medulla in SHR and potentially participates in hypertension development.

The main physiological stimulus for catecholamine secretion from adrenal medulla is acetylcholine (released by preganglionic neurons) which induces depolarization of chromaffin cells and subsequent increase in their intracellular calcium (Burgoyne 1991). Catecholamine release is modulated by substances such as neuropeptide Y, angiotensin II, substance P, cholecystokinin or adrenocorticotropic hormone (Mravec 2005, Guérineau 2020). It was hypothesized that adrenal medulla might play a role as a sensory organ and thus the non-cholinergic stimulation of chromaffin cells might participate in more complex regulation of catecholamine release (Mravec 2005). Catecholamine release evoked by the stimulation of cholinergic receptors as well as the membrane depolarization was described to be augmented in perfused adrenal glands of SHR in comparison to those of WKY rats (Lim et al. 2002, Bomfim et al. 2017). This was caused by both enhanced calcium signaling (de Pascual et al. 2013) and faster exocytosis of more vesicles as well as by greater quantal catecholamine content in hypertensive rats (Miranda-Ferreira et al. 2008). Moreover, the adrenal medulla of SHR contains a greater area of the norepinephrine releasing cell islets. Moreover, the increased number of both norepinephrine granules and vesicles was demonstrated in SHR from the prehypertensive stage to adulthood as compared to WKY rats (Tabei et al. 1988). Thus, the local alterations of catecholamine biosynthesis, storage, release, reuptake and degradation in chromaffin cells might also contribute to the enhanced release of epinephrine and/or norepinephrine from adrenal medulla in hypertensive rats. These aspects will be discussed in detail in the following parts of this review.

Catecholamine biosynthesis

The scheme of catecholamine biosynthesis, storage, release, reuptake and degradation in chromaffin cells is shown in the Figure 1. The catecholamine biosynthesis starts with an import of amino acid L-tyrosine and its hydroxylation by the enzyme tyrosine hydroxylase (TH, encoded by Th gene; Nagatsu et al. 1964). For catecholamine biosynthesis, TH enzyme requires Fe²⁺, molecular oxygen and the regulatory cofactor tetrahydrobiopterin (Nagatsu et al. 1964) which is synthesized by guanosine triphosphate cyclohydro-lase 1 (encoded by Gch1 gene) and recycled by quinoid dihydropteridine reductase (encoded by Qdpr gene; Thöny et al. 2000). Tyrosine hydroxylation is a rate-limiting step of catecholamine synthesis and it is a subject of complex regulation, including direct inhibition of the enzyme by catecholamines, post-translational modifications and changes in the transcription of Th gene (for review see Tekin et al. 2014). 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 the enzyme dopamine β-hydroxylase (DBH, encoded by Dbh gene) to form norepinephrine (Friedman and Kaufman 1965). The last enzyme phenylethanolamine N-methyl transferase (PNMT, encoded by Pnmt gene), which synthesizes epinephrine from norepinephrine, can be predominantly found in chromaffin cells of adrenal medulla, whereas extra-adrenal PNMT is expressed in a small number of epinephrine-producing neurons in the central nervous system and in some non-neuronal cells of the heart. Morphological studies showing the greatest expression of PNMT and/or epinephrine in the peripheral portion of the medulla, closest to the cortex, suggest that glucocorticoids are critical for efficient epinephrine synthesis in the chromaffin cells (Wong et al. 1987, Wong et al. 2003). It was shown that the expression and activity of catecholamine biosynthetic enzymes in chromaffin cells is regulated in a stimulus-specific
manner by several mechanisms including acetylcholine (released from sympathetic innervation), glucocorticoids and Ang II (Livett and Marley 1993, Stachowiak et al. 1990, Wong 2006). Other experiments using a combination of stress with various interventions (including hypophysectomy, preganglionic denervation of sympathetic nerves or the adrenal medulla, treatment with hormones or neural agents, e.g. ACTH, glucocorticoid, acetylcholine etc.) further suggest that the induction of Th gene may be primarily mediated by the neural activity, whereas the regulation of Pnmt gene is dependent mainly on hormonal influences (Axelrod and Reisine 1984, Viskupic et al. 1994).

Fig. 1. The scheme of catecholamine biosynthesis, storage, release and degradation in chromaffin cells of the adrenal medulla. The amino acid L-tyrosine is imported into the cytoplasm of chromaffin cells by L-type amino acid transporter (LAT) and converted to L-3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH). TH requires the regulatory cofactor tetrahydrobiopterin (BH4) which is synthesized by guanosine triphosphate cyclohydrolase (GCH) and recycled by quinoid dihydropteridine reductase (QDPR). L-DOPA is converted by L-DOPA decarboxylase (DDC) to form dopamine (DA). DA is transported to the chromaffin vesicles by the vesicular monoamine transporter (VMAT). The enzyme dopamine β-hydroxylase (DBH) catalyzes DA conversion to norepinephrine (NE). The final step of biosynthesis is performed in the cytoplasm by phenylethanolamine N-methyl transferase (PNMT) which synthesizes epinephrine (EPI) from NE. The content of chromaffin vesicles (NE, EPI, granins, neuropeptide Y (NPY) and ATP) is released from the cells by the process of exocytosis. Monoamine oxidases (MAO) and catechol-O-methyltransferase (COMT) remove catecholamines from the cytoplasm (after vesicular leakage).

Many studies have been published concerning the catecholamine biosynthesis in the adrenal glands of SHR with established hypertension, but their results are quite contradictory (see Table 1). The mRNA and protein expression of Th and Pnmt genes in the adrenal gland of adult SHR was reported to be higher (Kumai et al. 1994, Reja et al. 2002a, Reja et al. 2002b, Nguyen et al. 2009) or lower than in adrenals of WKY rats (Moura et al. 2005, Grundt et al. 2009, Vavřínová et al. 2019a). Moreover, we observed the downregulated adrenal expression of other enzymes involved in catecholamine biosynthesis (Ddc and Dbh) as well as the enzymes producing cofactor tetrahydrobiopterin (Gch1, Qdpr) for TH in adult SHR compared to WKY rats (Vavřínová et al. 2019a). The studies concerning adrenal content of dopamine, norepinephrine and epinephrine also provided rather conflicting results (Lee et al. 1991a, Korner et al. 1993, Moura et al. 2005, Vavřínová et al. 2019a, Vavřínová et al. 2019b). The inconsistent results might be caused by extreme susceptibility of the catecholaminergic system to stressful conditions and the differences in stress response between SHR and WKY rats. It is well known that acute stress elevates plasma levels of catecholamines and induces the
Table 1. The overview of papers evaluating the adrenal mRNA expression, protein expression and the enzyme activity of genes involved in catecholamine biosynthesis in spontaneously hypertensive rats (SHR). Gene symbols referring to mRNA expression are italicized, whereas all letters are in upper-case when describing protein expression or enzyme activity. The information about anesthesia, euthanasia and other important factors can be found in a note section. Symbols ↓, ↑ and = represent lower, higher or similar expression in SHR, respectively, compared to Wistar-Kyoto controls, if not stated otherwise. AG, adrenal gland; AM, adrenal medulla; IP, intraperitoneal; qPCR, quantitative real-time PCR; sqPCR, semiquantitative real-time PCR; w, weeks; WB, western-blot.

| Reference          | Age | Parameter (method) | Tissue | Gene change in SHR | Note                                |
|--------------------|-----|--------------------|--------|--------------------|-------------------------------------|
| Friese et al. 2005 | 4w  | mRNA (chip)        | AG     | Dbh, Pnmt          | ↓ Anesthesia not specified          |
|                    | 2w  | Enzyme activity    | AG     | TH, DBH, PNMT      | ↓ Tail-cuff (timepoint not specified) |
|                    |     |                    |        | TH, DBH, PNMT      | Decapitation                        |
|                    | 8w  |                    |        | TH                 | = Euthanasia not specified          |
|                    |     |                    |        | DBH, PNMT          | = Euthanasia not specified          |
|                    | 14w |                    |        | TH                 | ↑ Euthanasia not specified          |
| Grundt et al. 2009 | 20w | mRNA (qPCR)        | AG     | Th                 | ↓ Stress-naive Decapitation         |
| Grundt et al. 2009 | 20w | mRNA (qPCR)        | AG     | Th                 | = Immediately after tail-cuff       |
|                    |     |                    |        |                    | Decapitation                        |
| Jirout et al. 2010 | 6w  | mRNA (chip)        | AG     | Th, Ddc, Dbh, Pnmt | ↓ Brown-Norway control              |
| Kumai et al. 1994  | 25w | mRNA (Northern blot) |       | Th                 | ↑ Tail-cuff (timepoint not specified) |
|                    |     | Enzyme activity    | AM     |                    | Anesthesia not specified            |
|                    |     |                    |        |                    | Euthanasia not specified            |
| Kumai et al. 2001  | 15w | Protein (WB)       | AM     | Th                 | ↑ Tail-cuff (timepoint not specified) |
|                    |     | Enzyme activity    |        |                    | Pentobarbital anesthesia            |
|                    |     |                    |        |                    | Decapitation                        |
| Moura et al. 2005  | 5w  | Protein (WB)       | AG     | TH                 | ↓ Tail-cuff (timepoint not specified) |
|                    |     | Enzyme activity    |        |                    | Sodium pentobarbital                |
|                    | 12w | Protein (WB)       | AG     | TH                 | ↓ Tail-cuff (timepoint not specified) |
|                    |     | Enzyme activity    |        |                    | Sodium pentobarbital                |
|                    | 22w | Protein (WB)       | AG     | TH                 | ↓ Tail-cuff (timepoint not specified) |
|                    |     | Enzyme activity    |        |                    | Sodium pentobarbital                |
| Nagatsu et al. 1976| 3w  | Enzyme activity    | AG     | DBH                | ↑ Decapitation                      |
| Nagatsu et al. 1977| 16w | Enzyme activity    | AG     | TH, DBH            | ↑ Tail-cuff (timepoint not specified) |
|                    |     |                    |        |                    | Decapitation                        |
| Nguyen et al. 2009 | 16w | mRNA (sqPCR)       | AG     | Dbh                | = Repeated tail-cuff                |
|                    |     | Protein (WB)       |        |                    | Ketamine-xylazine anesthesia        |
|                    |     |                    |        |                    | Decapitation                        |
| O’Connor et al. 1999| 20-24w | Enzyme activity  | AG     | DBH, PNMT         | ↓ Tail-cuff (timepoint not specified) |
|                    |     |                    |        |                    | Anesthesia not specified            |
|                    |     |                    |        |                    | Euthanasia not specified            |
| Reja et al. 2002a  | 18w | mRNA (qPCR)        | AM     | Th                 | ↑ Tail-cuff 2 weeks before sampling |
|                    |     |                    |        |                    | Euthanasia by sodium pentobarbitone IP |
| Reja et al. 2002b  | 20w | mRNA (qPCR)        | AM     | Pnmt               | ↑ Tail-cuff 2 weeks before sampling |
|                    |     |                    |        |                    | Euthanasia by sodium pentobarbitone IP |
expression of catecholamine biosynthetic enzymes in adrenal medulla (Kvetnansky et al. 2004, Kvetnansky et al. 2009). By contrast, the chronic stress induced by social isolation decreases basal expression of *Th* gene (Gavrilovic et al. 2008), but chronically stressed animals exhibit higher plasma epinephrine levels and the augmented induction of adrenal *Th* expression when exposed to a novel stressor (McCarty et al. 1988, Gavrilovic et al. 2008). Adult SHR acutely exposed to immobilization show higher plasma levels of norepinephrine and epinephrine compared to WKY rats (Kvetnansky et al. 1979). Moreover, the increase in adrenal mRNA expression of *Th*, induced by 25 min of mild stress due to tail cuff measurement of blood pressure, was found to be greater in adult SHR compared to WKY rats (Grundt et al. 2009). These findings are in agreement with the above mentioned exaggerated activation of sympathoadrenal preganglionic fibers observed in SHR subjected to physical or psychological stressors (Zhang and Thorén 1998). Actually, several research groups, which reported on the hyperactivation of the adrenomedullary system in SHR, exposed the rats acutely or repeatedly to the stressful procedures such as tail-cuff BP measurement or repeated intraperitoneal administration of drugs (Korner et al. 1993, Kumai et al. 1994, Nguyen et al. 2009). It should be realized that the use of various types of anesthesia (sodium pentobarbital, ketamine-xylazine, isoflurane, etc.) as well as methods of anesthesia (decapitation, cervical dislocation, overdose by anesthetics, etc.) might influence the results, but the details concerning these procedures are often not included in the papers (see notes in Table 1). Besides the differences in the adrenomedullary system, SHR exhibited many other signs of their vulnerability to stress, including higher stress-induced plasma corticosterone levels (Kvetnansky et al. 1979) or ACTH levels (Behuliak and Vavřínová unpublished data). Furthermore, adult stress-naive SHR showed adrenal hypertrophy, increased locomotion (Vavřínová et al. 2019a, Vavřínová et al. 2019b), hyperthermia (Hajós and Endberg 1986) and thymic atrophy (Suzuki et al. 1999), that might be considered as the hallmarks of adaptation to chronic stress (Ulrich-Lai et al. 2006). Brown et al. (1988) demonstrated that intracerebroventricular administration of corticotropin-releasing hormone (CRH) produced a greater increase of plasma epinephrine in SHR suggesting that the central stress pathways might trigger the augmented activation of the adrenomedullary system in this rat strain.

The adrenomedullary system might be prone to the greater activation already in the prehypertensive stage. Similar to adult SHR, catecholamine biosynthetic pathway was described to be downregulated, unchanged or upregulated in adrenal gland of 4-week-old prehypertensive SHR compared to WKY rats (Kvetnansky et al. 1979). Accordingly, the increased cardiac index and heart rate was observed in conscious partially restrained 4-week-old SHR compared to WKY rats (Smith and Hutchins 1979, Behuliak et al. 2015, Vavřínová et al. 2019a). It seems that the central stress pathways are hyperresponsive in prehypertensive SHR because the exaggerated CRH-induced ACTH response and higher corticosterone levels were described in this strain already at the age of 5-6 weeks (Hattori et al. 1986, Hashimoto et al. 1989, Sterley et al. 2011). Indeed, the acute central administration of tranquilizing agent muscimol (GABA type A receptor agonist) reduced sympathoadrenal activity, lumbar sympathetic nerve activity and BP, the effects being more pronounced in

| Vavřínová et al. 2019a | 4w mRNA (qPCR) AM | Dbh, Pnmt ↓ | Isoflurane |
|----------------------|----------------|---------------|-----------|
|                      | Protein (WB) AG | Th, Ddc, Qdpr equal | Exsanguination |
|                      |                | Gch1 ↑ |   |
|                      |                | DDC, DBH ↓ |   |
|                      |                | TH, PNMT |   |
|                      | 24w mRNA (qPCR) AM | Th, Ddc, Dbh, Pnmt, Qdpr, Gch1 ↓ |   |
|                      | Protein (WB) AG | TH, DDC, DBH ↓ |   |
|                      |                | PNMT |   |

| Vavřínová et al. 2019b | 22-24w mRNA (qPCR) AM | Th = | Daily IP injection of saline for 2 weeks |
|------------------------|------------------------|------|----------------------------------------|
|                        |                        | Ddc, Dbh, Pnmt ↓ | Isoflurane |
|                        |                        | Exsanguination |   |
SHR than in WKY rats (Unger et al. 1984, Allen 2002). Moreover, chronic treatment with tranquilizing drug diazepam (positive allosteric modulator of the GABA type A receptors) given from the newborn period markedly reduced BP and vascular resistance in SHR to the levels only slightly above those found in WKY rats (Schieken 1979). Thus, the genetic predisposition of SHR might determine their vulnerability to stress and these rats probably perceive usual care and handling as more stressful. This state of chronic stress can contribute to the above mentioned alterations of the adrenomedullary system in SHR (the decreased basal expression of catecholamine biosynthetic enzymes but the pronounced induction of the expression and the augmented catecholamine release under stress conditions). The alternative explanation is that the downregulation of catecholamine biosynthetic pathway might be a consequence of the high blood pressure, e.g. a compensatory mechanism counteracting the hyperactivity of sympathetic nervous system in SHR. Indeed, SHR with transgenically overexpressed Dbh gene exhibited higher plasma levels of epinephrine and norepinephrine as well as higher blood pressure compared to non-transgenic SHR controls (Pravenec et al. 2016). Guanethidine-induced sympathectomy for 14 days increased the mRNA expression of Th, Dbh and Pnmt genes in adrenal medulla and elevated plasma levels of epinephrine of adult SHR and WKY rats. However, these effects were more pronounced in WKY rats than in SHR, which does not support the idea that the sympathetic hyperactivity is a cause of the downregulation of catecholamine biosynthetic pathway in SHR (Vavřínová et al. 2019b).

Taken together, the available evidence indicates that the catecholamine biosynthetic pathway is downregulated at different levels, i.e. mRNA expression, protein expression and the catecholamine content in the adrenal glands of stress-naive SHR with established hypertension when compared to WKY rats. However, the acute or chronic stressful stimuli cause a more pronounced activation of the adrenomedullary system in SHR, which should be taken into account during the design of experiments and also during the interpretation of results.

Catecholamine vesicles

The speed and the effectiveness of catecholamine release from the chromaffin cells of adrenal medulla depend on the immediate availability of vesicles filled with high content of catecholamines. The filling of catecholaminergic vesicles is mediated by vesicular monoamine transporters (VMAT) of two types (Blakely and Edwards 2012). Colocalization study showed that VMAT1 (encoded by Slc18a1 gene) is widely expressed in all rat adrenal chromaffin cells, while VMAT2 (Slc18a2 gene) is co-localized with TH but not with PNMT enzyme (Tillinger et al. 2010). This suggests that VMAT2 is expressed in norepinephrine- but not in epinephrine-synthesizing chromaffin cells. The mRNA expression of both Vmat1 and Vmat2 was reported to be decreased in adrenal medulla of adult SHR compared to WKY rats (Vavřínová et al. 2019a). The literature concerning expression of Vmat genes (and other genes related to catecholamine vesicles, reuptake or degradation which are discussed hereafter) in adrenal gland of SHR is summarized in Table 2. Our research group reported unchanged mRNA expression of Vmat1 and lower expression of Vmat2 in adrenal gland of prehypertensive SHR (Vavřínová et al. 2019a), whereas Friese et al. (2005) demonstrated the attenuated expression of Vmat1 and the augmented expression of Vmat2. The inconsistent results might be caused either by different methods of measurement (quantitative real-time PCR vs. microarray analysis) or by different sampling of tissue (adrenal medulla vs. whole adrenal gland). It would be desirable to verify the expression of Vmat2 in prehypertensive SHR since the augmented expression of this gene might be a sign of the transition of adrenal chromaffin cells from epinephrine-producing to norepinephrine-producing phenotype.

Catecholamine storage vesicles of the adrenal medulla contain remarkably high concentrations of chromogranins (encoded by Chga and Chgb genes), secretogranin (Scg2 gene), neuropeptide Y (Npy gene) and adenosine triphosphate (ATP). These molecules might influence the amount of catecholamines ready for exocytosis or when released they can mediate feedback regulation of catecholamine release from chromaffin cells (Burnstock 2014). Granins stabilize the vesicle core osmotically and they are involved in the regulation of exocytosis (Zhang et al. 2011). Chga knockout mice had the decreased size and number of chromaffin granules as well as the reduced adrenal content of epinephrine and norepinephrine (Mahapatra et al. 2005). In the adrenal medulla of prehypertensive SHR, the mRNA expression of Chga, Chgb and Scg2 was similar or slightly decreased compared to WKY rats (Friese et al. 2005, Vavřínová et al. 2019a). We reported the attenuated mRNA
expression of $\text{Chga}$, $\text{Chgb}$ and $\text{Scg2}$ genes in the adrenal medulla of adult SHR (Vavřínová et al. 2019a), whereas the increased mRNA and protein expression of $\text{Chga}$ was shown in the adrenal medulla of adult SHR by O’Connor et al. (1999) and Nguyen et al. (2009). Nevertheless, the latter two studies used the repeated tail-cuff measurement of BP, which might influence the results of these studies. Thus, similarly with the genes involved in the catecholamine biosynthesis, the basal expression of granins seems to be downregulated in adrenal medulla of SHR, but the expression might be activated by stressful conditions such as tail-cuff measurement use.

**Table 2.** The overview of papers evaluating the adrenal mRNA expression, protein expression and the enzyme activity of genes related to catecholamine vesicles, reuptake or degradation in spontaneously hypertensive rats (SHR). Gene symbols referring to mRNA expression are italicized, whereas all letters are in upper-case when describing protein expression or enzyme activity. The information about anesthesia, euthanasia and other important factors can be found in a note section. Symbols ↓, ↑ and = represent lower, higher or similar expression in SHR, respectively, compared to Wistar-Kyoto controls, if not stated otherwise. AG, adrenal gland; AM, adrenal medulla; IP, intraperitoneal; NB, northern blot; qPCR, quantitative real-time PCR; RIA, radioimmunoassay; sqPCR, semiquantitative real-time PCR; w, weeks; WB, western-blot.

| Reference | Age | Parameter (method) | Tissue | Gene change in SHR | Note |
|-----------|-----|--------------------|--------|--------------------|------|
| Friese et al. 2005 | 4w | mRNA (chip) | AG | $\text{Vmat1, Scg2, Maob}$ $\text{Chga, Chgb, Maoa, Net}$ $\text{Vmat2, Comt}$ | ↓ = ↑ | Anesthesia not specified Euthanasia not specified |
| Guffroy and Strollin Benedetti 1984 | 14-17w | Enzyme activity | AG | MAOA, MAOB | = | Cervical dislocation |
| Higuchi et al. 1993 | 6w | mRNA (NB) | AG | $\text{Npy}$ | ↓ | Decapitation |
| | 12w | mRNA (NB) | AG | $\text{Npy}$ | ↓ | Decapitation |
| | 17w | mRNA (NB) | AG | $\text{Npy}$ | ↑ | Decapitation |
| Jirout et al. 2010 | 6w | mRNA (chip) | AG | $\text{Npy, Comt}$ $\text{Vmat1, Chga, Chgb, Scg2, Maoa, Maob}$ | ↓ = = | Brown-Norway control Cervical dislocation |
| Nguyen et al. 2009 | 16w | mRNA (sqPCR) | AG | $\text{Chga}$ | ↑ | Repeated tail-cuff Ketamine-xylazine anesthesia Decapitation |
| O’Connor et al. (1999) | 4w | Protein (WB) | AG | CHGA | ↑ | Tail-cuff (timepoint not specified) |
| | 20-24w | mRNA (NB) | AG | $\text{Chga, Chgb}$ | ↑ | Anesthesia not specified |
| | | Protein (WB) | AG | CHGA | ↑ | Euthanasia not specified |
| Reja et al. 2002b | 18w | mRNA (qPCR) | AM | $\text{Net}$ | ↑ | Tail-cuff 2 weeks before sampling Euthanasia by sodium pentobarbitone IP |
| Tsunoda and Imai 2004 | 20-25w | Enzyme activity | AG | COMT | = | Diethyl ether anesthesia |
| Vavřínová et al. 2019a | 4w | mRNA (qPCR) | AM | $\text{Vmat2, Npy}$ $\text{Vmat1, Chga, Chgb, Scg2, Net, Maoa, Maob, Comt}$ $\text{Vmat1, Vmat2, Chga, Chgb, Scg2, Npy, Net, Maoa}$ $\text{Comt}$ $\text{Maob}$ | ↓ = = ↓ = ↑ | Isoflurane Exsanguination |
Catecholamine storage vesicles of the adrenal medulla contain remarkably high concentrations of chromogranins (encoded by Chga and Chgb genes), secretogranin (Scg2 gene), neuropeptide Y (Npy gene) and adenosine triphosphate (ATP). These molecules might influence the amount of catecholamines ready for exocytosis or when released they can mediate feedback regulation of catecholamine release from chromaffin cells (Burnstock 2014). Granins stabilize the vesicle core osmotically and they are involved in the regulation of exocytosis (Zhang et al. 2011). Chga knockout mice had the decreased size and number of chromaffin granules as well as the reduced adrenal content of epinephrine and norepinephrine (Mahapatra et al. 2005). In the adrenal medulla of prehypertensive SHR, the mRNA expression of Chga, Chgb and Scg2 was similar or slightly decreased compared to WKY rats (Friese et al. 2005, Vavřínová et al. 2019a). We reported the attenuated mRNA expression of Chga, Chgb and Scg2 genes in the adrenal medulla of adult SHR (Vavřínová et al. 2019a), whereas the increased mRNA and protein expression of Chga was shown in the adrenal medulla of adult SHR by O’Connor et al. (1999) and Nguyen et al. (2009). Nevertheless, the latter two studies used the repeated tail-cuff measurement of BP, which might influence the results of these studies. Thus, similarly with the genes involved in the catecholamine biosynthesis, the basal expression of granins seems to be downregulated in adrenal medulla of SHR, but the expression might be activated by stressful conditions such as tail-cuff measurement use.

Neuropeptide Y (NPY) is a peptide stored together with the catecholamines in the adrenal medulla which acts as a co-transmitter, a neuromodulator and a neurohormone (Lymeropoulos et al. 2016). NPY administered intravenously caused prolonged BP increase, which was augmented in SHR compared to WKY rats (Miller and Tessel 1991). Westfall et al. (1990) demonstrated that NPY potentiated the vasoconstriction of the mesenteric arterial bed induced by phenylephrine, angiotensin II and arginine vasopressin. This effect was enhanced in SHR compared to WKY rats. On the other hand, NPY decreased norepinephrine release from the mesenteric arterial bed evoked by periartrial nerve stimulation and this NPY action was attenuated in SHR (Westfall et al. 1990). Only few studies reported about the effect of NPY on chromaffin cells and their results are contradictory. NPY co-released due to cholinergic stimulation inhibited the parallel catecholamine secretion from cultured rat chromaffin cells (Shimoda et al. 1993). By contrast, Cavadas et al. (2006) showed that NPY increased catecholamine release from the cultured mouse chromaffin cells, but the constitutive catecholamine release from adrenal medulla was elevated in NPY Y1 receptor knockout mice. Furthermore, it was reported that stress-induced increase of catecholamine release is prevented in NPY knockout mice (Wang et al. 2013). Apart from the effects on the catecholamine release NPY is also involved in the regulation of catecholamine biosynthesis. Hong et al. (1995) showed that acute intravenous NPY administration increased mRNA expression of Th gene in rat adrenal medulla. On the other hand, NPY Y1 receptor knockout mice exhibited higher TH activity in the adrenal glands and the incubation with NPY decreased Th promoter activity in Y1 receptor expressing cells (Cavadas et al. 2006). In NPY knockout mice, basal TH immunoreactivity was increased in adrenals compared to wild-type animals suggesting that NPY exerts tonic inhibitory action on Th expression (Wang et al. 2013). In contrast, NPY knockout mice exhibited smaller stress-induced increase in the adrenal TH immunoreactivity than wild-type mice (Wang et al. 2013). Thus, the regulation of adrenal medulla by NPY is very complex and further investigation would be desirable. The decreased mRNA expression of Npy gene was found in adrenal gland of young as well as adult SHR (Higuchi et al. 1993, Vavřínová et al. 2019a). The contribution of lower NPY expression to the observed alterations in the adrenomedullary system in SHR remains to be elucidated.

**Catecholamine reuptake and degradation**

Catecholamine uptake at the neuroeffector junction is an important mechanism for the regulation of the synaptic norepinephrine concentrations. The catecholamine reuptake by norepinephrine transporter (NET, encoded by Slc6a2 gene) was enhanced in the blood vessels of SHR where it possibly compensates greater norepinephrine release from the sympathetic nerve endings (Whall et al. 1980, Hano and Rho 1989). On the other hand, the adrenal medulla is an endocrine organ and thus there is no reason for reuptake of released epinephrine and norepinephrine. Accordingly, the drugs using NET to enter the target cell (e.g. tyramine, 6-hydroxydopamine or guanethidine) work in the sympathetic
nerve endings but they do not influence the adrenal medulla (Thoenen and Tranzer 1973, Johnson and O’Brien 1976, Wakade and Wakade 1984). However, NET was surprisingly present in epinephrine- but not in norepinephrine-producing cells of rat adrenal medulla (Phillips et al. 2001). Moreover, NET was shown to be localized primarily in the cytoplasm rather than in the cell membrane (Kippenberger et al. 1999, Phillips et al. 2001). Reja et al. (2002b) reported higher mRNA expression of Net in the adrenal medulla of adult SHR compared to WKY rats. By contrast, we observed lower mRNA expression of Net in the adrenal medulla of adult SHR, but similar Net expression in 4-week-old SHR and WKY rats (Vavřínová et al. 2019a). However, the role of NET in rat adrenal chromaffin cells was not explained yet. Thus, it is not clear whether the altered mRNA expression of Net in adrenals of adult SHR might have any physiological effect.

Catecholamines are degraded by the enzymes monoamine oxidases (MAO, encoded by Maoa and Maob genes) or catechol-O-methyltransferase (COMT, encoded by Comt gene). Epinephrine is converted by COMT to more stable metabolite. Norepinephrine can be converted by both MAO and COMT and thus several metabolites can be produced, e.g. 3,4-dihydroxymandelic acid, normetanephrine, vanillylmandelic acid etc. Sympathetic nerves contain only MAO, while adrenal medulla and other non-neural tissues contain both enzymes – MAO and COMT (Eisenhofer et al. 2004a). It was reported that adrenal medulla is a source of about 90% of circulating metanephrine and 30% of normetanephrine. However, this is rather a consequence of intracellular catecholamine metabolism following the leakage of norepinephrine and epinephrine from the chromaffin storage granules to the cytoplasm (MAO and COMT protecting the chromaffin cells from catecholamine toxicity) than the clearance of catecholamines from the extracellular space (Eisenhofer et al. 1995a, b). This process of catecholamine leakage was proposed to be an important mechanism for “gearing down” the requirement for necessary increases in catecholamine biosynthesis under the stress conditions, which provides a capacity for a more extended range of sustainable rates of catecholamine release (Eisenhofer et al. 2004b).

The decreased catecholamine degradation was observed in both neural and in non-neural tissues of SHR (Masuda et al. 2006, Tsunoda and Imai 2004). In the adrenal gland of prehypertensive SHR, microarray analysis revealed more than 30-fold higher mRNA expression of Comt but lower expression of Maob gene compared to WKY rats (Friese et al. 2005). However, these strain differences were not confirmed by our recent study (Vavřínová et al. 2019a). In the adrenal medulla of adult SHR, slightly downregulated mRNA expression of Maoa, upregulated expression of Maob but unchanged expression of Comt was described (Vavřínová et al. 2019a). The activities of MAOA, MAOB and COMT were reported to be similar in the adrenals of adult SHR and WKY rats (Guffroy and Strollin Benedetti 1984, Tsunoda and Imai 2004). Taken together, the influence of catecholamine reuptake and degradation on the function of the adrenomedullary system is still not well understood. The studies concerning catecholamine reuptake and degradation are scarce and provide inconsistent results.

**Adrenergic receptors**

Adrenergic receptors are involved in the feedback regulation of catecholamine release. The activation of α2-adrenergic receptors inhibits catecholamine release from the sympathetic terminals, neurons in the brainstem as well as from the adrenal medulla (Brede et al. 2003, Gilsbach et al. 2009, Urban et al. 1995), thus exhibiting a hypotensive effect. As the adrenal medulla is an endocrine organ, the physiological role of α2-adrenergic receptors-mediated negative feedback in chromaffin cells is still questionable. However, PC12 rat pheochromocytoma cell line, which does not express α2-adrenergic receptors, secretes abnormal catecholamine quantities (Taraviras et al. 2002). The specific subtypes of α2-adrenergic receptors prevailing in the adrenal glands are still unknown and it seems there could be differences between species. In adrenal gland of the rat, mRNA expression of α2A, α2B and α2C was detected (Moura et al. 2011, Behuliak and Vavřínová unpublished results). All three types were expressed similarly in the adrenal medulla of young as well as of adult SHR and WKY rats and the α2A-mediated inhibition of catecholamine release was comparable in normotensive and hypertensive rats (Moura et al. 2011). By contrast, Reja et al. (2002a) reported lower mRNA expression of α2A-adrenergic receptor subtype in adrenal medulla of adult SHR than in WKY rats. Furthermore, Friese et al. (2005) described decreased mRNA expression of α2C- and unchanged expression of α2A- and α2B-adrenergic receptor subtypes in the adrenal gland of
prehypertensive SHR. However, the comparison of mRNA expression results from various laboratories is quite complicated by the use of different conditions of quantitative real-time polymerase chain reaction and different reference genes for data normalization, which might influence the results (Vavřínová et al. 2016).

β-adrenergic receptors facilitate norepinephrine release from the sympathetic terminals (Guimarães and Moura 2001). The stimulation of β-adrenergic receptors increases catecholamine release from bovine chromaffin cells (Parramón et al. 1995). β1-adrenergic receptors mediated the slow potentiation of Ca2+ currents into the rat chromaffin cells. By contrast, β2-adrenergic receptors caused the fast inhibition of Ca2+ currents in the rat adrenal medulla (Cesetti et al. 2003). Both mechanisms might be involved in the autocrine regulation of chromaffin cells, but their impact on catecholamine release has not been studied up to now. In rats with heart failure, the chronic treatment with β1-adrenergic receptor blocker bisoprolol reduced catecholamine overproduction in the adrenal medulla (Rengo et al. 2012). Lower mRNA expression of β2- but unchanged expression of β1- and β2-adrenergic receptor subtypes was reported in adrenal gland of young SHR (Fries et al. 2005). However, no physiological study determining the function of β-adrenergic receptors in the adrenal medulla of SHR has been published so far. Taken together, the study on the role of adrenal adrenergic receptors in the regulation of catecholamine release from chromaffin cells of SHR would be desirable since these receptors might alter calcium signaling and contribute to the enhancement of catecholamine release in SHR subjected to physical or psychological stressors.

Adrenal demedullation

There is clear evidence that the adrenomedullary system participates in the development of high blood pressure in SHR, but it is not a crucial cause of hypertension in this model. Adrenal demedullation performed in 4-week-old prehypertensive SHR attenuated but did not prevent the development of high BP in these rats (Borkowski and Quinn 1983, Borkowski 1991), the effect being reversed by epinephrine supplementation (Borkowski 1991). Chronic as well as acute adrenal demedullation decreased BP responses to the electrical stimulation in pithed rats, whereas it did not affect vascular smooth muscle contractility to phenylephrine (Borkowski and Quinn 1983). This suggests that epinephrine might potentiate norepinephrine release from the sympathetic nerve terminals. Indeed, a combination of sympathectomy with adrenal demedullation (Lee et al. 1991a, Lee et al. 1991b) or with α1-adrenergic blockade (Korner et al. 1993) completely prevented the development of high blood pressure in SHR. Moreover, epinephrine produced by the adrenal medulla might be involved in vascular hypertrophy and remodeling in SHR (Lee et al. 1991a, Lee et al. 1991b, Korner et al. 1993). These early structural and functional changes of cardiovascular system induced by pronounced activation of the adrenomedullary system (in cooperation with sympathoneural system) in immature SHR are probably irreversible because the effects of adrenal demedullation became insignificant since the age of 7 weeks (Borkowski 1991). Thus it seems that the adrenomedullary system does not actively contribute to the maintenance of high blood pressure in adult SHR. This is also in line with the age-dependent downregulation of catecholamine biosynthetic pathway described in the adrenal medulla of SHR with established hypertension (Vavřínová et al. 2019a). However, the adrenomedullary system might substitute the function of the suppressed sympathoneural system in both young and adult rats. The increased adrenal catecholamine content and the elevated plasma levels of epinephrine were demonstrated in rats treated neonatally or in adulthood with the sympatholytic drug guanethidine (Korner et al. 1993, Tipton et al. 1984, Vavřínová et al. 2019b). The activation of the adrenomedullary system in sympathectomized rats is probably mediated centrally because peripheral sympathectomy by 6-hydroxydopamine increased Fos immunoreactivity in many brain nuclei, including PVN (Callahan et al. 1998). Thereby, the activation of the adrenomedullary system might oppose the blood pressure lowering effects of the treatment targeting peripheral sympathetic system in SHR, being one of the reasons for the resistance of adult SHR to the treatment of hypertension.

Conclusions

Spontaneously hypertensive rats (SHR) exhibit numerous abnormalities in the adrenomedullary system from the hyperactivity of brain centers regulating sympathetic outflow, through the exaggerated activation of sympathoadrenal preganglionic neurons, the altered morphology of adrenal medulla, up to the local changes in catecholamine biosynthesis, storage and degradation in...
chromaffin cells. Although it is quite difficult to highlight one particular abnormality that would be responsible for the development and maintenance of high blood pressure in SHR, the present evidence suggests that this hypertensive strain is highly prone to various stressful stimuli. This is apparent already from the prehypertensive stage and hence the repeated excessive activation of the adrenomedullary system and the enhanced catecholamine release can promote other pathological changes observed in SHR, including the potentiation of norepinephrine release from the sympathetic nerve terminals or the structural changes of vascular arteries. Indeed, hypertension development is attenuated in animals subjected to adrenal demedullation before the age of 7 weeks and the combination of demedullation with neonatal sympathectomy normalized blood pressure of SHR to the level of normotensive controls. The deleterious effects of the stress are widely accepted and therefore SHR could be a suitable model for studying the connection between the stress susceptibility and the development of cardiovascular diseases. On the other hand, the contribution of the adrenomedullary system to the maintenance of high blood pressure in adult SHR seems to be minimal. Actually, the expression of genes involved in catecholamine biosynthesis and genes related to catecholamine vesicles is downregulated in adult stress-naïve SHR, but their adrenomedullary system is still more responsive to stressful conditions. Moreover, the activation of the adrenomedullary system is one of the mechanisms opposing the blood pressure lowering effects of treatment targeting peripheral sympathetic system in SHR. This finding points out to the interconnection of particular systems involved in the regulation of blood pressure and their mutual substitution. It also suggests that the treatments targeting central regulation of blood pressure might be effective for the lowering of blood pressure in hypertension in general.

**Conflict of Interest**

There is no conflict of interest.

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