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Spectral heart rate variability and selected biochemical markers for autonomic activity in rats under pentobarbital anesthesia

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ABSTRACT

Introduction: Autonomic nervous system (ANS) function can be evaluated by analysis of heart rate variability (HRV) and plasma concentration of noradrenaline (NA). Recent studies identified potential, biochemical markers of the ANS activity, including selected co-transmitters, released from either sympathetic (e.g. neuropeptide Y – NPY) or parasympathetic (e.g. vasoactive intestinal peptide – VIP) fibers.

Aim: The aim of the study was to analyze HRV recordings and to determine plasma level of NA, NPY and VIP in 3–12 months males and females rats under pentobarbital anesthesia. Moreover, our goal was to determine overall and gender-dependent correlation between the HRV indices and abovementioned compounds.

Material and methods: The experiment included 36 rats with different autonomic tone related to age and gender. Spectral HRV analysis was applied and NA, NPY and VIP were measured by ELISA.

Results and discussion: Male rats were characterized by significantly higher values of selected HRV indices: total power (TP), very low frequency (VLF) and high frequency (HF) and plasma concentrations of both analyzed neuropeptides comparing to female ones. Similar to the overall assessment, both males and females showed significant correlations between NA and TP, VLF and LF. Moreover, male rats (but not female ones) presented with significant moderate correlations between NPY and LF, VIP and TP, HF and normalized HF (nHF).

Conclusions: Our preliminary findings imply that NA correlates with global autonomic activity (TP) and with the values of sympathetically-driven components (VLF and LF). Furthermore, VIP seems to correlate with specific measures of parasympathetic drive (HF and nHF), but only in male rats.

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1. Introduction

Autonomic nervous system (ANS) is responsible for rapid and appropriate adaptation to constantly changing extrinsic and intrinsic conditions, and along with the endocrine system plays a role in maintaining body homeostasis. Normal autonomic function, underlying rapid compensation and maintenance of homeostasis, may be disrupted during the course of many diseases; this is expressed clinically as autonomic neuropathy, manifesting as pleotropic, sensory and motor symptoms from various organs.[1,2] A model condition for vegetative neuropathy is diabetes mellitus; the severity of diabetic neuropathy is an important marker of the disease progression.[2] Therefore, there is a need for reliable and accurate methods for functional assessment of the ANS, that would be suitable for clinical use. Unfortunately, the availability of such methods is currently fairly limited. While there are some direct methods for autonomic activity determination (e.g. autonomic fiber potential recording with an aid of electrophysiology techniques), most available tests are based on indirect assessment of the ANS-controlled processes. These methods were a subject of many review papers,[3,4] including one our publication.[5] The so-called ANS tests based on the analysis of cardiovascular reflexes in response to controlled stimulation (e.g. orthostatic stress, prolonged exercise, enhancement of respiratory sinus arrhythmia) constitute a group of commonly used methods for the assessment of autonomic function, available since 1980s. Comparison of an abnormal response observed in a given patient with an expected normal reflex is a simple way to detect a vegetative neuropathy. The exact methodology of the ANS tests (especially, those included in the so-called conventional Ewing test battery) was already presented in many review articles.[3,4] A complementary gold standard of the autonomic function assessment is still the analysis of heart rate variability (HRV), based on the measurement of cyclic modulation of sinus rhythm 'R-R' intervals by the ANS-controlled mechanisms.[6] ECG recording and subsequent spectral analysis of HRV enable one to study sympathetic and parasympathetic influences on the ECG curve, associated with the phenomena occurring in the ANS at variable frequencies. This constitutes the basis for identification of the following three main components: high frequency (HF) component (0.15–0.50 Hz), associated mainly with parasympathetic modulation of heart rate in a respiration-dependent manner, low frequency (LF) component (0.04–0.15 Hz), reflecting the influences of both ANS arms on heart rate, related to short-term baroreceptor-mediated regulation of blood pressure, and very low frequency (VLF) component (0.003–0.040 Hz), associated with various, still not fully understood, physiological processes – cyclic activity of the renin–angiotensin–aldosterone (RAA) system, autonomic thermoregulatory mechanisms.[7,6] Another analyzed parameter is total power (TP) of the HRV spectrum, i.e. overall distribution of R–R intervals depending on modulation of the sinus node activity by the three abovementioned principal components of heart rate. In view of the controversies around the interpretation of VLF component etiology, an additional step of the HRV analysis is normalization of the spectrum. Resultant secondary spectrum is deprived of VLF component, and enables one to calculate two normalized parameters, normalized (nLF) and normalized (nHF), which in line with the available guidelines[6] reflect pure sympathetic and pure parasympathetic activity, respectively. Analyzing the HRV indices determined at rest and comparing them to those recorded during the Ewing tests, one can conclude on sympathetic and parasympathetic activity in a given patient.

Also some biochemical parameters of the blood plasma were proposed as laboratory surrogate markers for the ANS activity. To this date, the only widely accepted biochemical surrogate marker for sympathetic activity is plasma concentration of noradrenaline (NA).[8,9] However, even interpretation of this marker raises many controversies, as shown in our review paper presenting currently available methods for the ANS assessment.[5] Therefore, other compounds which are functionally related to the ANS activity and can be determined in plasma, e.g. autonomic neurotransmitters, are a subject of extensive research. A pool of compounds that are released to the synaptic spaces of the autonomic fibers and undergo complex clearance mechanisms (reuptake, enzymatic cleavage) may eventually reach peripheral circulation. This refers particularly to neuropeptide co-transmitters, such as neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP). Both of them are released together with principal sympathetic and parasympathetic neurotransmitters, NA and acetylcholine, respectively. However, their half-lives are longer which allows for their determination in the blood plasma.[10] Therefore, plasma levels of NPY and VIP are likely to reflect sympathetic and parasympathetic activity, respectively. However, to confirm this hypothesis, one would need to find a relationship between NPY or VIP and HRV-based measures of sympathetic and parasympathetic activity.

2. Aim

The aim of our study was to estimate resting, short-lasting HRV recordings and plasma level of NA, NPY and VIP in heterogeneous in terms of age (3–12 months) and gender (male and female) group of rats subjected to general anesthesia evoked by pentobarbital.

Consequently, our main goal was to analyze correlations between the spectral HRV indices of global autonomic activity (TP), sympathetic (LF, nLF) and parasympathetic activity (HF, nHF), neuropeptides (NPY, VIP) and NA in healthy rats under pentobarbital anesthesia. Finding a relationship between the HRV indices and biochemical markers of autonomic function would expand the available options of the ANS assessment onto laboratory parameters.

3. Material and methods

3.1. *Ethical issues*

The protocol of this study was approved by the First Local Ethics Committee for Animal Experiments in Krakow. All procedures were performed in accordance with European Union legislation (2010/63/EU) and Polish regulations on the

Table 1 – Body weight and resting heart rate in study rats.

Lifespan of the study rats, months		Body weight, g	Heart rate, bpm
3	Male	282.86 ± 12.00	359.27 ± 15.47
	Female	207.00 ± 25.98	331.90 ± 9.13
	Total	259.70 ± 39.78	354.27 ± 22.92
4	Male	359.40 ± 38.27	376.46 ± 31.95
	Female	191.80 ± 16.80	360.05 ± 21.51
	Total	283.67 ± 94.44	369.17 ± 27.54
6	Male	450.83 ± 59.65	343.28 ± 12.33
	Female	248.80 ± 16.27	359.48 ± 13.35
	Total	373.10 ± 116.06	346.84 ± 15.12
8	Male	460.33 ± 52.00	359.87 ± 19.81
	Female	243.14 ± 10.43	352.66 ± 21.58
	Total	386.60 ± 114.28	358.19 ± 19.53
10	Male	479.22 ± 38.56	360.99 ± 19.72
	Female	287.25 ± 11.41	351.58 ± 22.63
	Total	393.33 ± 101.16	357.11 ± 20.91
12	Male	516.00 ± 26.89	342.85 ± 17.76
	Female	276.43 ± 21.13	346.03 ± 20.64
	Total	426.77 ± 119.78	344.13 ± 17.87

protection of animals used for scientific or educational purposes.

3.2. *Experimental design*

The study included a group of 3–12-month-old albino Wistar rats from the central animal house at the Pharmaceutical Faculty, Jagiellonian University Medical College. A total of 36 adult rats (equal proportion of males and females) were examined, among them 3-, 4-, 6-, 8-, 10- and 12-month-old animals (3 males and 3 females per each age group). The body weight and the resting heart rate of the study animals are given in Table 1.

Before reaching the appropriate age, the rats were kept at the central animal house. Subsequently, they were transferred to a local animal house at the Department of Pathophysiology and subjected to a 10-day quarantine to let them adapt to the new housing conditions. During this period, the rats were housed under a 12/12 h light to darkness cycle, 3 individuals of the same sex per standard cage, with unlimited access to food (Labofeed, Kcynia, Poland) and water. ECG recordings were obtained in the morning, under general anesthesia with intraperitoneal pentobarbital sodium (55 mg/kg b.w.) (Morbital, Biowet, Puławy, Poland). Pentobarbital sodium is often used anesthetic agent for producing anesthesia in animal research into autonomic control of the cardiovascular system due to the fact that cardiovascular and autonomic effects of this agent have already been described[11–14] (see below in 'Study limitation'). The applied pentobarbital dose resulted from our previous experience and performing other experiments requiring the development of the general anesthesia in study rats. The applied pentobarbital dose also meets the literature guidelines,[15] which recommend use of 30–60 mg/kg b.w. to induce the general anesthesia in rats.

The recording was preceded by a removal of abdominal fur and application of a standard ECG gel (Żelpol, Centrum Medicum, Łódź, Poland). 20-min ECG recordings were obtained with disposable Ag/AgCl pediatric electrodes (EK-S30 Sorimex PSG, Poland) and PowerLab 4/30 system (AD Instruments, Australia). During the recording, the animals were kept under a heating lamp to avoid hypothermia. The first 5-min segment of each recording was used to calibrate the ECG signal, and then proper 15-min recordings were obtained and subjected to spectral analysis of HRV with AD Instruments software (Chart v 5.4.2 for Mac OS X Version 10.1.2.). According to the general HRV guidelines,[6] short-term ECG recording should be subjected to the spectral (frequency) domain analysis. Time-domain analysis is recommended mostly for long-term (e.g. 24-h) ECG recordings. Therefore we subjected obtained ECG recordings to the spectral HRV analysis. The analysis included routinely determined spectral parameters, i.e. TP (ms^2), VLF (ms^2), LF (ms^2) and HF (ms^2) components. The spectrum bands for each component ($0.18 < VLF < 0.28 < LF < 0.78 < HF < 3$) were consistent with those used in previous studies of HRV in rats (Aubert et al.:[16] $0.19 < LF < 0.74 < HF < 2.5$ and Goncalves et al.:[17] $0.10 < LF < 1.0 < HF < 3.0$). Moreover, the secondary parameters, nLF [n.u.] and nHF [n.u.], were calculated to normalize the primary HRV spectra.

After the recordings were complete, another dose of anesthetic (Morbital, 200 mg/kg b.w. intraperitoneally) was administered to each animal, and blood samples were collected from the heart upon thoracotomy in under deep general anesthesia; however, before the detection of cessation of the vital functions. The finally applied dose also meets the criteria published in the guidelines which recommend the use of pentobarbital in an amount greater than 120 mg/kg b.w. in order to make euthanasia in rats.[15]

3.3. *Biochemical assay*

Immediately after collecting the blood and its centrifugation, plasma was frozen and stored until biochemical analysis. The samples were examined immunoenzymatically (ELISA) for NA, NPY and VIP concentrations with an aid of commercially available ELISA kits for NA (LDN; Labor Diagnostica Nord, GmbH & Co. KG; Nordhorn, Germany) NPY and VIP (EIA Kit CE Mark Certified, Phoenix Pharmaceuticals, USA) determination, in line with the manufacturers' instructions. The results were expressed in ng per mL (ng/mL).

3.4. *Statistical analysis*

HRV indices and biochemical parameters were analyzed separately in males and females and the significance of gender-specific differences in HRV indices was verified with the parametric Student's *t*-test; the intergroup differences were considered significant at $P \leq 0.05$.

In the main part of the experiment, we analyzed correlations between the following pairs of biochemical parameters and HRV indices:

NA–TP, NA–VLF, NA–LF, NA–nLF
NPY–TP, NPY–VLF, NPY–LF, NPY–nLF
VIP–TP, VIP–VLF, VIP–HF, VIP–nHF

Pearson coefficients of correlation (r) were determined for the whole study group (an overall assessment – without

gender differentiation) and separately for males and females. The results were interpreted as follows:

|r| less than 0.3 – weak correlation,
|r| between 0.3 and 0.5 – moderate correlation,
|r| between 0.5 and 0.7 – strong correlation,
|r| between 0.7 and 0.9 – very strong correlation,
|r| more than 0.9 – nearly ideal correlation between a HRV measure and a biochemical parameter.

4. Results

4.1. *Spectral analysis of HRV*

Male rats presented with significantly higher values of TP, VLF and HF components than females. There was an apparent, albeit insignificant, tendency to different LF values in males and females. The two groups did not differ significantly in terms of their normalized HRV parameters, nLF and nHF.

Detailed descriptive statistics for all analyzed HRV indices, as well as the results of statistical analysis comparing the values of these parameters in males and females are presented in Table 2.

4.2. *Biochemical parameters*

Similar to HRV indices, male rats presented with significantly higher plasma concentrations of both analyzed neuropeptides than females. Furthermore, male rats showed apparently higher plasma concentrations of NA but this difference did not prove to be significant on statistical analysis.

Detailed descriptive statistics for all analyzed biochemical parameters, as well as the results of statistical analysis comparing their values in males and females are presented in Table 3.

Table 2 – Comparison of the spectral HRV analysis in study rats.

	Male rats	Female rats	*P* value
TP, ms^2	58.41 ± 29.88	39.61 ± 28.43	0.01
VLF, ms^2	36.17 ± 24.72	25.20 ± 21.36	0.03
LF, ms^2	8.43 ± 6.25	6.45 ± 5.75	NS
HF, ms^2	16.79 ± 15.92	11.11 ± 8.68	0.02
nLF, n.u.	45.91 ± 17.41	49.98 ± 13.96	NS
nHF, n.u.	54.09 ± 17.41	50.02 ± 13.96	NS

NS, non-significant.

Table 3 – Comparison of the calculated concentrations (ng/mL) of the estimated biochemical markers in study rats.

	Male rats	Female rats	*P* value
NA	10.29 ± 6.77	8.42 ± 7.51	NS
NPY	0.07 ± 0.06	0.04 ± 0.02	0.04
VIP	0.64 ± 0.47	0.31 ± 0.17	0.001

NS, non-significant.

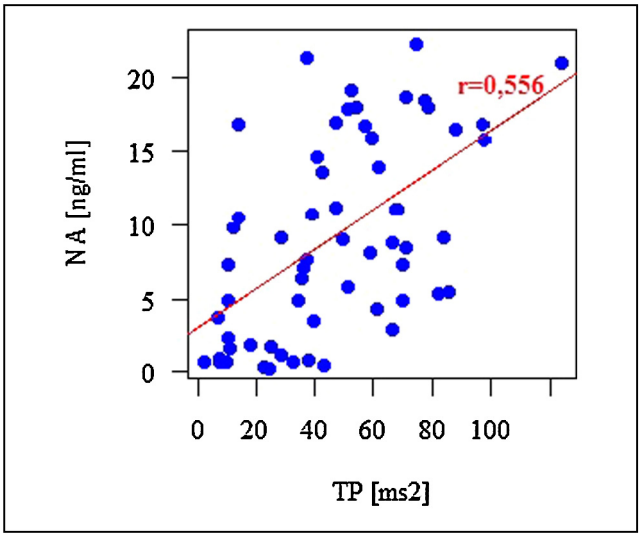

Fig. 1 – Correlation between HRV total power (ms^2) and NA concentration (ng/mL) in the overall assessment (both males and females).

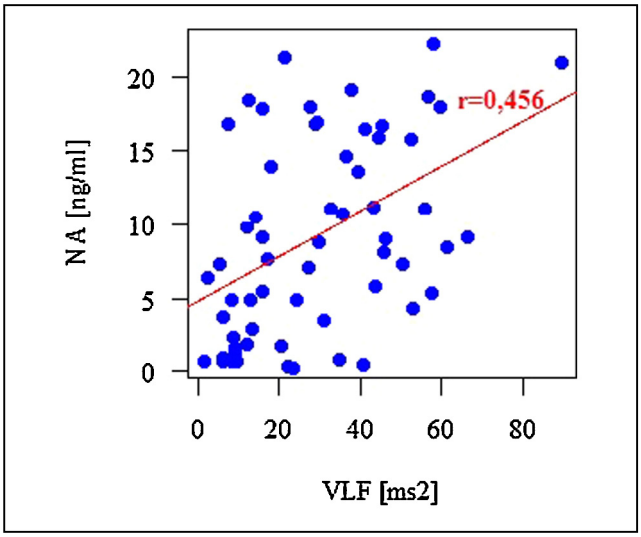

Fig. 2 – Correlation between HRV power of the VLF component (ms^2) and NA concentration (ng/mL) in the overall assessment (both males and females).

4.3. *Correlations between HRV indices and biochemical parameters*

In the overall assessment, a significant strong positive correlation between NA concentration and TP ($r = 0.556$) was found for the whole study group, along with a moderate positive correlations between LF and NPY ($r = 0.408$), VLF and NA ($r = 0.456$), LF and NA ($r = 0.480$). These relationships are depicted in Figs. 1–4. No significant correlations were observed for the remaining pairs of HRV indices and biochemical parameters.

Similar to the whole study group, also both males and females showed significant correlations between NA concen-

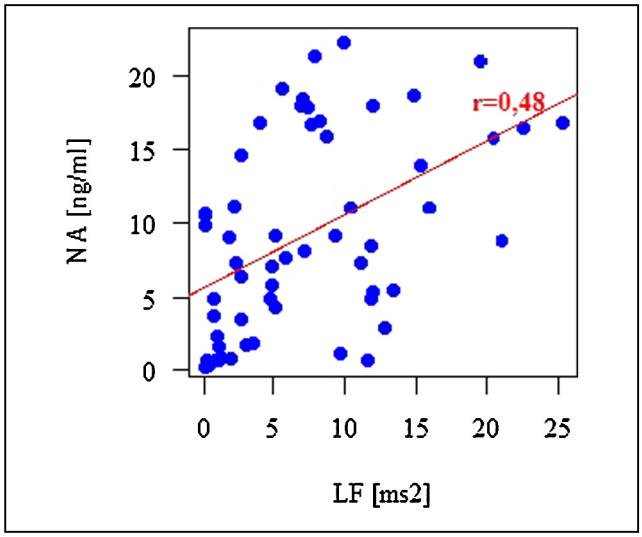

Fig. 3 – Correlation between HRV power of the LF component (ms^2) and NA concentration (ng/mL) in the overall assessment (both males and females).

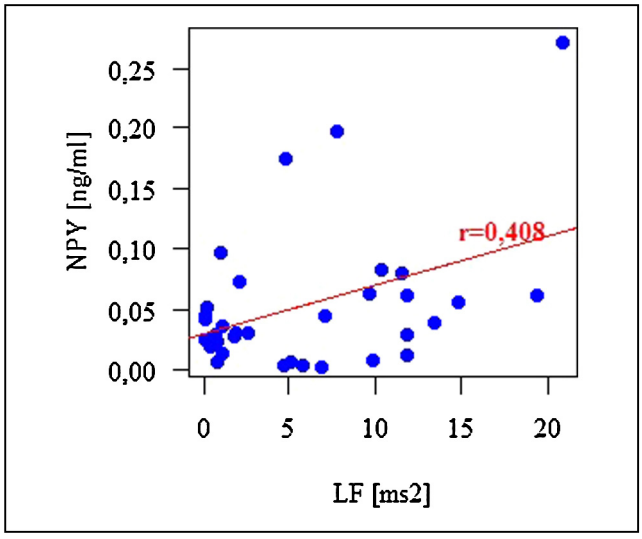

Fig. 4 – Correlation between HRV power of the LF component (ms^2) and NPY concentration (ng/mL) in the overall assessment (both males and females).

tration and TP, VLF and LF values. Moreover, male rats presented with significant moderate correlations between NPY and LF, VIP and TP, HF and nHF. Interestingly, similar associations were not found in females.

Detailed results of correlation analysis, separately for males and females, are presented in Table 4.

Table 4 – Correlations (Pearson *r* value) between selected spectral HRV components and biochemical markers calculated in male and female rats.

Biochemical marker	HRV component	Male rats	Female rats
NA	TP	0.605	0.614
	VLF	0.442	0.580
	LF	0.529	0.430
	nLF	NS	NS
NPY	TP	NS	NS
	VLF	NS	NS
	LF	0.358	NS
	nLF	NS	NS
VIP	TP	0.311	NS
	VLF	NS	NS
	HF	0.415	NS
	nHF	0.382	NS

NS, non-significant.

5. Discussion

5.1. *General reasoning*

Our main findings include positive correlations between plasma NA concentrations, total power of the HRV spectrum and its two components, VLF and LF. These relationships were observed both in the whole study group and separately in males and females. Moreover, we found a significant positive correlation between NPY concentrations and LF HRV component values in male rats. Furthermore, plasma concentrations of another polypeptide, VIP, correlated positively with TP of the HRV spectrum, and – conversely to NA – with its high frequency component, also after the normalization thereof (nHF). In contrast, no significant correlations were found between concentrations of the analyzed neuropeptides and HRV indices in female rats. It should be underlined that the abovementioned relationships were revealed in a heterogeneous in terms of age and gender group of anesthetized animals. The selection of age-differentiated group was associated with the results of our previous experiment, focused on the evaluation of the changes in autonomic activity in the following months of life in study rats.[18] Analyzing spectral HRV parameters and plasma NA level, we revealed that rat ANS aging was associated with global autonomic activity decrease, although without selective impairment of individual (sympathetic/parasympathetic) ANS component. Moreover, in the overall assessment, plasma NA level as well as both TP and all non-normalized HRV components demonstrated a tendency for reduction when compared the first (2nd) and last (12th) months. In the case of nLF and nHF, a trend of nLF predominance in the 2nd and 3rd month was revealed while an inverse relation was observed from the 6th month on, with nHF superiority. Overall, males reached comparable or slightly higher NA and non-normalized HRV values compared to females, although most differences were not statistically significant. A parallel decline of LF (starting from the 10th month) and HF (from the 6th month) was demonstrated in both male and female animals. Female rats had a little more stable nLF and nHF course in the study time.[18]

Due to the observed trend of a reduction in global autonomic activity with age in rats, revealed in the previously mentioned experiment, we intentionally enrolled into the study described in this manuscript age-differentiated group of animals (2–12 months) in order to obtain population reflecting the average autonomic activity in the studied time.

Clinical importance of the abovementioned findings becomes obvious in the context of available guidelines for HRV interpretation[7,6] and in view of published data on physiological neuropeptide-mediated co-transmission within the ANS.

As mentioned in the Introduction, spectral analysis of HRV is based on the expression of autonomically-controlled heart rate variability as a function of frequency. In line with widely accepted international guidelines for HRV interpretation,[6] TP of the spectrum should be considered a marker of global autonomic activity. HF component, associated with respiratory sinus arrhythmia, is a measure of parasympathetic activity, whereas LF, reflecting both sympathetically- and parasympathetically-driven processes, reflects the activity of both ANS arms. Although the underlying mechanisms of VLF component are complex and still not fully understood, according to many authors this parameter reflects primarily the sympathetic drive.[19] However, there is also some evidence suggesting contribution of parasympathetic mechanisms to this component, since administration of atropine was shown to result in a decrease in the VLF values[20] and an opposite effect was observed after administration of pyridostigmine.[21] As already mentioned in the Introduction, due to ambiguity of VLF interpretation, primary HRV spectrum needs to be normalized; consequently, primary results of HRV analysis are 'rewritten' upon exclusion of VLF values to obtain two normalized parameters, nLF and nHF. In line with the published interpretation guidelines, these two measures should be considered specific markers for sympathetic and parasympathetic activity, respectively.[6]

A concept of plasma NA determination originates from 1980s and is based on the assumption that after its release from the sympathetic efferent endings (especially in blood vessels), a fraction of this neurotransmitter is not a subject of re-uptake or other forms of synaptic clearance, but reaches peripheral circulation from which is eventually eliminated.[22] Previous studies showed that plasma concentration of NA reflects changes in sympathetic activity associated with short-term regulatory mechanisms of arterial blood pressure and heart rate,[8,9] also those activated in response to pharmacologic intervention.[23] However, the relationship between plasma NA concentration and sympathetic activity is not straightforward. As mentioned above, actual plasma level of NA reflects not only the rate of its release to circulation, associated with activity of the efferent sympathetic fibers, but also the rate of NA clearance from the plasma. Consequently, lower rate of NA elimination can be misinterpreted as a sympathetic overactivity. Moreover, a release of NA in response to a broadly defined stressors and resultant sympathetic activation is organ-specific, which stays in opposition to generalized interpretation of global sympathetic drive. Finally, upon its release, NA may likely interact with presynaptic α-2-adrenergic receptors of the sympathetic endings, thus inhibiting its further release.[8,9] In view of all these concerns, plasma concentration of NA should be considered solely a surrogate marker for sympathetic activity, rather than an accurate laboratory measure thereof. Nevertheless, unavailability of other laboratory markers for the ANS function, justifies determination of plasma NA for this purpose.

Determination of plasma neuropeptide concentrations constitutes another, evidently innovative, concept of laboratory autonomic activity markers.

NPY is a 36-amino acid peptide, synthesized and released by both central and peripheral neurons. It exerts pleotropic biological effects, agonizing membrane receptors NPY-Y 1–5 in various tissues. This compound is involved in central regulation of food ingestion and neuroendocrine mechanisms. Moreover, NPY exerts peripheral vasoconstrictive effect and contributes to cardiac and vascular remodeling.[24] Vasoactive intestinal peptide is a 28-amino acid peptide, similar to NPY characterized by pleotropic activity. Aside from its principal, primarily vasodilating effect (justifying its name), VIP causes relaxation of smooth muscles within the gut, stimulates intestinal secretion and exo- and endocrine activity of the pancreas. Furthermore, VIP is involved in central regulation of pituitary hormone release.[25]

Both VIP and NPY are also present within autonomic nerve fibers. VIP is a co-transmitter, stored in parasympathetic fibers together with acetylcholine (ACh),[10,26] and NPY is co-released with NA from sympathetic fibers.[10,27] Aside from NPY or VIP, both sympathetic and parasympathetic fibers release other co-transmitters: adenosine triphosphate (ATP), nitric oxide (NO), calcitonin gene-related peptide (CGRP) or substance P (SP).[10] Consequently, Dale's theory, based on the 'one nerve-one neurotransmitter' concept,[28] apparently does not apply to the ANS.

Presence of NPY and VIP within the autonomic fibers, as well as their co-release along with the principal neurotransmitters, NA and ACh, respectively, underlied the concept of their application as novel laboratory markers for the ANS activity. A growing body of evidence documents functional assessment of the ANS on the basis of plasma neuropeptide concentrations.[29–32] Since these compounds are also released from autonomic fibers innervating the salivary glands and thus can be detected in saliva, also this body fluid can be examined aside from the blood plasma.[33–35]

Analysis of correlations between HRV indices and biochemical parameters in the whole study group (both males and females), conducted in line with the abovementioned interpretation guidelines, showed that global sympathetic activation reflected by TP values was accompanied by changes in NA concentration. The fact that we also found a correlation between LF values and NA concentrations supports a hypothesis on the primarily sympathetic background of this HRV measure. A significant association between VLF values and NA level simplies that also this HRV parameter is primarily sympathetically driven. Further, the fact that VLF and LF contribute to the vast majority of the HRV spectrum, explains a correlation between TP and NA.

Furthermore, we found an overall correlation between LF values and NPY concentrations, i.e. between the HRV component and biochemical parameter reflecting sympathetic activity. This relationship was also observed in males but not in females. The values of another two spectral components (non-nHF and nHF) of male rats, both being specific markers for parasympathetic activity, correlated significantly with plasma concentrations of VIP, a co-transmitter released from parasympathetic fibers. Moreover, we found a significant

correlation between TP and VIP – which implies that the latter can be also considered a measure of global autonomic activity.

Importantly, our findings are also consistent with clinical data. In one previous study, El-Sayed et al.[36] evaluated autonomic neuropathy in patients with rheumatic disorders (systemic lupus erythematosus and juvenile idiopathic arthritis), using conventional tests for autonomic function, as well as plasma concentrations of NPY and VIP. The tests for autonomic function showed that both patients with systemic lupus and individuals with juvenile arthritis presented with non-selective mixed neuropathy involving both arms of the ANS. This was associated with a statistically significant decrease in plasma concentrations of the analyzed neuropeptides. Moreover, the same study showed an inverse correlation between severity of the vegetative neuropathy, expressed on a clinical scale based on the results of the Ewing tests, and VIP and NPY levels. Furthermore, in some patients a decrease in concentration of the analyzed neuropeptides preceded development of clinically evident autonomic neuropathy. According to the authors of this study,[36] plasma concentrations of VIP and NPY should be recommended as accurate laboratory markers for autonomic function, suitable for evaluation and monitoring of vegetative neuropathy and adding to clinical assessment with the Ewing test battery.

5.2. *Study limitations*

The results of our experiment were obtained under general anesthesia, so the reasoning must take into account this fact and be made through the prism of the potential impact of the applied anesthetic agent on the cardiovascular system. The pentobarbital may affect both ECG signal and subsequent HRV analysis and the release of the assayed NA and neuropeptides. According to Baum et al.[37] plasma pentobarbital concentration is regarded to be inversely related to NA concentration. Furthermore, pentobarbital levels are demonstrated to be negatively related to heart rate and mean blood pressure. These observations suggest the possibility that pentobarbital used as an anesthetic agent, inhibits the sympathetic nervous system. Because pentobarbital anesthesia affects plasma catecholamine concentrations (and as a consequence – the concentration of the NPY, a co-transmitter released with NA from adrenergic terminals), the regimen used in animal models requires consideration when interpreting data potentially influenced by the sympathetic nervous system. Moreover, Hanamoto et al.[38] confirmed that pentobarbital-associated catecholamine release is a dose-dependent phenomenon. Small doses of the anesthetic agent cause an increase in sympathetic activity whereas high doses of pentobarbital inhibit the release of catecholamines and reduce the sympathetic tension.[38] In our cautious and tentative estimation, the influence of the applied in our experiment pentobarbital on the sympathetic activity seemed to be moderately accentuated due to the maintaining of the resting heart rate of the study rats within physiological limits. According to literature the resting heart rate in rats vary in the range of 250–500/min[39] therefore no significant tachy- or bradycardia was observed in any of the study animal.

Moreover, due to a relatively small number of examined animals, our hereby presented experiment should be considered a pilot study. Discrepancies in the results for the whole study group versus the findings for males and females might result from too small size of the sample (a total of 18 male rats and the same number of female rats). Therefore it justifies further research on markedly larger populations of animals, also conscious but restrained ones to exclude the potential influence of the anesthetic agent on the cardiovascular system. However, also the effects of other, non-statistical confounders (e.g. hormonal factors) cannot be excluded as a reason behind the lack of expected correlations between plasma neuropeptide concentrations and HRV indices in female rats being a subject of this study.

6. Conclusions

In conclusion, this study showed that in rats under pentobarbital anesthesia the values of some HRV indices correlate with plasma concentrations of neuropeptides (VIP, NPY), and confirmed an association between plasma NA and most components of the HRV spectrum.

In our opinion, the hereby presented preliminary results imply that both techniques, HRV analysis and biochemical determination of neuropeptides being co-transmitters in autonomic fibers, may be suitable for complementary assessment of the ANS activity. This justifies further pre-clinical and clinical research on NPY and VIP as potential markers for autonomic function.

Conflict of interest

None declared.

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