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# Microglia and brain angiotensin type 1 receptors are involved in desensitising baroreflex by intracerebroventricular hypertonic saline in male Sprague-Dawley rats

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ABSTRACT

High salt diet alters cardiovascular control by increasing concentration of sodium ions ($Na^+$) in cerebrospinal fluid (CSF) and is a risk factor for hypertension. Hypernatremic conditions activate microglia and upregulate renin-angiotensin system in the brain. Thus, we checked if chronic elevation of CSF $Na^+$ affects neural control of circulatory system via microglia and brain angiotensin type 1 receptors (AT1Rs).

Normotensive adult male Sprague-Dawley rats received two-week intracerebroventricular (ICV) infusion of either isoosmotic saline (0.9% NaCl); hyperosmotic saline (5% NaCl); 5% NaCl with minocycline – inhibitor of microglia; 5% NaCl with losartan – AT1R blocker. Fluid intake, urine output, and urinary $Na^+$ excretion were measured before and during ICV infusions. At the end of ICV infusions, blood pressure and heart rate were recorded in awake rats at rest, in response to acute air jet stressor, during pharmacological evaluation of baroreflex, and after autonomic ganglia blockade. CSF and blood were collected for evaluation of $Na^+$ concentration.

Baroreflex was blunted in rats ICV infused with 5% NaCl. ICV treatment with losartan or minocycline prevented decrease in baroreflex sensitivity. Hemodynamic parameters at rest, in response to acute stressor and autonomic ganglia blockade were similar in all groups. Neither treatment affected water intake, urine output and urinary $Na^+$ excretion. ICV infusion of 5% NaCl resulted in higher concentration of $Na^+$ in CSF than in control group (0.9% NaCl) and in plasma. Our results indicate that chronic ICV infusion of hyperosmotic saline blunts baroreflex in normotensive rats and this desensitization is mediated by microglia and AT1Rs.

## 1. Introduction

Epidemiological studies show that increasing daily sodium intake is progressively associated with an increase in arterial blood pressure and cardiovascular risk, and even a modest decrease in salt intake translates into significantly lower blood pressures both in normotensive and hypertensive people (O'Donnell et al., 2015).

One of the postulated mechanisms responsible for the pro-hypertensive effects of high sodium diet is an increase in concentration of sodium ions ($Na^+$) in the cerebro-spinal fluid (CSF), which in turn alters functioning of neural circuits involved in the brain control of the cardiovascular system, that is manifested by baroreflex desensitization, sympathoexcitation and eventual development of hypertension (He et al., 2013; Huang et al., 2001; Bunag and Miyajima, 1984). In this context, it is important to note that high sodium diet leads to an increased concentration of $Na^+$ in CSF of salt-sensitive Dahl rats (Huang et al., 2009a; Huang et al., 2004). Furthermore, there is a cross-talk between renin-angiotensin system (RAS) and CSF $Na^+$ concentration. High salt diet upregulates angiotensin type 1 receptors (AT1Rs) in the hypothalamic paraventricular nucleus (PVN), a key cardiovascular centre of the brain (Su et al., 2017). In turn, inhibition of RAS components in the brain prevents attenuation of the baroreflex sensitivity as well as augmentation of pressor and tachycardic responses induced by systemic or central administration of hypertonic saline (Bealer, 2003a; Budzikowski and Leenen, 2001).

A number of clinical studies shows that elevated arterial blood pressure and decreased baroreflex sensitivity are associated with chronic low-grade inflammation and increased plasma concentration of proinflammatory cytokines (PICs) (Smykiewicz et al., 2018; Chae et al., 2001; Adlan et al., 2017; Subha et al., 2016).

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Experiments in laboratory rodents indicate that PICs participate in setting the pro-hypertensive and sympathoexcitatory milieu in the central nervous system (Smykiewicz et al., 2018; Shi et al., 2010; Segiet et al., 2019; Haspula and Clark, 2018). Infusions of tumour necrosis factor (TNF), interleukin 1 beta (IL-1β) or interleukin 6 (IL-6) into the cerebral ventricles, PVN or the subfornical organ (SFO) lead to attenuation of the baroreflex sensitivity, sympathoexcitation and increase in arterial blood pressure (Segiet et al., 2019; Helwig et al., 2008; Ufnal et al., 2006; Zera et al., 2008; Lu et al., 2009; Wei et al., 2015; Zera et al., 2016; Wei et al., 2013). In this light, it has been recently shown that TNF decreases excitation threshold and increases the firing rate in neurons located in the SFO (Simpson and Ferguson, 2017), suggesting direct effect of PICs on excitability of neural networks involved in the cardiovascular control.

Several lines of evidence point to the close interaction between PICs and activation of RAS in hypertensive rats and rats with heart failure. Specifically, inhibition of TNF in the brain decreases expression of RAS in the hypothalamus and attenuates development of hypertension induced by chronic administration of angiotensin II (Ang II) (Sriramula et al., 2013). Additionally, in rats with post-infarction heart failure, the regulation of blood pressure by brain AT1Rs depends on centrally acting endogenous TNF (Zera et al., 2015) and ICV infusion of this cytokine sensitizes these rats to centrally administered Ang II (Zera et al., 2008). In addition, ICV administration of IL-1β triggers increase in arterial blood pressure, which, at least partially, depends on activation of brain AT1Rs (Lu et al., 2009; Wei et al., 2015; Ufnal et al., 2005). Moreover, IL-1β administered into the lateral cerebral ventricles sensitizes normotensive rats to pressor action of centrally infused Ang II (Ufnal et al., 2006).

The main source of PICs in the central nervous system are microglia (Shi et al., 2010; Colonna and Butovsky, 2017). The microglia express AT1Rs (Biancardi et al., 2016), which upon stimulation by Ang II promote polarization of these cells towards proinflammatory phenotype and activation in the PVN (Biancardi et al., 2016; Labandeira-Garcia et al., 2017). Several studies indicate that inhibition of AT1Rs expressed by microglia reduces secretion of PICs (Haspula and Clark, 2018; Benicky et al., 2009).

A growing body of evidence shows that high salt diet or exposure to hyperosmotic stimuli activate microglia and induce expression of TNF and IL-1β in the hypothalamic supraoptic nucleus (SON) and the PVN of normotensive Sprague-Dawley, spontaneously hypertensive, and salt-sensitive Dahl rats (Summy-Long et al., 2008; Li et al., 2015; Qi et al., 2016; Nakagawa et al., 2013). Furthermore, chronic inhibition of IL-1β in the PVN with specific antibody prevents pro-hypertensive effect of the high sodium intake in salt-sensitive Dahl rats (Qi et al., 2016).

Minocycline is an antibiotic from tetracycline group that easily penetrates the blood-brain barrier, inhibits microglial activation and exhibits strong anti-inflammatory properties. The intrabrain administration of minocycline was shown to decrease expression of PICs in the central nervous system and to alleviate increase of arterial blood pressure in rats with Ang II-induced hypertension (Shi et al., 2010). It has been recently shown that centrally administered minocycline reverses upregulation of the RAS in the circumventricular organs and sensitization to Ang II induced by high-fat diet in rats (Xue et al., 2016). Furthermore, systemic administration of minocycline limits development of hypertension in rats and humans (Yang et al., 2015), which is being evaluated in a clinical trial in humans (NCT02133885).

In the present study we aimed at finding out if elevation of $Na^+$ concentration in CSF induced by chronic intracerebroventricular infusion of hypertonic saline affects arterial blood pressure, pressor response to acute stressor, and baroreflex control of the heart rate in normotensive rats. We also checked if pharmacological inhibition of microglia by minocycline and antagonizing the AT1Rs may prevent cardiovascular effects of the chronic exposure to centrally administered hypertonic sodium.

## 2. Methods

### 2.1. Animals

We did experiments on normotensive Sprague-Dawley male rats at the age of 12 weeks and weighting between 250 and 300 g at the beginning of the study. Animals were housed in cages with the 12: 12 h light/dark cycle, with unlimited access to a standard rat pellet diet and tap water. All surgical procedures were performed under anaesthesia with intraperitoneal ketamine (100 mg/kg; Bioketan, Vetoquinol Biowet, Poland) and xylazine (10 mg/kg; Xylapan, Vetoquinol Biowet, Poland). After each surgery, animals received benzathine penicillin intramuscularly (30,000 IU; Debecylina, Polfa Tarchomin, Poland).

The study was conducted according to domestic regulations and the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The experimental protocol was approved by the Local Ethics Committee for Animal Experimentation at the Medical University of Warsaw.

### 2.2. Study protocol

The animals were randomly assigned to 4 groups receiving a two-week lasting intracerebroventricular (ICV) infusion of either: (1) isoosmotic saline (5 μL/h, 0.9% NaCl) – control group (C); (2) hyperosmotic saline (5 μL/h, 5% NaCl) – HS group; (3) hyperosmotic saline together with minocycline – microglia inhibitor (5 μL/h + 5 μg/h, 5% NaCl + minocycline) - HS-Mino group; or (4) hyperosmotic saline together with losartan – AT1R blocker (5 μL/h + 12.5 μg/h, 5% NaCl + losartan) – HS-Los group. At the end of chronic ICV infusions, rats were instrumented with arterial and venous catheters and mean arterial pressure (MAP) and heart rate (HR) were recorded in awake, freely moving animals under the following conditions: (1) at rest; (2) during application of acute stressor in the form of air puff directed at the rat's head; (3) during intravenous infusion of phenylephrine and sodium nitroprusside for pharmacological testing of the baroreflex function; (4) during autonomic ganglia blockade with hexamethonium. Finally, CSF and blood were collected for evaluation of CSF and plasma concentration of $Na^+$.

Water intake, urine output, and urinary sodium excretion were measured in metabolic cages before and at the end of ICV infusions. Fig. 1 presents the time sequence of metabolic measurements, surgical

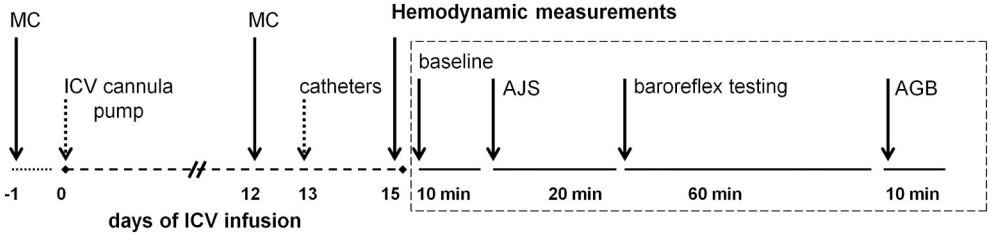


**Fig. 1.** Study protocol.
MC – metabolic cage measurements; AJS – air jet stressor; AGB – autonomic ganglia blockade.

procedures and hemodynamic recordings.

### 2.3. Surgical procedures

#### 2.3.1. Osmotic mini-pumps

Osmotic mini-pumps (model 2ML2 with Brain Infusion Kit 2, Alzet, Durect Corporation, Cupertino CA, USA) were prepared according to the manufacturer's instructions. In brief, the pump was connected to L-shaped cannula via polyethylene catheter and filled with either iso-osmotic saline (0.9% NaCl) (Polpharma, Poland), hyperosmotic saline (5% NaCl) (Polpharma, Poland), hyperosmotic saline (5% NaCl) together with minocycline (Sigma-Aldrich, Europe) or hyperosmotic saline (5% NaCl) together with losartan (Santa Cruz Biotechnology, Dallas, TX, USA). Then, the pump was primed in 37 °C warm sterile saline for 24 h. To implant the L-shaped cannula and osmotic mini-pump, each rat was placed in a stereotaxic apparatus (Kopf Instruments, Europe), a 3 cm cut was made in the sagittal plane from the inter-auricular line caudally and the surface of the skull was exposed around the crossing of the sagittal and coronal sutures. Next, a hole was drilled in the skull according to the following coordinates: −1.2 mm posterior to bregma, −1.8 mm laterolateral from sagittal suture, diameter 0.5 mm. Subsequently, the L-shaped cannula was inserted into the lateral cerebral ventricle with its tip at −3.5 mm dorsoventral from the skull's surface. The pedestal of cannula was fixed to the skull with cyanoacrylic glue. Then, the pump was implanted subcutaneously in the interscapular region and the wound was closed with sutures (Zera et al., 2015). The ICV infusion rate provided by the pump was 5 μL/h and it was shown previously that such infusion of 5% NaCl solution elevates $Na^+$ concentration in the CSF (Huang et al., 2001). Minocycline was infused at the rate of 5 μg/h and losartan at the rate of 12.5 μg/h. The dose of minocycline was based on studies, in which it effectively inhibited expression of PICs in the brain and resulted in preventing development of hypertension (Shi et al., 2010; Xue et al., 2016). The dose of losartan was based on studies, in which centrally administered losartan effectively prevented blood pressure increase induced by ouabain and decreased sympathoexcitation in rats with heart failure (Huang et al., 2009b; Huang and Leenen, 1999).

#### 2.3.2. Venous and arterial catheters

After 13 days of ICV infusion, intravascular catheters made of polyurethane tubings (intravascular part: inner diameter – 0.30 mm, external diameter – 0.64 mm, length - 35 mm; extravascular part: inner diameter – 0.64 mm, external diameter - 1.00 mm; Scientific Commodities, Inc., Lake Havasu City, AZ, USA) were inserted into the femoral artery and femoral vein for MAP and HR measurements and for intravenous infusions, respectively. Each catheter was filled with heparin dissolved in sterile saline (100 IU/ml; WZF Polfa S.A., Poland). The procedure is described in detail elsewhere (Zera et al., 2016; Zera and Ufnal, 2005). The hemodynamic measurements were conducted 48 h after the insertion of catheters.

### 2.4. Metabolic cages

Each animal was individually housed in a metabolic cage (Tecniplast, Italy) for 24 h, one day before implantation of osmotic mini-pumps and again after 12 days of ICV infusion, a day before implantation of intravascular catheters. Rats in metabolic cages had unrestricted access to chow and water. We measured the following parameters: quantity of ingested food and water, urine output. Concentration of urinary sodium in 24-h urine collection was measured with a flame-photometric method (Dobrowolski et al., 2007) and 24 h sodium excretion was calculated by multiplying urine sodium concentration by urine output.

### 2.5. Hemodynamic measurements

We recorded hemodynamic parameters two days after insertion of catheters. The arterial catheter was attached to blood pressure transducer, amplifier and analog-digital converter (MP100 system, Biopac Systems, Goleta, CA, USA) connected to a PC station for on-line recording of blood pressure signal. The venous catheter was connected to the microsyringe (SGE Analytical Science, Australia) mounted in the syringe infusion pump (Model 2, Harvard Apparatus, Holliston, MA, USA) for administration of drugs. After stabilization of hemodynamic parameters, resting blood pressure was recorded for 10 min and this was followed by application of acute stressor, pharmacological testing of the baroreflex and ganglionic blockade.

#### 2.5.1. Air jet stressor

Air jet was applied on the rat's head for 2 s from the laboratory made device providing peak air flow of 5 L/s. Maximal MAP increase in response to the stimulus was evaluated based on 3 consecutive hemodynamic cycles with the highest blood pressure values (Ufnal et al., 2008). The acute jet stressor is used to assess activity of central sympathoexcitatory pathways, as it activate defence mechanisms (Huang et al., 2001; Ufnal et al., 2008).

#### 2.5.2. Baroreflex testing

When MAP and HR returned to the control values ( ± 10% of the baseline values) within 20 min after the air-jet stressor, the baroreflex function was pharmacologically tested. In order to produce a ramp increase of MAP phenylephrine (Sigma-Aldrich, Europe) was intravenously infused at an increasing rate (from 20 up to 200 μg/kg/min) over 1–2 min. After HR and MAP returned to control values ( ± 10% of the baseline values), approximately after 20 min, sodium nitorprusside (Sigma-Aldrich, Europe) was intravenously administered at an increasing rate (from 20 up to 200 μg/kg/min) to generate a ramp decrease in MAP (Drapala et al., 2014).

Infusion of sodium nitroprusside always followed infusion of phenylephrine in order to minimize the effects of vasoactive neurohormones (vasopressin, Ang II, catecholamines) on the cardiovascular reflexes, which are released upon lowering of blood pressure.

#### 2.5.3. Ganglionic blockade

When MAP and HR returned to ± 10% of baseline values after the administration of sodium nitroprusside, autonomic ganglia were blocked with hexamethonium (Sigma-Aldrich, Europe) intravenously infused (20 mg/kg/min) for 1 min and the measurements were continued. The blood pressure response to blockade of autonomic ganglia was used to assess sympathetic-mediated vasoconstriction (Drapala et al., 2014; Bealer, 2003b).

### 2.6. Euthanasia, CSF and blood sample collection

After hemodynamic measurements, all rats were euthanized with an overdose of ketamine and xylazine. In a subgroup of 8 animals ICV infused with hypertonic saline and in 6 animals ICV infused with isotonic saline, immediately after obtaining surgical level of anaesthesia, the cisterna magna was accessed via suboccipital puncture with a 24 gauge needle connected to 1000 μL syringe and 80–110 μL of clear CSF samples were collected. Next, blood samples were taken by puncturing the heart and blood was centrifuged at 6000 rpm at 4 °C.The concentration of $Na^+$ was determined in the plasma and CSF with a flame-photometric method (Jenway PFP7, Essex, UK) (Dobrowolski et al., 2007; Dobrowolski et al., 2015). Each sample was evaluated twice and an arithmetic average was used for further calculations.

The placement of ICV cannula in the lateral cerebral ventricle was verified post mortem by injecting 5 μL of Evans Blue via the ICV L-shaped cannula and visual inspection of staining of the ventricles in gross coronal brain sections. Only animals with evident presence of blue

**Table 1**
Baseline hemodynamic parameters in rats receiving chronic ICV infusions.

| Group | Control ($n = 6$) | HS ($n = 6$) | HS-Los ($n = 6$) | HS-Mino ($n = 6$) |
|---|---|---|---|---|
| Baseline hemodynamic parameters | | | | |
| MAP (mm Hg) | 123 ± 9.4 | 116 ± 2.4 | 120 ± 1.2 | 121 ± 2.4 |
| HR (beats/min) | 372 ± 6.5 | 363 ± 13.1 | 356 ± 8.5 | 375 ± 6.5 |
| Baroreflex function parameters | | | | |
| Lower plateau (beats/min) | 242 ± 9.4 | 206 ± 16.7 | 215 ± 10.6 | 251 ± 11.4 |
| Upper plateau (beats/min) | 541 ± 17.6 | 553 ± 34.3 | 542 ± 10.6 | 528 ± 5.7 |
| HR range (beats/min) | 299 ± 17.6 | 347 ± 36.7 | 326 ± 7.3 | 277 ± 14.3 |
| BP-50 (mm Hg) | 113 ± 2.4 | 113 ± 7.3 | 117 ± 2.4 | 116 ± 1.6 |
| Maximal gain (1/min/mm Hg) | 5.2 ± 0.24 | 3.8 ± 0.24* | 4.8 ± 0.24 | 5.6 ± 0.49 |
| Spontaneous baroreflex sensitivity | | | | |
| sBRS (ms/mm Hg) | 2.9 ± 0.26 | 2.3 ± 0.26* | 2.8 ± 0.27 | 2.7 ± 0.18 |
| Spectral analysis of systolic blood pressure | | | | |
| VLF (mm $Hg^2$) | 5.4 ± 0.7 | 6.8 ± 2.0 | 4.4 ± 0.4 | 4.3 ± 0.8 |
| LF (mm $Hg^2$) | 8.3 ± 0.8 | 9.4 ± 2.1 | 6.1 ± 1.5 | 8.1 ± 0.7 |
| HF (mm $Hg^2$) | 0.4 ± 0.2 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 |

The top panel of the table presents baseline mean arterial pressure (MAP) and heart rate (HR) at the beginning of measurements, before the onset of tests to evaluate hemodynamic responses to air-jet stress, baroreflex testing and autonomic ganglia blockade.
The second panel presents parameters of the baroreflex function: lower plateau, upper plateau, HR range, mean arterial blood pressure at the midpoint of the HR range (BP-50) and maximal gain of the baroreflex.
The third panel presents spontaneous baroreflex sensitivity (sBRS) evaluated with the sequence method.
The bottom panel presets power spectra of frequencies of systolic blood pressure. VLF- very low frequency; LF – low frequency; HF – high frequency.
Data are expressed as mean ± SEM.
Control – control group; HS – hyperosmotic saline group; HS-Los – hyperosmotic saline group with losartan infusion; HS-Mino – hyperosmotic saline group with minocycline infusion.
* $p < 0.05$ - HS versus Control, HS-Los, and HS-Mino.

staining of the cerebroventricular system were included in the present study.

### 2.7. Analyses of haemodynamic parameters

#### 2.7.1. Air jet stress

The resting MAP and HR were averaged over 5 min interval preceding the air-jet stressor. The maximum MAP and HR during the air-jet stress were averaged from three consecutive hemodynamic cycles with the highest pressures during stress response.

#### 2.7.2. Baroreflex function

The baroreflex function was evaluated by fitting a four parameter logistic curve to the MAP-HR relationship, according to the equation:

$$y = a/(1 + exp(-(x - xo)/b) + y0$$

with the following parameters: y - HR; x – MAP; a - range of HR; x0 – MAP at midpoint of the curve; b - gain coefficient; y0 - lower plateau of HR changes (Kent et al., 1972). For each animal, at least 30 observations (pairs of MAP values with corresponding HRs) were used to fit the curve. The analysis was performed using SciDavis software. The gain of the baroreflex was obtained from the derivative of the above equation:

$$gain = (a/b)\, exp\, ((x - xo)/b)/(1 + exp((x - xo)/b))\text{^}2.$$

The baroreflex function was measured by the parameters of the logistic curve (lower and upper HR plateau, HR range, MAP at the midpoint of the curve (BP-50)) and maximum value of the gain function defined above (maximal gain).

#### 2.7.3. Spontaneous baroreflex sensitivity

In addition to pharmacological evaluation of the baroreflex, the sequence method as described by Bertinieri (Bertinieri et al., 1985) was used to estimate spontaneous baroreflex sensitivity (sBRS) function. For sBRS analysis, pulsatile arterial pressure was sampled at 500 Hz during baseline 10 min recording and exported to text file. The analysis was done in Analyzer module of HemoLab software (version 20.7, Harald Stauss Scientific, Iowa City, IA, USA) (Stauss et al., 2006).

#### 2.7.4. Autonomic ganglia blockade

To evaluate hemodynamic response to ganglionic blockade with hexamethonium, MAP and HR were averaged over 1 min intervals before and after administration of hexamethonium. Maximal decrease of MAP and corresponding HR were compared to pre-infusion values.

#### 2.7.5. Spectral analysis of systolic blood pressure

Fast Fourier transform (FFT) power spectral analysis of systolic blood pressure was used to indirectly estimate sympathetic outflow to the cardiovascular system (Oliveira-Sales et al., 2014). The range of frequencies were set to very low (VLF) (0.02–0.2 Hz), low (LF) (0.2–0.6 Hz) and high frequency (HF) (1–4 Hz), respectively (Stauss, 2007). For FFT power spectral analysis, the pulsatile arterial pressure was sampled at 500 Hz during baseline 10 min recording, exported to text file analysed with Batch Processor module of HemoLab software. The VLF band is associated with thermoregulatory and hormonal regulation of arterial blood pressure, whereas the LF band is associated with sympathetic vascular drive (Oliveira-Sales et al., 2014; Stauss, 2007).

### 2.8. Statistical analysis

All numerical values are reported as means with standard error of means (SEM). The box plots present continuous data with interquartile range (25–75%), median value represented by a line, mean value by a plus mark, and whiskers indicating minimum and maximum of the data set.

One-way analysis of variance (ANOVA) was used to compare MAP and HR in all groups at rest, before baroreflex testing and before ganglionic blockade. Repeated measure one-way ANOVA was used for detecting significant changes of hemodynamic parameters from the pre-intervention values in response to air-jet stress and ganglionic blockade in a given group. One-way ANOVA was used to compare the maximal gain and parameters of the logistic curve describing the baroreflex function between groups, sBRS, and power spectra of FFT analysis. Tukey post-hoc test was used to determine significant differences between specific groups. All statistical analyses were performed in Statistica ver. 12.5 (StatSoft Inc., Tulsa, OK, USA). Value of $p < 0.05$ was considered significant.

## 3. Results

### 3.1. Hemodynamic parameters at rest

The hemodynamic parameters were similar in control and treatment groups at baseline. Chronic two-week ICV infusion of either 0.9% saline, 5% saline, 5% saline with minocycline or 5% saline with losartan had no effect on resting MAP or HR (Table 1).

### 3.2. Hemodynamic response to acute stressor

In all groups the air-jet applied on the rat's forehead resulted in blood pressure increase, however, neither treatment changed the maximum increase of MAP. The changes in HR varied greatly in all

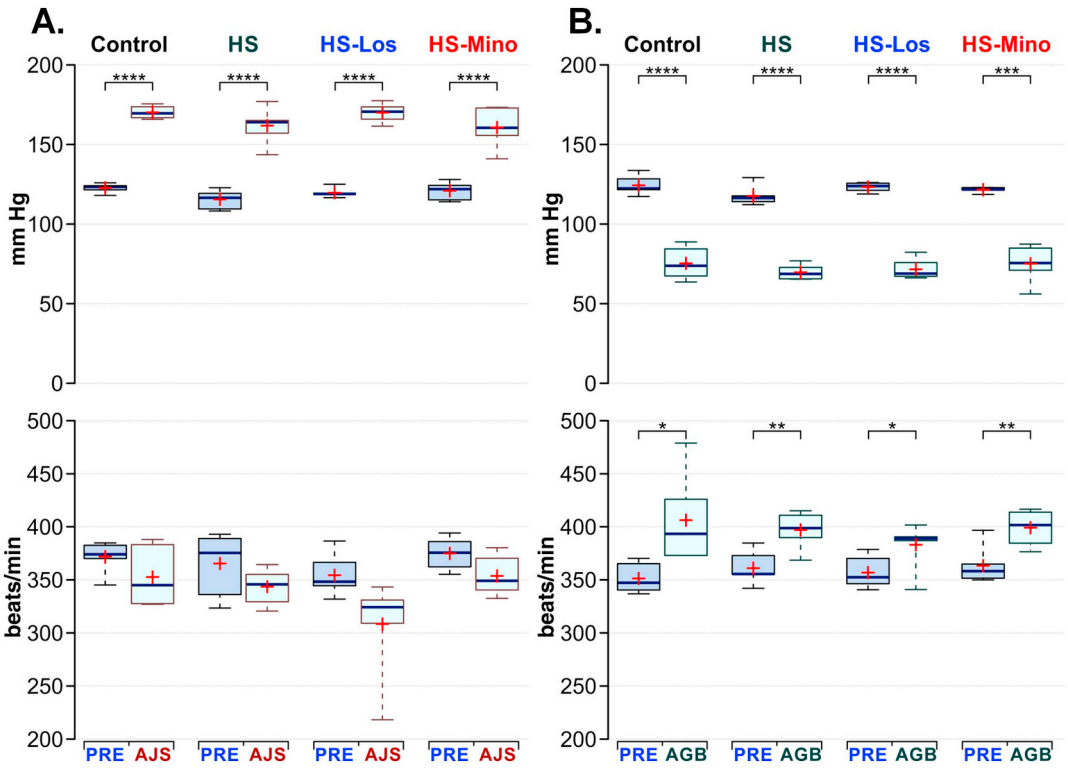


**Fig. 2.** A. Hemodynamic response to air-jet stress.
Maximal changes of mean arterial pressure (MAP) and heart rate (HR) in response to air-jet stress (AJS).
B. Hemodynamic response to autonomic ganglia blockade.
Maximal changes of MAP and HR in response to autonomic ganglia blockade (AGB) with hexamethonium.
Control – control group; HS – hyperosmotic saline group; HS-Los – hyperosmotic saline group with losartan infusion; HS-Mino – hyperosmotic saline group with minocycline infusion.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

groups and the differences between treatments were insignificant (Fig. 2).

### 3.3. Baroreflex function

Analysis of the baroreflex parameters with one-way ANOVA showed a significant difference in the sensitivity and gain of the baroreflex function ($p < 0.05$). Post hoc analysis showed that chronic ICV infusion of hyperosmotic saline led to a decrease in sensitivity and gain of the baroreflex function in comparison to animals treated with isoosmotic saline ($p < 0.05$). Coadministration of microglial inhibitor minocycline with hypertonic saline prevented attenuation of sensitivity and gain of the baroreflex in comparison to the rats receiving hyperosmotic saline alone ($p < 0.05$). Similarly, coadministration of AT1R blocker losartan with hyperosmotic saline infusion also prevented decrease in baroreflex gain and sensitivity ($p < 0.05$) (Table 1 and Fig. 3).

One-way ANOVA confirmed significant differences in sBRS between groups ($p < 0.05$), with ICV infusion of hyperosmotic saline causing attenuation of the baroreflex sensitivity in comparison to control, minocycline and losartan groups ($p < 0.05$) in the post hoc analysis (Table 1).

### 3.4. Ganglionic blockade and spectral analysis of systolic blood pressure

Blockade of autonomic ganglia with hexamethonium resulted in a rapid and significant decrease of MAP with corresponding increase in HR in all groups (Fig. 2). The changes of MAP and HR from pre-hexamethonium values were similar in all groups. Furthermore, one-way ANOVA did not reveal significant differences in the VLF, LF and HF between groups (Table 1).

### 3.5. Metabolic cages

The water intake, urine output, food intake and urinary sodium output were similar in all groups before the beginning of chronic intrabrain infusions. Chronic ICV administration of hyperosmotic saline, hyperosmotic saline with minocycline or hyperosmotic saline with losartan for 12 days resulted in insignificant differences among treatments. The changes in water intake, urine output, food intake and urinary sodium output from preinfusion values were insignificant. In addition, there were no significant differences between treatments (Table 2).

### 3.6. Sodium ions in CSF and plasma

Concentration of $Na^+$ in the CSF was significantly higher in rats receiving chronic infusion of 5% NaCl than in rats infused with 0.9% NaCl (155.9 ± 1.84 mmol/L vs 147.3 ± 1.67 mmol/L) ($p < 0.05$). Rats chronically ICV infused with 5% NaCl had also significantly higher concentration of $Na^+$ in the CSF than in the plasma (155.9 ± 1.84 mmol/L vs 136.5 ± 3.55 mmol/L) ($p < 0.05$). There were no significant differences in plasma $Na^+$ concentration between rats ICV treated with hyperosmotic saline (136.5 ± 3.55 mmol/L) and rats ICV treated with isoosmotic saline (139.1 ± 2.61 mmol/L).

## 4. Discussion

The key finding of our study is that in the normotensive male Sprague-Dawley rats chronic intracerebroventricular infusion of

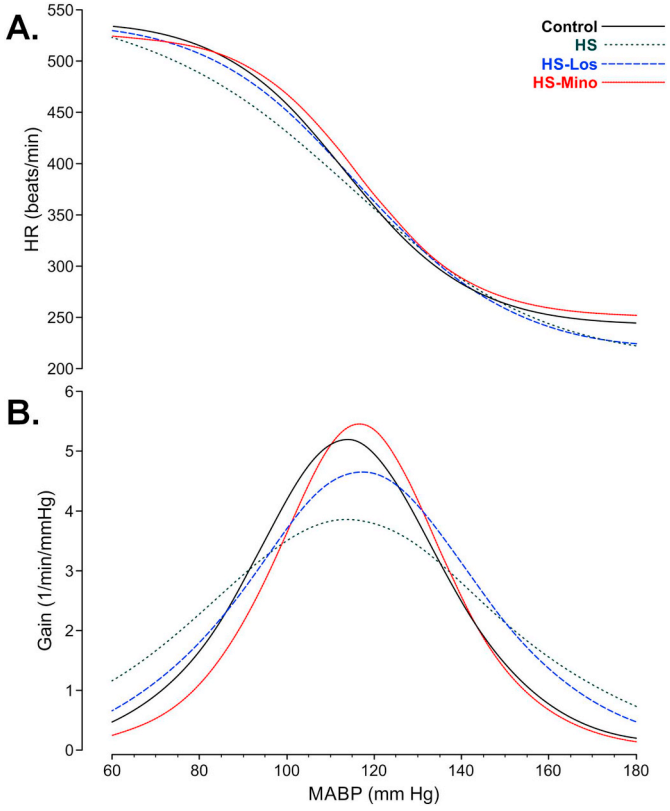


**Fig. 3.** Baroreflex function.
A. Baroreflex sensitivity.
B. Baroreflex gain.
MAP – mean arterial pressure; HR – heart rate; Control – control group; HS – hyperosmotic saline group; HS-Los – hyperosmotic saline group with losartan infusion; HS-Mino – hyperosmotic saline group with minocycline infusion.

hypertonic saline blunts the baroreflex sensitivity via mechanisms involving AT1Rs and microglia, as the treatment with intrabrain losartan, an AT1R antagonist, or intrabrain minocycline, an inhibitor of microglial activation, prevented attenuation of the baroreflex induced by hypertonic saline. Additionally, we show that in normotensive Sprague-Dawley rats, central infusion of hypertonic $Na^+$ solution for two weeks has no effect on resting arterial blood pressure, hemodynamic response to alerting stress, and sympathetic vasomotor tone maintaining arterial blood pressure. These findings are evidenced by similar increase in MAP in response to the air jet stress, comparable decrease in MAP after pharmacological blockade of autonomic ganglia and nonsignificant differences in LF band in FFT spectral analysis of systolic blood pressure variations.

In our study central infusion of hypertonic saline had no effect on sodium excretion and water balance evaluated at the 12th day of ICV infusions. This finding suggests that the cardiovascular effects of central sodium loading are not dependent on homeostatic mechanisms maintaining extracellular fluid (ECF) and blood volumes in normotensive rats. Furthermore, it is in line with our previous results, which showed that high salt diet has no effect on arterial blood pressure and blood volume in normotensive rats (Ufnal et al., 2011).

Several lines of evidence indicate that the pro-hypertensive effects of high sodium diet involve an increase in CSF concentration of $Na^+$, which alters functioning of the cardiovascular centres of the brain, eventually leading to sympathoexcitation, reduction of the baroreflex function and potentially hypertension (Blaustein et al., 2012). The air jet stressor acutely triggers centrally mediated sympathoexcitation and pressor effect (Huang et al., 2001; Budzikowski and Leenen, 2001). In salt-sensitive Dahl rats, chronic intracerebroventricular administration of hypertonic saline robustly enhanced pressor and sympathoexcitatory responses to air jet stress (Huang et al., 2001). Similarly, administration of high-salt diet for five weeks exacerbated hypertension and increased pressor responses to air jet stress in spontaneously hypertensive rats, but not in normotensive controls (Budzikowski and Leenen, 2001). In our study chronic ICV administration of hypertonic saline did not change the pressor response to the air jet stress, regardless of co-administered treatment with minocycline or losartan. Thus, our findings together with previously published results indicate that increased CSF $Na^+$ exerts an insignifcant/modest effect on acute pressor and sympathoexcitatory responses in normotensive rats.

In the present study, we found that chronic ICV administration of hypertonic saline decreases sensitivity of the baroreflex. This is in line with studies by Buñag and Miyajima in Sprague-Dawley rats and by Huang et al. in Dahl rats (Huang et al., 2001; Bunag and Miyajima, 1984). Huang et al. found that chronic ICV infusion of hypertonic saline administered at the same dose as in our study leads to a decrease in baroreflex sensitivity in both the salt-resistant and salt-sensitive Dahl rats, but the increase in arterial blood pressure due to ICV hypertonic saline infusion was modest and significantly smaller in salt-resistant rats in comparison to salt-sensitive Dahl rats (Huang et al., 2001). In line with the above study, we show that ICV infusion of 5% NaCl solution at the rate of 5 μL/h resulted in a significant increase of $Na^+$ concentration in the CSF, confirming previous findings, in which the same rate and concentration of NaCl solutions were used for chronic ICV infusions (Huang et al., 2001). Such an increase in CSF $Na^+$ levels reflects the increase in CSF concentration of $Na^+$ induced by high salt diet in the salt-sensitive Dahl rats, in which high sodium intake blunts the baroreflex function and increases arterial blood pressure via an

**Table 2**
Metabolism cages.

| Group | | Control ($n$ = 6) | HS ($n$ = 6) | HS-Los ($n$ = 6) | HS-Mino ($n$ = 6) |
|---|---|---|---|---|---|
| Body weight | Pre | 319 ± 11.8 | 308 ± 11.8 | 306 ± 6.1 | 323 ± 6.9 |
| (g) | Post | 334 ± 12.2 | 336 ± 13.5 | 338 ± 6.9 | 346 ± 17 |
| Food intake | Pre | 80 ± 9.8 | 86 ± 6.1 | 89 ± 5.3 | 79 ± 4.9 |
| (g/kg b.w.) | Post | 89 ± 11.0 | 79 ± 4.1 | 93 ± 4.5 | 73 ± 3.3 |
| Water intake | Pre | 105 ± 6.9 | 92 ± 4.1 | 105 ± 5.3 | 103 ± 10.6 |
| (ml/kg b.w.) | Post | 97 ± 5.7 | 95 ± 6.1 | 106 ± 6.1 | 92 ± 4.1 |
| Urine output | Pre | 28 ± 5.3 | 22 ± 1.6 | 25 ± 1.6 | 25 ± 4.5 |
| (ml/kg b.w.) | Post | 29 ± 5.7 | 27 ± 2.0 | 33 ± 4.1 | 29 ± 3.3 |
| Urinary Na + excretion | Pre | 3.1 ± 0.41 | 2.9 ± 0.24 | 3.7 ± 0.20 | 2.9 ± 0.33 |
| (mmol/kg b.w.) | Post | 2.9 ± 0.53 | 3.6 ± 0.37 | 3.2 ± 0.9 | 3.4 ± 0.4 |

The table presents body weight, food intake, water intake, urine output, and urinary sodium output before (Pre) the onset of intracrebroventricular (ICV) infusion and at the 12th day of ICV infusion (Post). Data are expressed as mean ± SEM.
Control – control group; HS – hyperosmotic saline group; HS-Los – hyperosmotic saline group with losartan infusion; HS-Mino – hyperosmotic saline group with minocycline infusion.

increased concentration of $Na^+$ in the CSF (Huang et al., 2009a; Huang et al., 2004).

In contrast to Buñag and Miyajima's observations, in our study chronic ICV infusion of hypertonic saline had insignificant effect on arterial blood pressure. There are several possible factors that may contribute to this discrepancy. The normotensive rats in our study were one month older than animals in the study by Buñag and Miyajima. It was shown that younger rats manifest greater pressor and sympathoexcitatory response to central infusions of $Na^+$ (Huang et al., 2001). Furthermore, environmental factors may affect salt-sensitive increase in arterial pressure. Specifically, qualitative differences in the maternal diet were shown to affect development of salt-dependent hypertension in the offspring rats (Geurts et al., 2015). Moreover, pregestational and gestational exposure to chronic stress promotes pro-hypertrophic changes in the cardiac muscle of the offsprings (Czarzasta et al., 2018). Finally, arterial blood pressure and heart rate in laboratory rodents are highly dependent on ambient temperature, even within typical ranges for experimental laboratories (Swoap et al., 2004). Taken together, it is likely that different environmental/maternal factors may play a role in lack of hypertensive effect of ICV hypertonic saline in our study.

One of the main mechanisms maintaining high blood pressure is increased vascular resistance, which in great part depends on increased sympathetic outflow to small arteries. The pharmacological blockade of autonomic ganglia with hexamethonium allows for evaluation of sympathetically mediated vasomotor tone maintaining arterial blood pressure (Drapala et al., 2014; Bealer, 2003b). In addition, the LF band obtained in the FFT spectral analysis of systolic blood pressure variations is associated with sympathetic vascular tone (Oliveira-Sales et al., 2014; Stauss, 2007). Our findings from both pharmacological assessment and spectral analysis indicate that in normotensive rats a two-week ICV administration of hypertonic saline had insignificant effect on sympathetically mediated vasoconstriction at rest. It is noteworthy that in telemetered rats with Goldblatt hypertension, increase in the arterial blood pressure after clipping of the renal artery precedes changes in vascular tone reflected in alterations of LF power spectrum, whereas decrease in sBRS occurs early, before the onset of hypertension (Oliveira-Sales et al., 2014). In this light, it is speculated that exposure of rats to hypertonic saline longer than for two weeks could lead not only to decrease in baroreflex sensitivity, but also to changes in both arterial blood pressure and sympathetically mediated vascular tone.

One of the main sources of PICs in the central nervous system are microglia (Shi et al., 2010; Colonna and Butovsky, 2017), which may be activated by high osmolarity and increased concentrations of $Na^+$. It was shown in normotensive rats that hypertonic saline administered subcutaneously triggers release of IL-1β from microglia and neurons in the SON (Summy-Long et al., 2008). Furthermore, in salt-sensitive Dahl rats, high sodium intake induces expression of IL-1β in the PVN, whereas chronic inhibition of the cytokine with specific antibody prevents pro-hypertensive effect of the high salt diet (Qi et al., 2016). Similar results were obtained in experiments on normotensive Sprague-Dawley rats, which showed that long-term high salt diet promotes expression of the key PICs, TNF and IL-1β, in the PVN (Li et al., 2015). In line with the above findings, it has been recently shown that high salt diet in the stroke-prone spontaneously hypertensive rats activates microglia of the PVN (Nakagawa et al., 2013).

A number of studies showed that PICs acting in the brain promote shift towards pro-hypertensive regulation of the cardiovascular system (Smykiewicz et al., 2018; Shi et al., 2010; Segiet et al., 2019; Haspula and Clark, 2018). Infusions of TNF or IL-1β into the carotid artery, cerebral ventricles or directly into the brain centres involved in the cardiovascular control, such as PVN or SFO, increase arterial blood pressure (Segiet et al., 2019; Ufnal et al., 2006; Zera et al., 2008; Lu et al., 2009; Wei et al., 2015; Zera et al., 2016; Wei et al., 2013). Furthermore, we recently showed that ICV infused TNF not only increases arterial blood pressure, but also decreases baroreflex sensitivity (Zera et al., 2016).

Results of our study indicate that desensitization of the baroreflex function induced by ICV infusion of hypertonic saline depends on activation of the microglia and release of PICs, as the treatment with ICV administered minocycline, an inhibitor of microglial activation, prevented changes in the baroreflex sensitivity. The intrabrain administration of minocycline at the same dose as in our study was shown to decrease expression of PICs in the central nervous system and to alleviate rise of arterial blood pressure in rats with Ang II-induced hypertension (Shi et al., 2010). Moreover, it has been recently shown that high-fat diet induces PICs and the RAS in the circumventricular organs, which is functionally manifested by sensitization of rats to Ang II, and the changes were reversed by centrally administered minocycline at the same dose as in our study (Xue et al., 2016). Furthermore, systemic administration of minocycline was shown to prevent the proinflammatory polarization of microglia and to limit development of hypertension both in rats and humans (Yang et al., 2015; Mi et al., 2018). Taken together, our results and the above discussed studies indicate that minocycline, an inhibitor of microglial activation, restores physiological control of the cardiovascular system.

It was shown previously by Budzikowski and Leenen that AT1Rs are involved in mediating pressor responses to acute increases in $Na^+$ concentration in the CSF of normotensive rats (Budzikowski and Leenen, 2001). In the present study we show that chronic ICV infusion of hypertonic saline leads to desensitization of the baroreflex, which is also dependent on the brain AT1Rs, as blocking of the receptors with losartan, a selective AT1R antagonist, prevented blunting of the baroreflex by central sodium loading. Since both minocycline and losartan prevented attenuation of the baroreflex in rats chronically ICV treated with hypertonic saline, it is plausible to assume that increase in CSF $Na^+$ content leads to activation of the microglia and AT1Rs and that both mechanisms are critically involved in the diminished function of the baroreflex.

In fact, several lines of evidence indicate that PICs and RAS expressed in the central nervous system act synergistically in the control of arterial blood pressure and in shifting cardiovascular regulation towards sympathoexcitation and withdrawal of the parasympathetic system. It has been recently shown that in rats with heart failure, the increased expression of PICs and RAS in the PVN is associated with desensitization of the baroreflex (Li et al., 2016). In heart failure rats, ICV infusion of TNF enhances pressor response to centrally administered Ang II (Zera et al., 2008) and chronic inhibition of the brain TNF prevents increased activity of AT1Rs (Zera et al., 2015). Furthermore, inhibition of brain TNF decreases expression of the RAS in the hypothalamus and attenuates development of hypertension induced by chronic administration of Ang II (Sriramula et al., 2013). In normotensive rats, increase of arterial blood pressure in response to ICV administration of IL-1β depends, in part, on activation of the brain AT1Rs (Lu et al., 2009; Wei et al., 2015; Ufnal et al., 2005). Moreover, intrabrain infusion of IL-1β also sensitizes the pressor response to centrally infused Ang II (Ufnal et al., 2006). In summary, there is a reciprocal interaction between PICs and RAS, which directs the regulation of the cardiovascular system towards hypertensive phenotype. The microglia express AT1Rs (Biancardi et al., 2016), which upon stimulation by Ang II robustly promote polarization of the microglia towards proinflammatory phenotype and activation in the PVN (Biancardi et al., 2016; Labandeira-Garcia et al., 2017). Several studies indicate that inhibition of AT1Rs on microglia reduces secretion of PICs (Haspula and Clark, 2018; Benicky et al., 2009). Here, we suggest that chronic central administration of losartan alleviates hypertonic saline-mediated activation of microglia.

Our study provides evidence, that central sodium loading, which may reflect the changes in the CSF under conditions of increased sodium intake in hypertensive subjects (Blaustein et al., 2012), leads to desensitization of the baroreflex function mediated by activation of both the microglia and stimulation of the AT1Rs.

## 5. Conclusions

Our results indicate that increased CSF $Na^+$ concentration decreases gain of the baroreflex and spontaneous baroreflex sensitivity without affecting arterial blood pressure in conscious, freely moving normotensive rats. Prevention of the baroreflex dysfunction by concomitant central administration of losartan or minocycline suggests that both Ang II and activation of microglia participate in the desensitizing action of high CSF $Na^+$ concentration on the arterial baroreflex.

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TZ conceived and designed the study, provided funding, conducted experiments, analysed the data, did sBRS and FFT spectral analyses, interpreted results, wrote the manuscript, approved the final version of the manuscript; AN conducted metabolism cages experiments, assisted in surgical procedures and hemodynamic measurements, analysed the data, reviewed the manuscript; AS analysed the data, reviewed the manuscript; PS analysed the data, reviewed the manuscript. Artur Nowiski's current address is at Department of Experimental Physiology and Pathophysiology, Laboratory of Centre for Preclinical Research, the Medical University of Warsaw, Banacha 1B, 02-097 Warsaw, Poland.

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Authors declare no conflict of interest related to the study.

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