Differences in Serum Steroid Hormones Concentrations in Spontaneously Hypertensive Rats (SHR) – an Animal Model of Attention-Deficit/Hyperactivity Disorder (ADHD)

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Summary

Spontaneously hypertensive rats are the most common animal model used to study attention deficit hyperactivity disorder (ADHD). The present study investigated the levels of steroid hormones in the bloodstream of hypertensive rats and its normotensive control strain, Wistar-Kyoto rats, to check if there are any hormonal differences between both strains at the onset of ADHD. Plasma samples were collected from young (5-week-old) and mature (10-week-old) male hypertensive and normotensive rats to determine the serum level of testosterone, 17β-estradiol, free estriol, progesterone, corticosterone and cortisol using ELISA kits. The results showed statistically significant increases in serum levels of testosterone and free estriol in 10-week-old hypertensive and normotensive rats when compared to 5-week-old animals. Moreover, the concentrations of progesterone, corticosterone and cortisol were significantly elevated in 10-week-old hypertensive rats when compared to 5-week-old animals of both strains as well as 10-week-old normotensive rats. Hormonal differences observed between 10-week-old hypertensive and normotensive rats were also accompanied by differences in the volumes of lateral ventricles as well as the third ventricle and cerebral aqueduct. In conclusion, elevated contents of progesterone, corticosterone and cortisol in hypertensive rats may be associated not only with ADHD but also with developing hypertension. This question needs further study.

Key words

Hormones • ADHD • Spontaneously hypertensive rats

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Introduction

It is estimated that attention-deficit/hyperactivity disorder (ADHD) affects 10 % of boys and 5 % of girls at elementary school age (Dulcan 1997). This disorder is a developmental condition of inattention and distractibility, with or without associating hyperactivity and it is also characterized by susceptibility to distraction (Nagui 2009). It has been reported that anatomical abnormalities in the brain could be attributable to symptoms of ADHD (Hsu *et al.* 2010). Neuroimaging studies of children's brains with ADHD have shown that the main putative brain regions involved in this condition are the prefrontal cortex (Zang *et al.* 2007), striatum (Scheres *et al.* 2007) and cerebellum (Castellanos *et al.* 1996). However, abnormalities have also been found in other brain regions, such as the anterior cingulate cortex

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(Zang *et al.* 2007) and substantia nigra (Romanos *et al.* 2010).

There is also data indicating that steroid hormones may play a role in the pathogenesis of ADHD. It is not surprising as they are engaged in the brain organization, plasticity and modulation of neurotransmitter system (McEwen 1992, Morris *et al.* 2004) and there is a sex bias in ADHD (Gaub and Carlson 1997). For example, it is suggested that higher prenatal testosterone (T) exposure is associated with a greater risk of developing disruptive behavior disorders. This suggestion is partly supported by King *et al.* (2010) who found that the exposure of spontaneously hypertensive rats (SHRs; an animal model of ADHD) to elevated T-levels during early development resulted in additional deficits in spatial memory. In addition, various neurocognitive effects of T on boys and girls with ADHD were observed and they were sex-specific (Wang *et al.* 2017). Finally, medical drugs such as methylphenidate, which is widely used to treat ADHD (Burcu *et al.* 2016), can potentially diminish T-levels and, in consequence, delay puberty onset (Ramasamy *et al.* 2014). Estrogens and progesterone (P_4) have also been proposed to play an important role in ADHD, because they are synthesized *de novo* in the cerebellum during critical developmental periods in rats (Dean and McCarthy 2008). In addition, a low level of estrogens in women with ADHD is associated with intensity of symptoms (Roberts 2016). Furthermore, estrogens can increase visual and place memory in rats (Luine *et al.* 2003) as well as attention in macaques (Shively and Bethea 2004), and both memory and attention are deficient in children with ADHD (Holmes *et al.* 2014). Finally, studies in gonadectomized male mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine or with methamphetamine-induced neurotoxicity have shown that estrogens are engaged in the neuroprotection of the nigrostriatal dopaminergic system (Dluzen 2000), which is dysfunctional in children with ADHD (Romanos *et al.* 2010). The participation of corticosteroids in the course of ADHD should also be considered since the level of cortisol (CT) was lowered in children with ADHD (Isaksson 2014). A similar observation was reported in SHR rats with regard to corticosterone (CTT) (King *et al.* 2010). It is worth mentioning that low levels of corticosteroids may indicate abnormalities in the activity of the hypothalamic–pituitary–adrenal (HPA) axis, which is involved in emotion, learning and attention (Smith 2006).

All of these findings suggest that serum concentrations of various steroid hormones should be altered in ADHD subjects. To verify this hypothesis, these concentrations were evaluated in SHRs, which are the best-validated animal model of ADHD (Sagvolden and Johansen 2012) with Wistar-Kyoto (WKY) rats serving as a control. The following hormones were tested: T, 17β-estradiol (E_2), free estriol (E_3), P_4, CTT and CT. Considering that in SHR rats the anatomical abnormalities in the brain associated with ADHD are observed in the juvenile animals (5-week-old) and they disappear in mature animals (10-week-old) (Hsu *et al.* 2010), the highest alterations in hormone serum levels due to ADHD should be expected before puberty (5-week-old animals). As mature SHRs are also one of the most common animal models of hypertension in humans (Louis and Howes 1990), hormonal changes after puberty (10-week-old animals) maybe associated with hypertension. Since ADHD is more common in boys than in girls (Gaub and Carlson 1997), male SHRs were chosen.

Methods

Animals

A total of twenty-four male rats were used in the present study. All of the animals were divided into four groups: (1) 5-week-old SHR rats (n=6); (2) 5-week-old WKY rats (n=6); (3) 10-week-old SHR rats (n=6), and (4) 10-week-old WKY rats (n=6). The mean (±SD) weight of animals in the individual groups was: (1) 120.08±6.30; (2) 115.08±4.65; (3) 269.58±8.48; and (4) 254.94±7.91. All experiments were carried out in accordance with the European Union Directive for animal experiments (2010/63/EU) and were approved by the Local Ethical Commission of the University of Warmia and Mazury in Olsztyn (no. 43/2014). The 3-week-old SHR and WKY rats were obtained from Charles River (Germany) and were transported to the animal house at the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences (Olsztyn, Poland) where they were housed in pairs or threes to prevent isolation stress. The temperature-controlled (21±1 °C) and ventilated (12-20 exchanges/h) animal room was maintained on a 12/12 h light/dark cycle (lights on from 6 a.m. to 6 p.m.). All animals were fed with a grain mixture (VRF1 diet; Charles River, Germany) and tap water *ad libitum*. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable scientific data.

Tissue preparation

Rats were deeply anesthetized with an intraperitoneal injection of pentobarbital (Biowet, Poland; 50 mg/kg), then, the abdomen was opened and blood was drawn from the vena cava into EDTA tubes (42110, FLMEDICAL, Poland) (Palombo *et al.* 2000). Blood samples were collected from all animals between 7 a.m. and 8 a.m. In each animal blood was taken within time shorter than 3 min to avoid the initiation of the pituitary stress response (Vahl *et al.* 2005). After collection of blood samples, all animals were transcardially perfused with saline (0.9 %) followed by 4 % paraformaldehyde (pH 7.4; 1040051000, Merck, Germany) in phosphate-buffered saline (PBS; P5493, Sigma Aldrich, Germany).

Following perfusion, the brains were carefully dissected out from the skulls and post-fixed by immersion in the same fixative for 24 h, washed three times in 0.1 M phosphate buffer (pH=7.4, 4 °C) and then stored for 3-5 days in graded solutions (10 %, 20 % and 30 %) of sucrose (363-117720907, ALCHEM, Poland) in 1xPBS at 4 °C until they sunk. Finally, the brains were frozen and then coronally sectioned at a thickness of 10 µm using a cryostat (HM525 Zeiss, Germany). The sections were stored at -80 °C until further processing.

Immunoenzymatic determination (ELISA) of steroid hormone concentrations in rat serum

The measurements of steroid hormones: testosterone, estradiol, free estriol, progesterone, corticosterone and cortisol in rat plasma were done with an ELISA test according to the manufacturer's instructions. All ELISA tests were produced by DRG Instruments (Germany; Table 1). The absorbance in ELISA test plate was measured by plate reader TECAN infinite M200 PRO (Austria) at the wavelength λ=492 nm.

Table 1. List of ELISA kits used for the determination of steroid hormones concentrations in rat serum.

ELISA kit	Catalog number and manufacturer	Intra Assay Variation CV [%]	Inter Assay Variation CV [%]
Testosterone ELISA	EIA-1559. DRG Instruments (Afify *et al.* 2010)	3.593 %	7.126 %
Estradiol ELISA	EIA-2693. DRG Instruments (Chistyakov *et al.* 2010)	8.970 %	10.870 %
Free Estriol ELISA	EIA-1612. DRG Instruments (Klocke *et al.* 2014)	3.930 %	7.530 %
Progesterone ELISA	EIA-1561. DRG Instruments (Inegbenebor *et al.* 2009)	6.416 %	6.630 %
Corticosterone ELISA	EIA-4164. DRG Instruments (Kazemi *et al.* 2011)	3.096 %	6.010 %
Cortisol ELISA	EIA-1887. DRG Instruments (Kalshetti *et al.* 2015)	5.630 %	6.930 %

Immunohistochemistry

Brain sections were processed for DAB immunohistochemistry using primary antisera and species-specific secondary antibodies. All staining procedures were carried out in humid chambers (Immuno Slide Staining Trays, R64001-E, Pyramid Innovation Ltd., UK) and at room temperature.

DAB immunohistochemistry

The sections designated for morphometric and stereological procedures (every 25th section in the single brain) were processed for a routine immunoperoxidase labeling using DAB as a chromogen (Dako Liquid DAB + Substrate Chromogen System, K3468, Denmark). After triple-washing cold PBS, the sections were pre-incubated for 30 min in 0.3 % H_2O_2 diluted in methanol and then for 60 min with a solution of 10 % normal horse serum (diluted in PBS). The sections were then incubated overnight with a solution of primary antibodies directed towards neuron-specific nuclear protein NeuN (pan-neuronal marker; Anti-NeuN Antibody, clone A60, MAB377; Merck Millipore, Poland; working dilution

1:1,000). The antibodies were diluted in PBS containing Triton X-100 (0.3-0.5 %) and 1 % normal horse serum. In the next step, after triple-washing in cold PBS, the sections were incubated for 60 min with ImmPRESS Reagent, washed in cold PBS and incubated with a 3.3-diaminobenzidine substrate–chromogen solution (ImmPRESS™ UNIVERSAL REAGENT Anti-Mouse/Rabbit IgG PEROXIDASE, MP-7500; Vector Laboratories, Inc.; Burlingame, CA, USA). Finally, the sections were rinsed in tap water, dehydrated through graded alcohol series (POCH, Poland), cleaned in xylene and mounted in DPX (DPX Mountain for histology; 44581, Sigma Aldrich, Germany).

Controls

The antibody against neuron-specific nuclear protein NeuN used in the present study is an excellent marker for neurons in the central and peripheral nervous systems (Mullen *et al.* 1992). To test the secondary antibody specificity, the omission and replacement of all primary antisera by non-immune sera or PBS was applied. No observable immunoreactions had proven specificity.

Stereological analyses

Volumetric measurements were done using image-analysis software Fiji (Madison, USA). The following structures were taken into consideration in the WKY and SHR rats at each matched age: lateral ventricles (left and right) and third ventricle together with cerebral aqueduct. Measurements were done on evenly spaced sections arranged from the rostral to the caudal extent of the brain. Every 25th section was stained using DAB method and antibody against NeuN protein from the level where the prefrontal cortex arrived to the end of the cerebellum. All these sections were then digitalized with magnification 5.0 x using PathScan Enabler IV Histology Slide Scanner (Praha, Czech Republic). On each digital slice from the bregma 2.52 (Paxinos and Watson 2005) the boundaries of the individual brain ventricles (right and left lateral ventricles as well as 3rd ventricle in conjunction with cerebral aqueduct) were outlined by a mouse-driven cursor. The number of sections analyzed per specific ventricle depended on the brain size and these numbers were as follows: right and left lateral ventricles: 25-28 and 3rd ventricle with conjunction of cerebral aqueduct: 29-32. Lengths differences were mostly due to the natural variability among subjects as well as strain and age volumetric differences. The total volumes of the individual brain ventricles were calculated according to the formula proposed by DeVito *et al.* (1989), in which the total volume of a structure (Vo) is the sum of the subvolumes of the sections through the structure (Vn). The outlined areas depicting boundaries of the individual brain ventricles on the studied sections with the thickness 250 µm (space between sections) were subvolumes.

Preparation of images

In the first step, all NeuN stained sections were digitalized using PathScan Enabler IV Histology Slide Scanner (Praha, Czech Republic) receiving images with a quality of 5.0 x objective. These digital images were slightly modified to optimize the image resolution, brightness and contrast using CS4, version 11.0, software (Adobe Systems Inc., San Jose, CA, USA).

Statistical analysis

The statistical differences between groups of data (WKY vs. SHR at each matched age) were analyzed by one-way ANOVA followed by a Tukey test (* $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$) using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA).

Results

The serum steroid hormone concentrations in the SHR and WKY rats

A significant increase of the serum content of T (Fig. 1A) and E_3 (Fig. 1C) was noted in the 10-week-old SHR and WKY rats when compared to the juvenile animals. However, in both age groups, the T contents did not differ between SHR and WKY rats (Fig. 1A). Moreover, the concentrations of P_4 (Fig. 1D), CCT (Fig. 1E) and CT (Fig. 1F) were significantly elevated in 10-week-old SHR rats when compared to 5-week SHR and 10-week-old WKY rats (Figs 1D-F). No statistically significant differences in the serum levels of E_2 were found between age groups or between the strains at any of the ages studied (Fig. 1B).

The volumetric measurements of the brain ventricular system in the SHR and WKY rats

The brain ventricular system is enlarged in SHR rats when compared to that in the WKY rats (Figs 2-3). The volumes of the lateral ventricles in 5-week-old SHR rats (Figs 2A and 3B) are approximately one-third larger than in WKY rats (Figs 2A and 3A). The volumetric difference increases with age and in 10-week-

old SHR rats these ventricles are two fold larger (Figs 2A and 3D) than in the WKY rats (Figs 2A and 3C). The total volumes composed of the third ventricles and cerebral aqueduct in the 5-week-old SHR were significantly greater than those of WKY rats (Fig. 2B). Moreover they also are one-quarter larger in the 10-week-old SHR rats compared to the 10-week-old WKY rats (Fig. 2B).

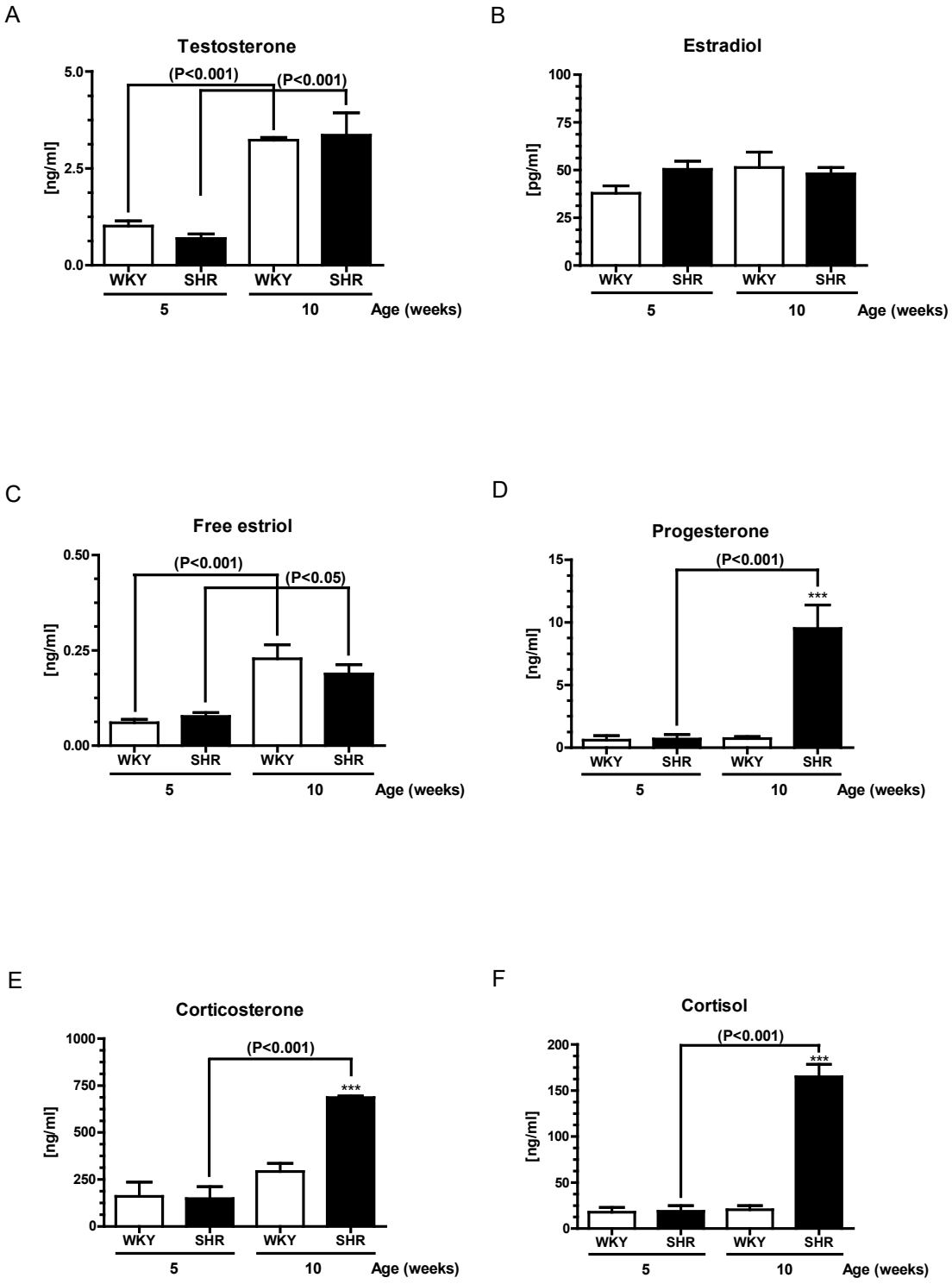


Fig. 1. Mean (±SEM) concentrations of serum testosterone (**A**), estradiol (**B**), free estriol (**C**), progesterone (**D**), corticosterone (**E**) and cortisol (**F**) in the 5 and 10 weeks old spontaneously hypertensive rat (SHR, n=6 in each group) and Wistar-Kyoto rat (WKY, n=6 in each group). *** – indicate differences (P<0.001) between the WKY and SHR rats. P<0.05; P<0.001 – indicates differences between the WKY and SHR before and after puberty.

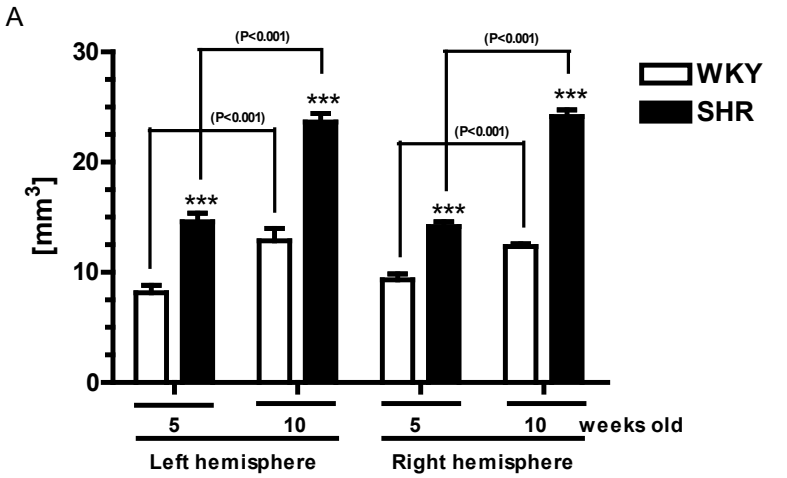
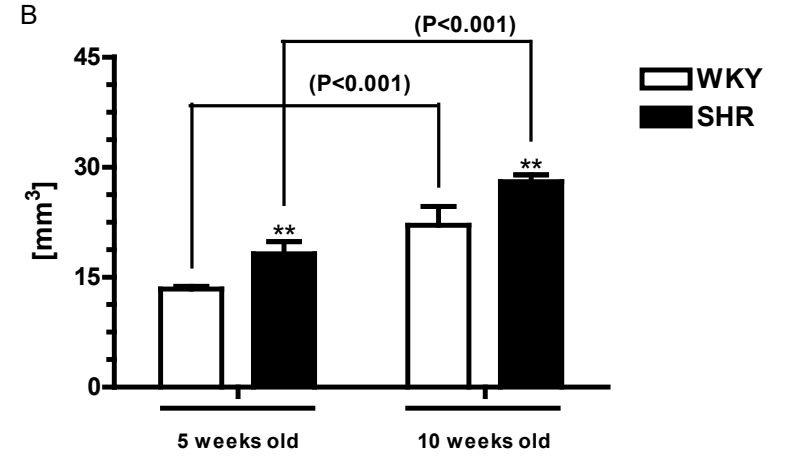


Fig. 2. The volumetric measurements of the brain ventricular system in the 5 and 10 weeks old spontaneously hypertensive rat (SHR, n=6 in each group) and Wistar-Kyoto rat (WKY, n=6 in each group) rats. (**A**) lateral ventricles, (**B**) third ventricles and cerebral aqueduct. Data were expressed as mean standard deviation (SD). **; *** – indicate differences (P<0.01; P<0.001) between the WKY and SHR rats. P<0.001 – indicates differences between the WKY and SHR before and after puberty.



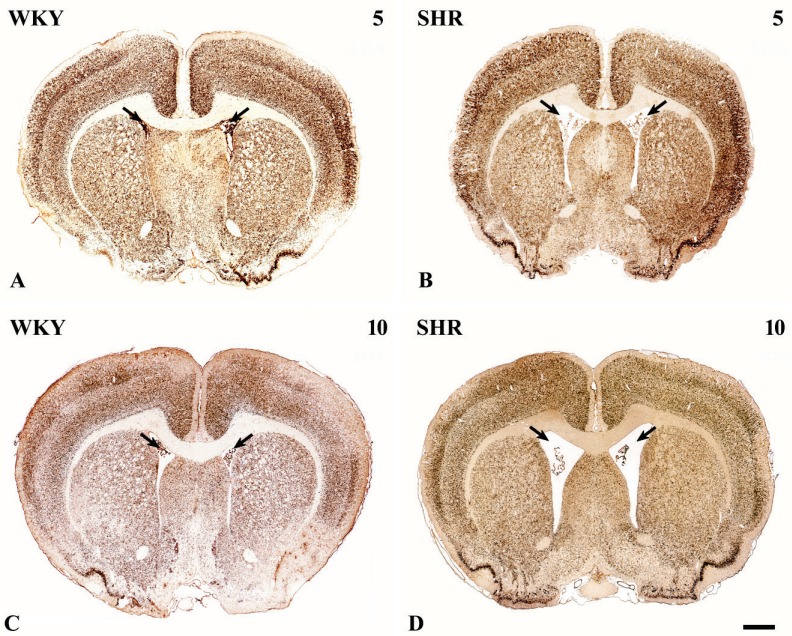


Fig. 3. Low-magnification photomicrographs of coronal sections through the brain of the WKY (**A** and **C**) and SHR (**B** and **D**) rats illustrating enlargement of the left and right lateral ventricles (arrows) in SHR rats. Note that the size differences are bigger in 10-weeks old animals (**C** and **D**) when compared to the 5-weeks old animals (**A** and **B**). Scale bar: 1 mm.

Discussion

The present experiment showed that the serum steroid hormone contents differed significantly between the SHR and WKY rats; however, these differences were only evident in 10-week-old animals. The most striking differences were observed in P_4, CCT and CT content, with subtle differences in the T and E_3 content. Moreover, the differences in serum steroid hormones levels in the 10-week-old SHR rats were accompanied by twofold greater volumes of the brain lateral ventricles in those animals when compared to the WKY rats. An enlarged ventricular system was previously reported in mature SHR rats (Bendel and Eilam 1992). Salerno *et al*. (1992) suggested that standing hypertension results in structural changes in the human brain, e.g. a rise in mean volumes of the right and left lateral ventricles.

Testosterone

The immunoenzymatic determination revealed a significant increase of the serum T-content in the 10-week-old SHR and WKY rats when compared to the juvenile animals. However, in both age groups, the T-contents did not differ between SHR and WKY rats. The first phenomenon, i.e. a significant increase in the serum T-content in the 10-week-old animals is not surprising and quite easy to explain. It is well known that the testosterone level in rats is low in the prepubertal period, but it increases significantly during puberty (Döhler and Wuttke 1975). 5-week-old SHR and WKY rats are prepubertal, while 10-week-old animals are postpubertal. The second phenomenon, i.e. the lack of differences in T-contents between SHR and WKY rats in both age groups, is more difficult to explain and very intriguing. There is consensus that 5-6-week-old SHR rats are in the course of ADHD while with age they develop hypertension (Reckelhoff *et al.* 1998). However, it seems that both of these syndromes have no or very little impact on the serum T-content according to present results. There are many studies in rats (Wall *et al.* 1992, Dorn *et al.* 2009) as well as a few in humans (Wang *et al.* 2017, Pompa *et al.* 2007, Yu and Shi 2009) which try to explain the exact role of T in the course of ADHD. However, these results are sometimes contradictory. For example, there is a hypothesis that prenatal T-exposure increases the risk of ADHD occurrence in boys (Martel and Roberts 2014). In support, some authors have revealed a significant positive relationship between T-concentration in saliva and aggressive behavior in adolescents (Pompa *et al.* 2007, Yu and Shi 2009). However, other authors did not find such a relationship (Dorn *et al.* 2009, Wang *et al.* 2017). There is also evidence that the serum T-levels were significantly higher in castrated juvenile and T-treated SHR rats than in WKY rats (Pompa *et al.* 2007). On the other hand, the salivary levels of this hormone in children with ADHD (boys and girls) did not change significantly in the group treated with methylphenidate or the untreated group (the intact group was not tested; Wang *et al.* 2017). It should be noted, however, that there was early androgen treatment in males (King *et al.* 2010). This fact coincides well with brain abnormalities and symptoms observed in SHR rats and ADHD patients (Castellanos *et al.* 1996, Castellanos *et al.* 2002, Sontag *et al.* 2011). The role of T in hypertension is also strongly postulated (Louis and Howes 1990, Yu and Shi 2009). For example, the serum T-level in the SHR male rats is high in the 12-week-old animals and is accompanied by high blood pressure (Reckelhoff *et al.* 1998). This observation corresponds with the results of Huisman *et al.* (2006), who reported that the serum T content was significantly higher in hypertensive humans of both sexes when compared to the normotensive controls. The lack of differences between 10-week-old SHR and WKY rats in the T-contents observed in the present study may be due to the fact that hypertension develops in 12-week-old SHR rats and, at that time, the T-level is much higher (Reckelhoff *et al.* 1998).

Estrogens

The results demonstrated that the serum levels of E_2 and E_3 do not differ in SHR and WKY rats. E_2 contents also do not differ in 5- and 10-week-old animals, but E_3 is significantly increased in 10-week-SHR and WKY rats. The roles of E_2 and/or E_3 in ADHD are poorly documented, although existing data suggest neuroprotective actions of both of these hormones (Sherwin 2002, Xiao and Becker 1994, Reaven and Chang 1992). For example, E_2 positively impacts some aspects of cognitive function (Sherwin 2002). Moreover, E_2 similarly to P_4, can lead to an increase in the dopamine level in the striatum of female rats, however, unfortunately this phenomenon was not observed in male rats (Xiao and Becker 1994). E_3 is considered to regulate blood glucose concentration (Yamabe *et al.* 2014) and, in this way, relieve symptoms of ADHD in SHR rats, as it has been previously proposed for P_4 and CT (Reaven and Chang 1992, Ryan and Enns 1988). The roles of

E_2 and/or E_3 in hypertension are rather limited as the blood pressure in the SHR rats is independent of estrogen (Reckelhoff *et al.* 1998).

Progesterone

The results demonstrated that the serum levels of P_4 do not differ in 5-week-old SHR and WKY rats or in 10-week-old WKY rats, but in 10-week-old SHR rats the level of this hormone is highly elevated. Such results would suggest rather the role of P_4 in development of hypertension, but some roles of this hormone in ADHD are also postulated. For example, it was reported that ADHD symptoms were significantly reduced in children treated with high doses of P_4 (Nadjafi 2010, Schilling 2014). The positive role of P_4 in ADHD may be supported by the results of Hsu *et al.* (2010) who found significant decrease in the striatal volume in the juvenile SHR rats (5-week-old) which was not observed in postpubertal animals (8-10-weeks-old). Similarly, significant differences in the caudate volume existing between ADHD children and healthy controls diminished with age studied (Castellanos *et al.* 2002). There is also a suggestion that P_4, together with CT, may relieve ADHD symptoms in another manner, namely by modulation of insulin resistance and, in this way, regulate glucose levels. This suggestion is supported by studies showing that both of these steroids lead to decreased maximum insulin binding and [^{14}C]3-O-methylglucose transport in cultured female virgin rat adipocytes (Ryan and Enns 1988). A similar effect was also observed in the juvenile and mature SHR rats where maximal insulin-stimulated glucose transport by isolated adipocytes was lower than in WKY rats (Reaven and Chang 1992). The role of P_4 in hypertension should also not be excluded. For example, it was reported that elevated levels of this hormone can exert antihypertensive effects in rats (Wambach and Higgins 1979). Interestingly, elevated P_4 content accompanied by enlargement of brain ventricles in the 10-week-old SHR rats observed in the present study coincides well with these results.

Cortisol and corticosterone

The patterns of serum CT and CCT contents observed in the present study were quite similar to that of P_4. Thus, the levels of both of these hormones did not differ in 5-week-old SHR and WKY rats or in 10-week-old WKY rats, but in 10-week-old SHR rats their levels were highly elevated. The lack of differences in CT and CCT contents between juvenile SHR and WKY rats is somewhat surprising because some authors have reported that children with ADHD (Isaksson *et al.* 2012) as well as 6-week-old SHR rats (King *et al.* 2010) had lowered CT or CCT levels when compared to non-affected individuals. It is generally known that CT is involved in a wide range of cognitive functions (Gaysina *et al.* 2012) which are deficient/disturbed in ADHD children compared to normal children (executive functions: selective inhibition, working memory and plan implementation; Liu and Wang 2015). These discrepancies may be due to the differences in the age of rats and/or human children or due to the time of sample collections (Kern *et al.* 1996, Buckingham 2006). The elevated levels of CT and CCT in the 10-week-old SHR rats are also interesting since no significant differences in the CT levels were found between adults with ADHD and healthy controls (Corominas-Roso *et al.* 2015). The elevated CT content (in combination with a high content of P_4) found in the present study was probably associated with alleviation of ADHD symptoms by this hormone, which was already discussed above (Ryan and Enns 1988). This assumption is supported by the fact that chronic adolescent CCT exposure reduces impulsive actions without any influence on their general cognitive function or attention ability in male rats (Torregrossa *et al.* 2012). On the other hand, excessive levels of CT and CCT in the mature SHR rats may also be associated with hypertension, as was previously reported in both rodents and humans (Yagil *et al.* 1996, Whitworth *et al.* 1998). For example, glucocorticoids may be influential in the regulation of blood pressure by stimulation of the phosphoinositide signaling system (Ohanian and Heagerty 1992). Another explanation may be autoinflammatory action, e.g. high levels of CT and CCT are connected with inflammatory response and immunosuppression (Coutinho and Chapman 2011) in which the main immunosuppressive and regulatory factors transform growth factor β (TGFβ). Interestingly, a decreased level of this cytokine was observed in spleens from 10-week SHR rats when compared to 5-week SHR rats and both age groups of WKY rats (unpublished data). The decreased level of TGFβ may lead to autoinflammatory action (Lifshitz and Frenkel 2013).

Conclusions

The present study, for the first time, demonstrated differences in the serum steroid hormone levels between SHR and WKY rats. Significant

differences in the serum levels between SHR and WKY rats were mostly observed after puberty. Thus, elevated contents of P_4, CT and CCT in SHR rats may be associated not only with ADHD, but also with developing hypertension, although this requires further study.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Author Contributions

Anna Kozłowska conceived and designed the experiments; Paweł Wojtacha performed the ELISA procedures; Anna Kozłowska, Maciej Równiak and Małgorzata Kolenkiewicz performed the immunohistochemical procedures, Anna Kozłowska analyzed the data and wrote the paper. Maciej Równiak and Meng-Li Tsai performed paper revision.

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