

The Effect of Hypertension on Adult Hippocampal Neurogenesis in Young Adult Spontaneously Hypertensive Rats and Dahl Rats

Adela Pistikova^{1,2¶*}, Hana Brozka^{1¶}, Michal Bencze⁴, Dominika Radostova^{1,2}, Karel Vales^{1,3}, Ales Stuchlik^{1*}

¹ Department of Neurophysiology of Memory, Institute of Physiology, Czech Academy of Sciences, Vídeňská 1083, 142 20, Prague, Czech Republic

² 2nd Faculty of Medicine, Charles University in Prague, V Úvalu 84, 150 06, Prague, Czech Republic

³ Department of Experimental Neurobiology, National Institute of Mental Health, Topolová 748, 250 67, Klecany, Czech Republic

⁴ Department of Experimental Hypertension, Institute of Physiology, Czech Academy of Sciences, Vídeňská 1083, 142 20, Prague, Czech Republic

¶These authors contributed equally to this work.

***Corresponding authors**

Email: adela.pistikova@fgu.cas.cz, ales.stuchlik@fgu.cas.cz

Short title: *The Effect of Hypertension on Adult Neurogenesis in Young Adult Rats*

Abbreviations

ACE inhibitor - angiotensin-converting-enzyme inhibitor

AT - angiotensin

BrdU - bromodeoxyuridine

CPS - cryoprotectant solution

DAB - diaminobenzidine

Dahl SR - salt resistant strain of inbred Dahl rats

Dahl SS - salt sensitive strain of inbred Dahl rats

DCX - doublecortin

DG - dentate gyrus

IP - intraperitoneal

MAP - mean arterial blood pressure

PBS - phosphate-buffered saline

SD - Sprague Dawley

SHR - spontaneously hypertensive rats

TBS - tris-buffered saline

Summary

The dentate gyrus of the hippocampus is one of the few places in the brain where neurogenesis occurs in adulthood. Nowadays, an increasing number of children and young adults are affected by hypertension, one of the factors in the development of cerebrovascular diseases and age-related cognitive deficits. Since these cognitive deficits are often hippocampus-dependent, it is possible that hypertension exerts this effect via decreasing adult neurogenesis which has been shown to be essential for a range of cognitive tasks. We used spontaneously hypertensive rats, which develop hypertension in the first weeks of life. Half of them were treated with the antihypertensive drug captopril. We found that the drug-induced lowering of blood pressure in this period did not affect the rate of adult neurogenesis. In a second experiment, we used another animal model of hypertension – salt-sensitive and salt-resistant strains of Dahl rats. A high-salt diet induces hypertension in the salt-sensitive strain, but not in the salt-resistant strain. The high-salt diet led to salt-induced hypertension, but did not affect the level of adult neurogenesis in the dentate gyrus of the hippocampus. We conclude that hypertension does not significantly affect the rate of hippocampal neurogenesis in young adult rats.

Keywords: adult neurogenesis, captopril, hypertension, Dahl rats, SHR, young animals.

Introduction

Hypertension is a chronic cardiovascular disease that is reaching epidemic proportions in western countries. Although hypertension is traditionally considered an ailment of old age, its prevalence is also increasing among children and youth (Falkner, 2010). Nowadays it is well established that the hippocampus is specifically sensitive to the alterations in blood pressure (Sabbatini et al., 2002, 2000). Importantly, hypertension can lead to the impairments in hippocampus-dependent processes such as learning and spatial orientation, even in the absence of clinical evidence of vascular damage (Harrington et al., 2000; Papademetriou, 2005). Studies show that hypertension decreases (Pietranera et al., 2006, 2008, 2010; Shih et al., 2016) but also increases (Hwang et al., 2008; Kronenberg et al., 2007; Perfilieva et al., 2001) adult neurogenesis. Thus question remains, however, if hippocampal sensitivity to blood pressure also includes alterations of adult neurogenesis in the dentate gyrus (DG).

Neuroscientists recognize a plethora of intrinsic and extrinsic factors that affect the rate of adult neurogenesis in the DG of the hippocampus. Incidentally, the same factors that decrease adult neurogenesis in the DG (chronic stress, aging and depression to list a few) are also associated with impaired cognitive performance (S. W. Lee et al., 2012; Song et al., 2006). Awareness of the factors affecting neurogenesis is therefore of utmost importance. Moreover, special attention should be paid to factors acting in youth, because this is the period when neurogenesis is greatest (Seki and Arai, 1995). High levels of neurogenesis in children and young adults could serve some important role in cognition since it co-occurs with the age where most learning takes place (Amrein and Lipp, 2009).

In the present study we tested the hypothesis that early hypertension is associated with a decrease of adult neurogenesis in the DG. Compared to most other studies we used younger animals in order to capture the time of greatest neurogenesis, and a time where neurogenesis may be most important to cognition (Amrein and Lipp, 2009). We used two complementary approaches: 1) A drug-

induced lowering of blood pressure in inbred spontaneously hypertensive rats (SHRs) treated with the antihypertensive drug captopril; and 2) An increase in blood pressure in a salt-sensitive strain of inbred Dahl (Dahl SS) rats on a diet with high salt content. Inbred salt-resistant Dahl (Dahl SR) rats were used as controls for the effect of salt intake on adult DG neurogenesis without a concomitant increase in blood pressure. SHR inbred rats are a commonly used model of essential hypertension, which develops during the first 3 months of life. Micro-anatomical changes such as increased ventricle size, increased astrogliosis and decreased hippocampal volume have been observed (Sabbatini et al., 2002, 2000). Captopril, an angiotensin-converting enzyme (ACE) inhibitor, successfully reduces the increase of blood pressure with no observed serious side-effects (Richer et al., 1984). The model used here for induced hypertension is an inbred Dahl SS rats. Hypertension develops in the Dahl SS rats after increase of salt intake. In contrast, the Dahl SR rats do not develop high blood pressure even when high-salt diet is administered (Rapp and Dene, 1985). Thus, these two strains are an ideal tool to explore the effects of salt-induced hypertension on adult neurogenesis (Dahl SS), as well as effects of high salt intake on adult neurogenesis without associated hypertension (Dahl SR). In both cases effects are thus examined between animals of the same genetic background. Moreover, we compared adult neurogenesis between these two strains. We labeled proliferating cells at one time point – two weeks prior to euthanasia, because two week old and older neurons are functionally important in learning and memory (Schmidt-Hieber et al., 2004). Our results indicate that neither the captopril treatment, hypertension induced by a high-salt diet, nor the high salt intake alone had an effect on adult neurogenesis.

Methods

Animals

Inbred animals (both SHRs and Dahl rats) from the breeding colony of the Institute of Physiology CAS arrived after weaning at the age of 28 days. Rats were housed in pairs or triplets in transparent

plastic cages under standard light conditions in an air-conditioned animal room with a 12/12 light dark cycle with lights on at 6 am, constant temperature and humidity (22 ± 1 °C; 50 %) and with food and water *ad libitum*. Rats were labeled with stripes on their tails for identification. All animal procedures were approved by the Local Animal Care Committee and complied with the Animal Protection Act of the Czech Republic, EU directive 2010/63/EC and NIH guidelines. The experiments used in this study were approved by the Committee for Proper Procedures in Animals and Welfare of the Institute of Physiology, Czech Academy of Sciences and by the Resort Committee of the Czech Academy of Sciences (Project of Experiments No. 136/2013).

In the first experiment we used 41 male SHR. Rats were randomized into control (n=20) and captopril-treated groups (n=21). Rats in the treatment group received captopril dissolved in the drinking water at 500 mg/l, while control animals received - tap water. The captopril solution or tap water was administered for six weeks.

In the second experiment we used Dahl SS (n=20) and Dahl SR (n=20) male rats. Half of the Dahl SS and half of the Dahl SR groups were fed a special high-salt diet (4% NaCl, Altromin C 1036). The remaining animals were fed a special low-salt diet (0.04% NaCl, Altromin 1324). These diets were administered for six weeks.

Bromodeoxyuridine labeling

To detect the proliferation and survival of newborn neuronal cells we administered bromodeoxyuridine (BrdU) to all SHRs and Dahl rats by intraperitoneal injection at a dose of 50 mg/kg dissolved in saline (20 mg/ml) two weeks before euthanasia (Garthe et al., 2009). Fig. 1 shows representative micrographs of BrdU+ cells in DG of hippocampus.

Blood pressure measurement

After six weeks of captopril medication or consumption of the salt diet we measured mean arterial blood pressure (MAP) in both SHRs and Dahl rats. MAP was measured under thiopental anesthesia (50 mg/kg) in the femoral artery using a catheter connected to a measuring device consisting of an MLT0380/D pressure transducer wired to a QUAD Bridge and PowerLab/8SP (AD Instruments Ltd, Australia). For visualization of experiment time line see the Fig. 2.

Tissue preparation

Immediately after blood pressure measurements animals were transcardially perfused by 0.1 M phosphate-buffered solution (PBS) and subsequently by 4 % formaldehyde. Following perfusions the brains were retrieved and kept in 4 % formaldehyde for 24 hours and cryoprotected in sucrose solutions (consecutively in 10, 20 and 30 %). Afterwards, brains were sectioned to 40 μ m coronal sections on a cryostat (Leica 1850) and stored in cryoprotectant solution (CPS) at -20 °C.

BrdU Immunocytochemistry

First, sections were transferred from CPS to Tris-buffered saline (TBS) and repeatedly rinsed. Sections were then placed into a solution of H₂O₂ and methanol in TBS to block endogenous peroxidase. Then, sections were washed in NaCl 0.9 % to decrease the buffering of tissue. To denature DNA, sections were incubated in 2.5 N HCl for 30 min at 38 °C. Next, sections were washed in PBS to eliminate background staining and quenched in blocking buffer (10 % donkey serum and 0.3 % Triton X-100 in PBS) for 1 hour. Immediately after, sections were incubated with a rat monoclonal anti-BrdU antibody (1:500; clone BU1/75, Serotec OBT0030) diluted in blocking buffer for 48 hours at 4 °C in a cold room. The next day, sections were repeatedly rinsed in PBS. After washing, sections were incubated with a biotinylated donkey anti-rat secondary antibody (1:500, Jackson/Dianova, 712-065-153) diluted in PBS with 3 % donkey serum and 0.3 % Triton X-100 for 2.5 hours. Sections were then rinsed in PBS and incubated with avidin/biotin complexes conjugated with horseradish peroxidase. 9 μ m/ml of each reagent (ABC elite kit standard, PK6100, Vector Laboratories, USA) was used diluted in

PBS for 1 hour. Next, sections were placed in diaminobenzidine (DAB) solution 0,25 mg/ml in PBS (DAB, Sigma Aldrich, D5905) with 0.01% H₂O₂ and 0.04% NiCl₂ followed by rinsing with tap water and PBS. Finally, sections were mounted on gelatin coated slides and cover-slipped (Neo-Mount®, Merck). BrdU+ cells were counted under a light microscope using a 40x objective. The upper focal plane was disregarded to avoid over-counting cells at the cutting surfaces. The whole area of the DG in each section was used to estimate total cell count. Specifically, BrdU+ cells were counted in each section bilaterally and this value was multiplied by six (sampling interval) to acquire an estimate of labeled cells for the whole DG.

Data analysis

Data was analyzed using SPSS software (SPSS Statistics for Windows, Version 23.0. USA). First, the distribution was assessed by the Kolmogorov-Smirnoff test. If data were not normally distributed we used a suitable transformation to achieve normally distributed data. Then, data were analyzed using an appropriate parametric test (analysis of variance (ANOVA) or t-test). Significance was accepted at a level of $p < 0.005$.

Results

Twelve control SHR animals and 13 captopril-treated animals were used in the analysis of MAP. Data was analyzed using an independent-sample t-test. We observed significantly lower MAP in the captopril-treated group compared to control group [$t(23)=10.200$, $p<0.001$] see the Fig. 3A. When analyzing the number of BrdU+ cells, data point were removed in they were more than three standard deviations from the average. This was most likely due to incorrectly performed intraperitoneal injections containing BrdU which resulted in an absence of correctly labeled brain tissue. Therefore 19 control animals and 17 captopril-treated animals were used in the analysis. The independent-sample *t*-test did not show any effect of captopril treatment on the number of BrdU+ cells [$t(34)=-1.298$, $p=0.203$] see the Fig. 3B.

Mean arterial blood pressure was recorded in 13 Dahl SS and 15 Dahl SR animals. Two-way analysis of variance was used to analyze the data - salt intake and rat strain were used as two independent variables. There was a significantly lower MAP in the Dahl SR compared to the Dahl SS rats [$F(1,24)=21.774$, $p<0.001$]. However, there was no overall significant change in MAP induced by the high salt diet [$F(1,24)=2.735$, $p=0.111$]. However, the interaction between genetic strain and salt diet was close to being significant [$F(1,24)=3.276$, $p=0.083$]. This suggests a strain-specific effect of salt on different Dahl rat strains, as has been reported in the literature (Rapp and Dene, 1985). Moreover, we analyzed the difference in MAP between the high and low salt diet separately in the SS and SR strains. In the SS strain, the independent t-test showed a significant increase in MAP for the group on a high salt diet compared to the low salt diet [$t(11)=2.435$, $p=0.033$]. In the SR strain there was no significant difference in MAP between the high salt and low salt diets [$t(13)=-0.114$, $p=0.911$] see Fig. 3C. The analysis of BrdU+ cell numbers was conducted on 15 SS and 13 SR animals. Data was analyzed using two-way ANOVA. There was no significant difference in the number of BrdU+ cells between the Dahl SR and SS strains [$F(1,23)=0.048$, $p=0.829$]. The effect of salt intake on the number of BrdU+ cells was also not significant [$F(1,23)=0.007$, $p=0.934$] see Fig. 3D. Moreover, there was no significant interaction between strains and type of salt diet [$F(1,23)=0.408$, $p=0.529$].

Discussion

In our experiments we found that hypertension in young adult rats did not affect the level of adult neurogenesis in the dentate gyrus (DG). In our first experiment we used SHRs that develop hypertension in the first months of their life. We found no differences in the number of BrdU+ cells in the DG of the hippocampus between untreated SHRs with hypertension and normotensive captopril-treated SHRs. In the second experiment we used two strains of Dahl rats, salt-sensitive (Dahl SS) and salt-resistant (Dahl SR). In Dahl SS rats there was no effect of hypertension on the number of BrdU+ cells. Similarly, in Dahl SR rats we also did not find any effect of high salt intake on adult

neurogenesis. Moreover, there was no difference in BrdU+ cell numbers between the Dahl SS and Dahl SR rats.

There have been several other experiments that have studied cell proliferation /survival in the DG of the hippocampus in hypertensive animals compared to normotensive control animals, but our study is the first one to focus on young adult rats. Pietranera and colleagues (2006) used two animal models of hypertension: SHRs (compared to normotensive Wistar-Kyoto rat) and deoxycorticosterone-salt-induced hypertension rats (the DOCA-salt model; compared to control rats without DOCA-salt treatment). In both models they found a reduction of adult neurogenesis in the DG of the hippocampus measured by the number of BrdU+ cells. Further studies by this group confirmed these findings (Pietranera et al., 2008, 2010). Another study, using mice, was conducted by Shih and colleagues (2016). In their experiment hypertension was induced by the constriction of one of the two renal arteries (2K1C hypertension). In mice that developed hypertension they observed a reduction in cell proliferation measured by the number of BrdU+ cells and a reduction in the number of immature neurons measured by doublecortin positive cells (DCX+) in the DG. Compared to our study, there are several methodological differences that could have contributed to the different results. Primarily, these studies used older animals and most of them compared two different genetic strains. Nonetheless, several other studies have found the opposite result - an increased level of adult neurogenesis in hypertensive rats. Perfilieva et al. (2001) compared the number of BrdU+ cells between SHRs and Sprague Dawley (SD) rats. Both cell proliferation and cell survival was increased in SHRs measured by the number of BrdU+ cells after one day or 30 days after BrdU application. Kronenberg and colleagues (2007) showed an increase in the number of DCX+ and BrdU+ cells in the DG both in SHR hypertensive strain and SHR stroke-prone hypertensive strain compared to their genetic control Wistar Kyoto rats. Hwang et al. (2008) measured proliferation using the number of DCX+ cells at various ages (1, 8 and 12 months) in SHRs compared to SD rats. At all ages the number of DCX+ cells in the DG was higher in SHRs. Since none of these latter experiments compared animals with the same genetic background, conclusions about the

relationship between hypertension and adult neurogenesis can hardly be made. These variations in adult neurogenesis may simply reflect strain differences, as differences in adult neurogenesis were found also between strains of mice (Kempermann et al., 1997; Kim et al., 2009) and rats (Perfilieva et al., 2001).

Although we did not find any alteration of adult neurogenesis after the treatment with ACE inhibitor captopril, some literature suggests that the reduction of angiotensin II levels or the blockade of angiotensin (AT) receptor 1 could alter adult neurogenesis by a blood-pressure-independent mechanism. It has been speculated that this effect is mediated by reduced activation of the AT₁ receptor, which is present on neurons, because inhibition of this receptor protects adult neurogenesis against stress (Ping et al., 2014). Moreover, ramipril, another ACE inhibitor, mitigated the radiation-induced reduction of adult neurogenesis (Jenrow et al., 2010). This observed effect was small but significant after 12 weeks of treatment. However, another study showed that ramipril treatment for 28 weeks did not alter neurogenesis (T. C. Lee et al., 2012). Similarly, L-158, an AT₂ inhibitor, does not seem to influence neurogenesis in the rat brain (Conner et al., 2011). On the contrary, the systemic administration of angiotensin II, similar to the observed increases of angiotensin II level during exercise, also increases adult neurogenesis, whereas blockade of the AT₁ receptor prevented this increase in adult neurogenesis (Mukuda et al., 2014). We did not observe any enhancing effect of captopril, which is in line with the results of studies that have utilized ACE inhibitors or blocked AT₁ receptors. Our study thus indirectly supports the results that show that angiotensin II does not significantly affect adult neurogenesis in the dentate gyrus.

Our study has two major limitations. First, we did not assess neurogenesis by BrdU at different stages of maturation but we selected the time of maturation that appears critical in learning and memory (Schmidt-Hieber et al., 2004). Second, we did not characterize BrdU⁺ cells as being neurons. However, literature is consistent that more than 80 percent of newly generated cells are neurons (Cameron and McKay, 2001; Leuner et al., 2010, 2007; Sisti et al., 2007; Waddell and Shors, 2008).

Conclusions

In both experiments, we found no effect of hypertension in young adult rats on adult neurogenesis in the DG of the hippocampus. Six weeks of a high-salt diet given to Dahl SS rats did not affect adult neurogenesis measured by BrdU incorporation into dividing cells. Accordingly, no effect of high salt intake was seen in Dahl SR rats. Similarly, there was no effect on BrdU incorporation in the SHR model of genetic hypertension compared to SHR treated with the antihypertensive drug captopril.

Acknowledgements

This work was supported by the Charles University in Prague, project GA UK 750214, GACR Center of Excellence P304/12/G069 and by Academic project M200111204. Institutional support for Institute of Physiology, CAS, was provided by RVO: 67985823. Institutional support for NIMH, CZ was provided by the project “Sustainability for the National Institute of Mental Health”, under grant number LO1611, with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the NPUJ program.

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Fig. 1. Micrographs illustrating BrdU+ cells in DG of hippocampus in SHRs.

(A) SHR with captopril treatment. (B) SHR control group. BrdU+ cells are shown by black arrows.

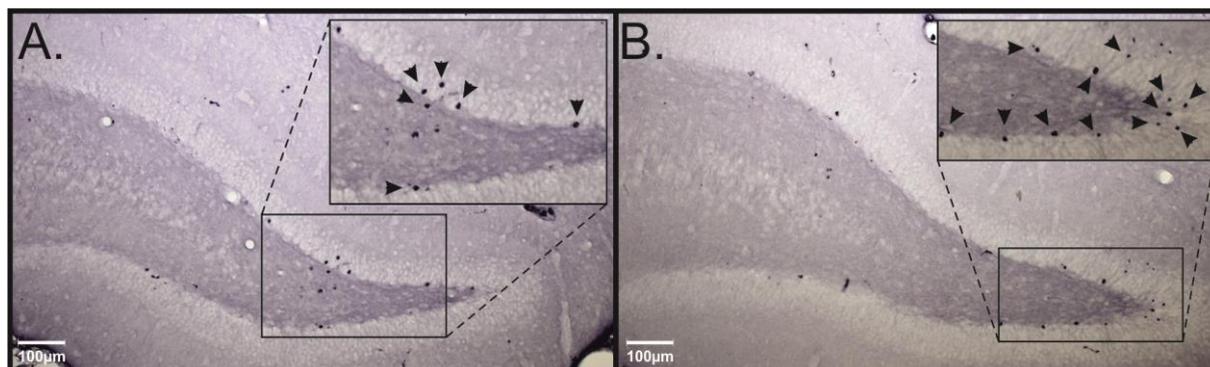


Fig. 2. Experimental scheme:

In the first experiment SHRs received a captopril solution or tap water for six weeks. In the second experiment half of the Dahl SS and half of the Dahl SR groups were fed a high-salt diet and the remaining rats were fed a low-salt diet. Rats received these special diets for six weeks. In both experiments we administered BrdU by i.p injection to all SHR and Dahl rats two weeks before sacrifice. After six weeks of captopril treatment or feeding special salt diets we measured arterial blood pressure (MAP) and perfused all rats transcardially for preparation of brain sections.

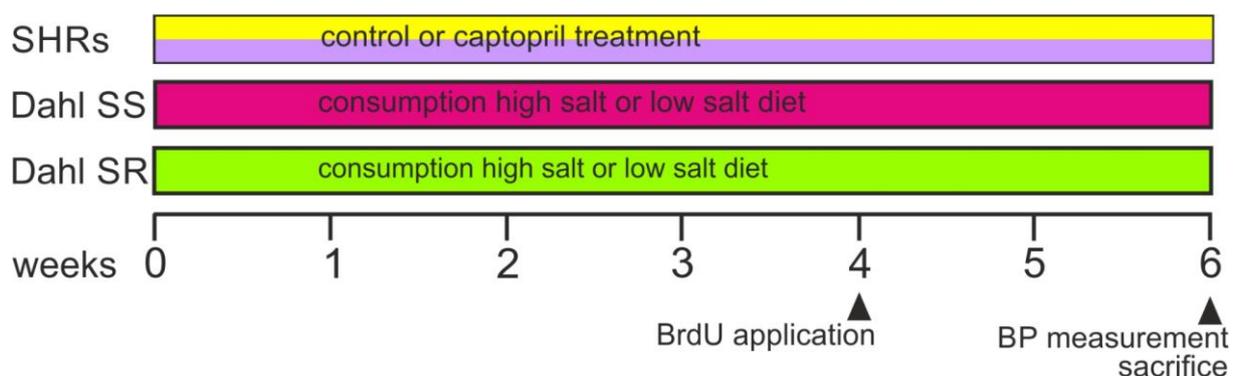


Fig. 3. Mean arterial blood pressure (MAP) and number of BrdU+ cells.

(A) Mean arterial blood pressure (MAP) in control and captopril-treated SHR groups. There was significantly lower MAP in the captopril-treated group ($p < 0.001$), denoted by *. (B) The number of BrdU + cells in control and captopril-treated groups. There was no difference between groups. (C) MAP in Dahl SS or Dahl SR with high-salt (HS) or low-salt (LS) diets. Only Dahl SS rats with the high salt diet had significantly increased MAP compared to Dahl SS with the low-salt diet ($p = 0.033$), denoted by*. (D) The number of BrdU + cells in Dahl SS or Dahl SR with high-salt (HS) or low-salt (LS) diets. There was no difference between groups. Data are given as means \pm S.E.M.

