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ORIGINAL ARTICLE

The Long-Term Sex-Specific Effects of Sub Chronic Prenatal Asphyxia on miR-15b-5p and miR-124-3p Levels in Limbic System of Rats and Possible Connection with Regulation of GABA-ergic System

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Abstract

Sub chronic prenatal asphyxia (SPA) refers to reduced levels of transport of nutrients, oxygen and metabolic waste products between the foetal and maternal organisms. This results in hypoxic conditions in foetal tissues, accumulation of toxic products of metabolism and disruption of acid-base balance. The most sensitive organ of the foetus is the brain. The effect of this prenatal risk factor is often observable in adulthood at the level of behavior and function of the higher nervous system. The experimental animals in our study were adult offspring of pregnant Wistar rats exposed to a lowered oxygen-containing environment (10.5% O_2) during 12 h on the 20th day of gestation (H12 group) or 8 h in the 19th and 20th day of gestation (H8 group). The inhibitory system is one of the key systems involved in the development, as well as sexual differentiation, of the brain. Using in situ hybridisation methodology with radio-labelled cDNA probes, we determined the mRNA levels transcribed from the *gad1* (GAD67), *gad2* (GAD65) and *slc6a1* (GAT1) genes in limbic neuronal structures. We noticed discrete gender differences between animals prenatally affected by asphyxia and control animals. We were also focused on detecting the presence of two miR molecules (miR-15b-5p and miR-124-3p) with the use of FISH methodology. Both regulatory miRs have been shown to be involved in the ability of tissues to resist or recover from hypoxic conditions. We were able to demonstrate the changed level of miR-15b-5p of prenatal asphyxia exposed rats. Which is, however, conditioned by sex.

Introduction

About 3% of births per year are associated with perinatal asphyxia, and about one million of these new-borns die globally. Between 20–50% of asphyxiated new-borns with hypoxic-ischaemic encephalopathy die within the new-born period, while up to 25% of the survivors will exhibit permanent neuropsychological handicaps, such as mental retardation, cerebral palsy, learning disability and epilepsy (Vannucci &

Hagberg 2004). There are two important molecular and cell cascades elicited by perinatal hypoxic insults. One leads to the removal of cells damaged by the reduced supply of oxygen, implying activation of ubiquitination, peroxisomal and caspase pathways, resulting in apoptosis or necrosis, and the latter encompassing mild to severe inflammation. The other cascade is activated to compensate for cell loss by multiple mechanisms of DNA repair. Naturally occurring nicotinamide, with its various mechanisms of action, could support these compensatory mechanisms if given systemically for therapeutic means. In any case, both cell loss and cell rescue eventually entail more or less subtle consequences on brain development, neuronal wiring, and neuron–glia interactions. These tentatively negative consequences are probably reinforced by additional negative impact during development (puberty and adolescence), resulting in psychiatric disorders, if not aggravating neurological deficits (Herrera-Marschitz *et al.* 2014).

Although no single animal model was found to be ideal for all research of brain injury due to hypoxia/ischaemia, some models are noticeably superior to others. The foetal sheep, new-born lamb and piglet models are well suited for the study of acute and subacute metabolic and physiologic endpoints, whereas the rodent and primate models can be used for long-term neurological and behavioural outcome experiments as well (Roohey *et al.* 1997). It was the long-term effect that was of interest to us. The laboratory rat *(Rattus norvegicus)* is one of the best-studied model organisms in neurophysiology. It matures quickly and has a relatively short life. For this reason, the rat model of prenatal asphyxia appeared to be the most appropriate for our use.

GABAergic interneurons are the only source of GABA and the main source of inhibition in the mammalian central nervous system. Depending on the brain region, they constitute 10–25% of the total number of cortical neurons, where they play a crucial role in controlling and orchestrating the activity of pyramidal neuron assemblies (Le Magueresse & Monyer 2013). An important feature of the development of GABAergic system is its long duration. The process of formation and maturation of the inhibitory neuronal system is best studied in mice. At prenatal stages (E14–E19) in the corticogenesis, GABA-positive cells can be observed mainly in the subplate, marginal and subventricular zone (SVZ). GABA-immunoreactive cells disappear from these regions between P0 and P8. GABA immunoreactivity is present at E14 also in the cortical plate but exhibits its mature pattern only postnatally. By P16–P21, the pattern of GABA immunoreactivity is similar to that of the adult brain (Del Rio *et al.* 1992; Le Magueresse & Monyer 2013).

We were interested in variations in gene expression of *gad1*, *gad2* and *slc6a1* genes in hippocampus, amygdala and thalamus. *Gad1* is a gene encoding the GAD67 protein, *gad2* is gene coding the GAD65 protein, and the *slc6a1* gene encodes the sodium- and chloride-dependent GABA transporter type 1(GAT1). Both variants of the enzyme glutamate decarboxylase (GAD65 and GAD67) catalyse a reaction in which it is converted from glutamate to GABA. They are formed mainly in inhibitory neurons and are a limiting factor in the regulation of neuronal inhibition in the brain (Olexová *et al.* 2016). GAT1, in turn, is the most widely used GABA transporter, which removes this neurotransmitter from intercellular space. It occurs on the surface of inhibitory neurons, but also on the surface of glia (Snow *et al.* 1992; Guastella *et al.* 1990).

Genes whose expression responds to hypoxia are regulated by miRNAs (Cao *et al.* 2019; Wu *et al.* 2020;

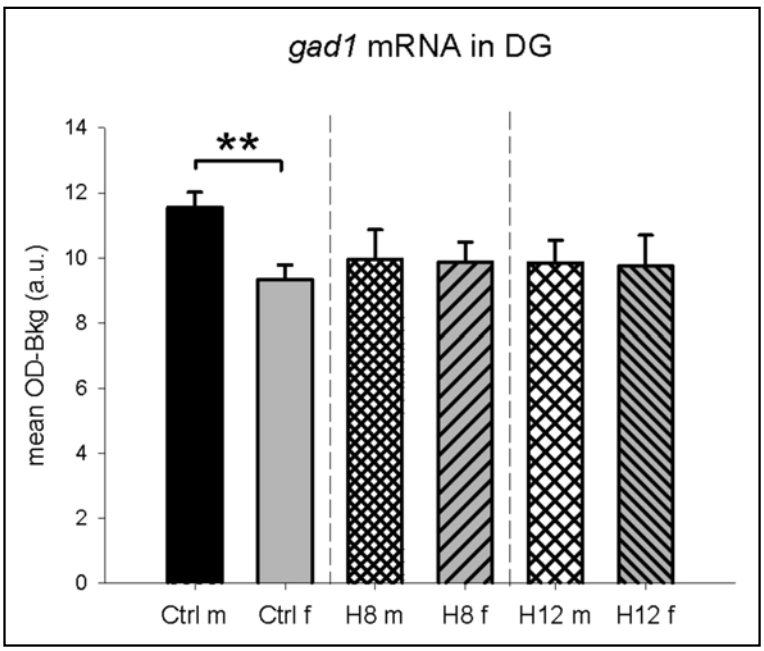

Fig. 1. Mean optical density (OD) of *gad1* signal in dentate gyrus (DG) of control rats (control; males (m), n = 7; females (f), n = 8), prenatal asphyxia-affected rats at E19 and E20 for 8 hours (H8; males (m), n = 7; females (f), n = 6) and prenatal asphyxia-affected rats at E20 for 12 hours (H12; males (m), n = 8; females (f), n = 8). ** df = 13, $p = 0.00507$, but evaluated solely by student t-test for each group of animals.

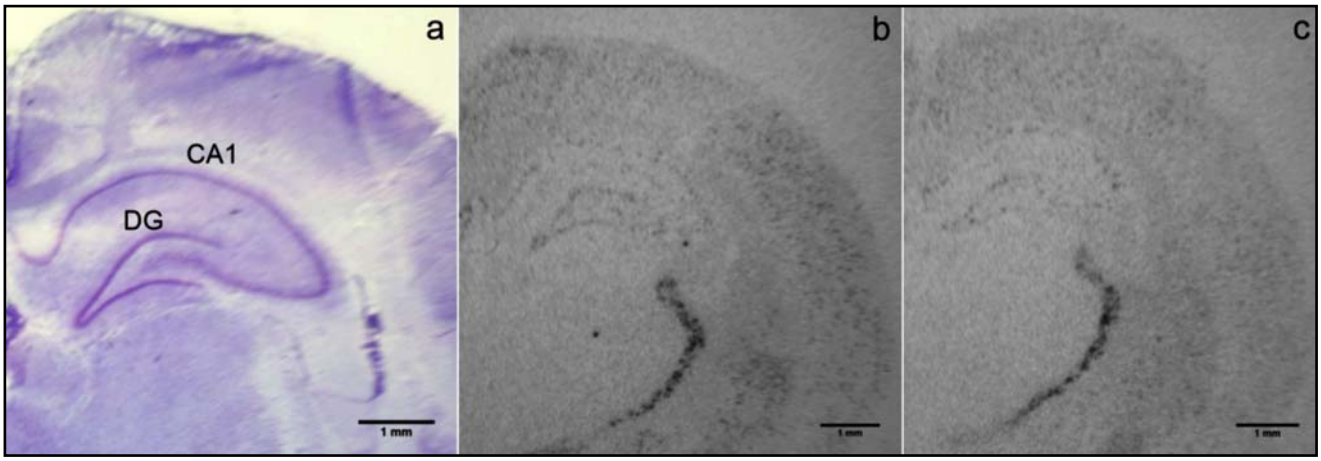

Fig. 2. Representative photographs of Nissl staining. (**a**; Nissl) With marked CA1 and dentate gyrus (DG) structures. The other two photographs show the ISH signal of the *Gad1* gene mRNA in the brain of a control male (**b**) and a female (**c**), respectively.

Wang *et al.* 2019). Micro RNAs (miRs) are small (20–22 nt) non-coding RNA molecules with a regulatory role in the cell (Loohuis *et al.* 2012). Mature miRs modulate gene expression by associating with argonaut-containing complexes to form the RNA induced silencing complex (RISC). By imperfectly or near-perfectly base pairing with sequences in the 3′ UTRs of target mRNAs. Mature miRs modulate gene expression through transcript destabilisation and translational attenuation (Huntzinger & Izaurralde 2011). Depending on their sequence, they are capable of complementary binding to particular mRNAs and in this way negatively regulate the production of new proteins in the cell, although the ability to switch from repression to activation of transcription has also been documented (Vasudevan *et al.* 2007).

Based on in silico analyses [TargetScan.org] we were able to identify the target mRNAs of specific genes, that are possible controlled by the two miRs evaluated by us in the experiment: the focus is on two molecules, miR-15b-5p and miR-124-3p. We wondered whether the model of animal asphyxia during pregnancy would leave demonstrable changes at the miR level in the brain of adult rats. Both miRs (miR-15b-5p and miR-124-3p), by their presence in the cell, are capable of regulating gene expression of genes involved in the physiological response to hypoxic conditions (Banasiak *et al.* 1999; Yamaguchi *et al.* 1999).

The asphyxia conditions simulated in our animal model coincide with the period of migration of progenitor cells. Some of these progenitor cells become inhibitory neurons in the brain. Through the expression of the *gad1* and *gad2* genes, and partly also of *slc6a1*, we can indirectly identify the effect of asphyxia or distinguish between single and repeated asphyxia, possibly to identify sexual specifics in the long-term response of nervous tissue to these pathological conditions. We hypothesise that the reduced level of oxygen in this critical time window may affect the amount and "condition" of these cells and the manner of their establishment in the still-forming brain. The aim was to find any type of consequences of this disrupted process in adulthood.

Material and methods

Animals

Virgin female Wistar/DV rats (weight 200–220 g, aged 3–4 months, n = 40) of monitored conventional breeding were obtained from the breeding station Dobra Voda (Bratislava, Slovak Republic, reg. No. SK CH 24016). These animals were housed in transparent plastic cages under standard laboratory conditions (12/12 h light–dark cycle, 21 ± 1 °C, 55 ± 10% humidity, food and water ad libitum). After 7 days of adaptation, the females were mated with males in the ratio 1 male: 3 females. The presence of spermatozoa in the vaginal smear was considered day 0 of gestation.

Subchronic perinatal asphyxia

Pregnant rats were exposed to a lowered oxygen-containing environment (reference calibrated gas Class I: 10.5% O_2 in 89.5% N_2 obtained from Linde Gas, Bratislava, Slovakia, EU) for 12 h/day in hermetically sealed hypoxic chambers during sensitive stages of brain development (day 20 of gestation, H12 group, n = 8), and for 8 h/day two days in a row (day 19 and 20 of gestation, group H8, n = 10). The generated CO_2 in the chamber was replaced every 30 min by reflow of the calibrated gas for 60 s (see in Ujhazy *et al.* 2013). As a control, we used the offspring of females (n = 9), whose pregnancy took place without intervention.

Offspring and withdrawal of samples

One part of the adult offspring at the age of 134 days (n = 60, males n = 30 and females n = 30, n = 10 for each combination of sex and asphyxia) were sacrificed by cervical dislocation. To eliminate the effect of mother or confound caused by siblings only one male and one female from the litter were used for

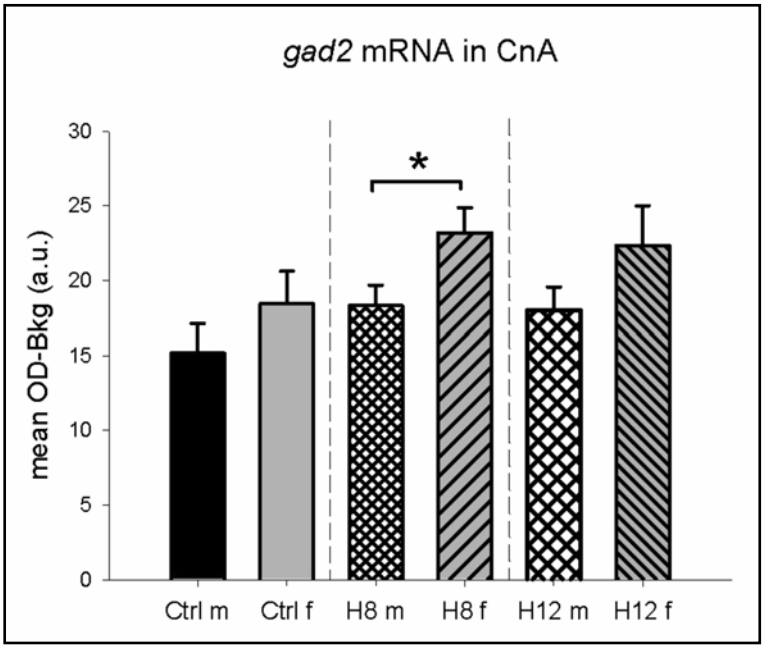

Fig. 3. Mean optical density (OD) of *gad2* signal in central nucleus of amygdala (CnA) of control rats (Control; males (m), n = 10; females (f), n = 10), prenatal asphyxia-affected rats at E19 and E20 for 8 hours (H8; males (m), n = 10; females (f), n = 10) and prenatal asphyxia-affected rats at E20 for 12 hours (H12; males (m), n = 10; females (f), n = 9).* *df* = 18, *p* = 0.037, but evaluated solely by student t-test for each group of animals.

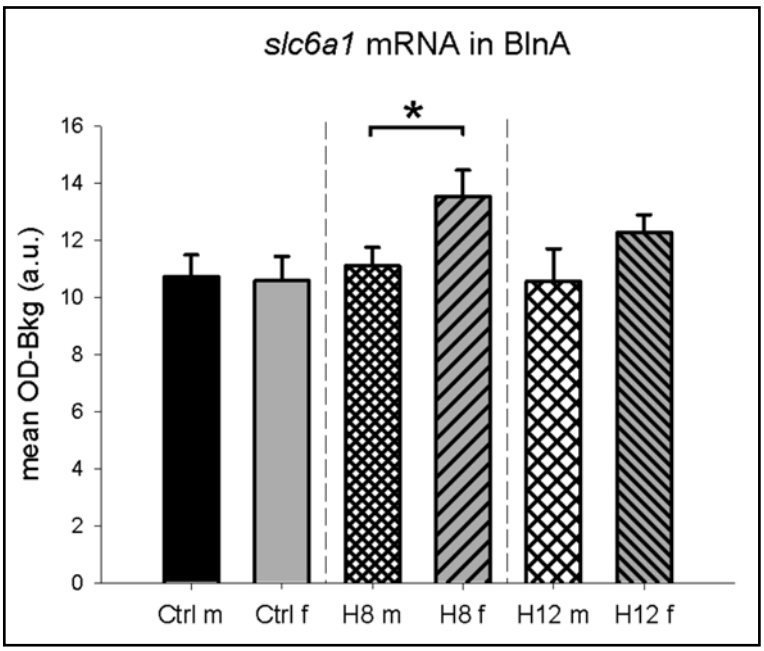

Fig. 4. Mean optical density (OD) of *slc6a1* signal in basolateral nucleus of amygdala (BlnA) of of control rats (Control; males (m), n = 10; females (f), n = 10), prenatal asphyxia-affected rats at E19 and E20 for 8 hours (H8; males (m), n = 10; females (f), n = 10) and prenatal asphyxia-affected rats at E20 for 12 hours (H12; males (m), n = 10; females (f), n = 10). * (*df* = 18, *p* = 0.046), but evaluated solely by student t-test for each group of animals.

experiment. The brains were removed and subsequently embedded in cryoprotective media (Cryomouth, Histolab AB, Göteborg, Sweden, EU). The brains were stored in –80 °C until cryosection. For fluorescent in situ hybridisation, with locked nucleic acid (LNA) modified probes for miR detection as well as standard radioisotopes stained cDNA probes for *gad1*, *gad2* and *slc6a1* mRNA we used cryo-sections from (Bregma 0.2 mm to –2.3 mm according to (Paxinos & Watson 1998) localisation. Cryo-sections (10 μm) include subventricular brain zone (SVZ), hippocampus with dentate gyrus (DG) and CA1, thalamus and amygdala, which are the areas of the brain that we focused primarily on. We used histological Nissl staining for morphological confirmation of the evaluated neuronal structures.

The in-situ hybridisation protocol

The in-situ hybridisation protocol for radio-labelled cDNA probes has been previously described elsewhere (Olexová *et al.* 2016). Briefly, consecutive frozen cryostat sections of the rat brain (10 μm) were mounted on adhesion slides (SuperFrost®Plus, Menzel-Gläser, Thermo Fisher Scientific Inc.) for subsequent hybridisation of chosen mRNAs. A synthetic 30-base oligonucleotide cDNA probe was used 5′- AGCCTTCTTCTG-CACCTTGACTACAAGGGT-3′ which was complementary to nucleotides 85–114 of *slc6a1* mRNA (Snow *et al.* 1992; Guastella *et al.* 1990); 5′- ATAGAGGTATTCAGC-CAGCTCCAAGCATTT - 3′ complementary to nucleotides 1621-1650 of *gad1* mRNA (Kobayashi *et al.* 1987; Najlerahim *et al.* 1990); 5′- ATACTCCATCATTCTG-GCTTTAATCACTGG - 3′ complementary to nucleotides 1665–1694 of *gad2* mRNA (Erlander *et al.* 1991). All sequences were checked with the use of BLAST in the EMBL sequence database for complementarity with non-specific rat RNA. None of the probes we used recognised different non-specific sequences with sufficient fidelity. Probes were 3′-end-labelled with [^{35}S] dATP (1200 Ci/mmol; Perkin Elmer Inc., USA) by terminal deoxynucleotidyl-transferase (Thermo Fisher Scientific Inc., Canada). Unincorporated radioactivity was removed with Sephadex G-50 micro columns (GE Healthcare, Little Chalfont, UK). The sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 5 min, rinsed twice in PBS (pH 7.4), acetylated in 0.25% acetic anhydride, in 0.8% triethanolamine, HCl 0.9%

NaCl (10 min), dehydrated through graded ethanol, delipidated in chloroform (5 min), and dipped in 100 and 95% ethanol. Finally, the slides were air-dried. Sections were hybridised with the labelled probe in 70 µl of hybridisation buffer per slide (50% formamide, 4× SSC, 1× Denhardt's, 10% dextran sulphate, 500 µg/ml sheared single-stranded DNA, 250 µg/ml yeast tRNA) which was mixed before use with 0.1 M dithiothreitol (DTT) with purified radiolabelled oligoprobe (1477388 cpm/µl for *slc6a1*, 638179 cpm/µl for *gad2* and 1568540 cpm/µl for *gad1*). The radiolabelled probe was diluted to a concentration of 4.5×10^6 counts/min/ml. Slides were incubated in humid boxes for 18 h at 41 °C. Post-hybridisation washes were processed by 1× SSC for 5 min at room temperature, then in 1× SSC for 1 h at 55 °C, and in 1× SSC for 1 h at room temperature. Afterwards, the slides were dipped in distilled water and 70% ethanol and air-dried. Slides with probes for *gad2*, *gad1* and *slc6a1* were exposed to Hyperfilm-Beta-Max (Eastman Kodak Company, Chalon-sur- Saône, France, EU) for 20 days. The time was determined empirically. The film was developed in a developer (LQN) and fixed in FOMAFIX (FOMA, Hradec Králové, Czech Republic, EU) solution. After exposition, the slides were stained with cresyl violet to determine the position and shape of the neuronal structures. Autoradiographs were captured with the use ofoptical magnifier and digital camera (ToupCamTm UHP USB, China) and were analysed using NIH software program Image J (v 1.50i NIH, LOCI University of Wisconsin, USA). On each slide, the relative density of individual structures was measured; the optical density (OD) of *slc6a1*, *gad2* and *gad1* mRNA was measured in dentate gyrus (DG) and CA1 in the hippocampus, central (CnA), basolateral (BlnA) and basomedial (BmnA) nucleus of the amygdala and also in the reticular nucleus of the thalamus (RtnT). Optical density signals were bilaterally but separately evaluated on each section. As an internal standard, the background density adjacent to each structure was captured and deducted from the densities of the region of interest (separately one each side). The mean of the left- and right-side values and from four to six slides per animal was taken as the final result.

In situ hybridisation with LNA probes and fluorescence visualisation

The hybridisation protocol for LNA probes on the frozen tissue section was modified

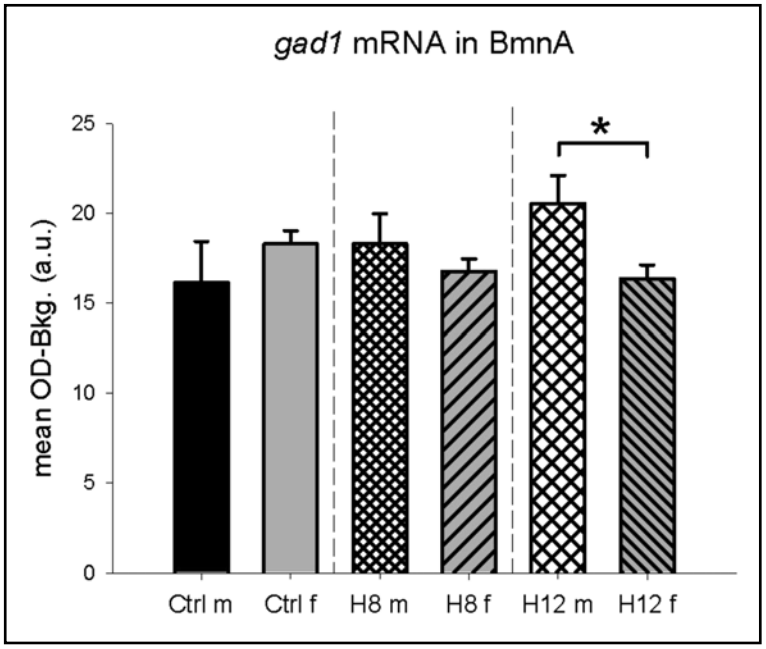

Fig. 5. Mean optical density (OD) of *gad1* signal in basomedial nucleus of amygdala (BmnA) of control rats (Control; males (m), n = 8; females (f), n = 10), prenatal asphyxia-affected rats at E19 and E20 for 8 hours (H8; males (m), n = 10; females (f), n = 8) and prenatal asphyxia-affected rats at E20 for 12 hours (H12; males (m), n = 9; females (f), n = 10).* *df* = 17, *p* = 0.023, but evaluated solely by student t-test for each group of animals.

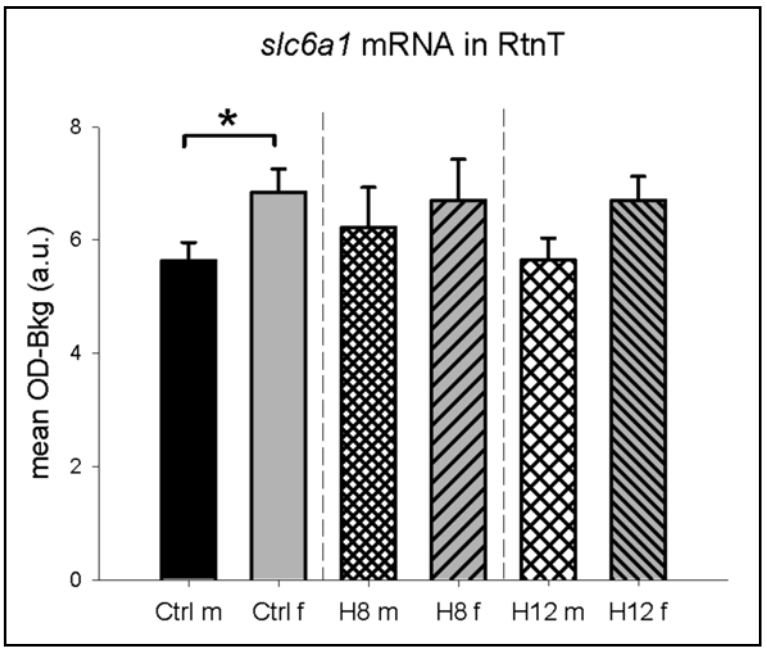

Fig. 6. Mean optical density (OD) of *slc6a1* signal in reticular nucleus of thalamus (RtnT) of control rats (Control; males (m), n = 10; females (f), n = 10), prenatal asphyxia-affected rats at E19 and E20 for 8 hours (H8; males (m), n = 8; females (f), n = 9) and prenatal asphyxia-affected rats at E20 for 12 hours (H12; males (m), n = 9; females (f), n = 10). * *df* = 18, *p* = 0.032, but evaluated solely by student t-test for each group of animals.

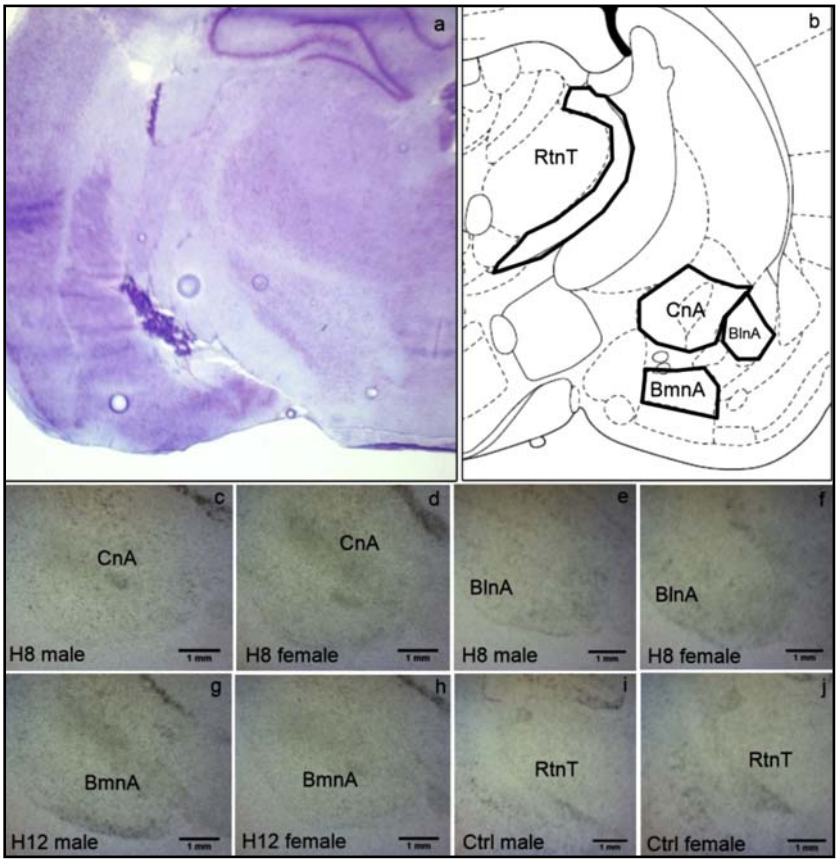

Fig. 7. Picture of Nissl staining (**a**) Nissl and a schematic drawing of the evaluated rat brain region, **b**) with marked central nucleus of amygdala (CnA), basolateral (BlnA), basomedial nucleus of amygdala (BmnA) and reticular nucleus of thalamus (RtnT). The representative pictures of *gad2* signal in CnA of H8 group (**c,d**), *slc6a1* signal in BlnA of H8 group (**e,f**), *gad1* signal in BmnA of H12 group (**g,h**) and *slc6a1* signal in RtnT of Control group (**i,j**).

according to (Silahtaroglu *et al.* 2007) and (Obernosterer *et al.* 2007). The hybridisation temperature and after hybridisation washes was adjusted according to the sequence of used probes (miR15b-5p:/5DiGN/TGTAAACCATGATGTGCTGCTA-3 and miR124-3p:/5DiGN/CATTCACCGCGTGCCTTA-3). Both used LNA probes (Qiagen, Düsseldorf, Germany, EU) where 5-end digoxin (DiG) modified. After post-hybridisation washes was the slides visualised by binding with primary anti-DiG antibodies (Thermo Fischer, Waltham, Massachusetts, USA, 1:300 in 1% goat serum and 0.01% saponin, for 1 hour) and subsequently by secondary anti-goat fluorescence antibodies (Alexa Fluor 490nm, Sigma-Aldrich, St-Louis, USA, 1:1000, for 30 min). The nucleus in slides was labelled with DAPI and covered with Fluoromont (Sigma-Aldrich, St-Louis, USA). The samples were captured with a fluorescent microscope (Zeiss Axio Scope.A1) at magnification 40x. For evaluation and relative quantification of fluorescent signals of labelled miRNAs, we used approximately three sections including lateralisation (average of 6 microscopic photos per sample). Images were evaluated independently by three persons in a double-blind study. The multi-point tool for positive cells counting in Image J software (ImageJ 1.50i, NIH, LOCI University of Wisconsin, USA) was used. The mean amount was normalised by an automatic analyse particle tool for all cells on the images (positive cells/all cells * 1000).

In silico analysis

In silico analysis was performed using the miRBase.org database, release 22.1, according to (Kozomara *et al.* 2019) and TargetScan.org version 7.2 released in March 2018 according to the methodology of (Agarwal *et al.* 2015). Only genes with cumulative weighted context score ≤ −0.2, or aggregate PCt ≥ 0.5 were included, excluding genes with only poorly conserved sites.

Statistical examination

The normality of the data distribution was verified using the Kolmogorov–Smirnov test. If the data distribution did not pass the test, we normalised it by its logarithmic function. The data were analysed by two-way ANOVA (asphyxia condition and sex as independent factors) and one-way ANOVA for asphyxia factor separately for each sex, followed by Tukey HSD post-hoc test. The sex differences were also evaluated within groups by Student´s t-test. The data are expressed as mean ± SEM. The significance limit of $p < 0.05$ was considered statistically significant.

Ethics statement

The experiments were performed in compliance with the Principles of Laboratory Animal Care issued by the Ethical Committee of the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, and the experimental design was approved by the State Veterinary and Food Administration of the Slovak Republic.

Results

Gad1, gad2 and slc6a1 mRNA in the limbic system

The two-way ANOVA statistical comparison of mRNA OD signals of *gad1*, *gad2* and *slc6a1* in DG showed no statistical differences between groups (Control, H8 and H12) and also between sexes (males vs. females), as well as boundaries between both factors (sex x group). Also, one-way ANOVA for each sex separately did not indicate a significant effect of prenatal asphyxia. The Student´s t-test implemented for animals from all experimental groups separately showed significant sex difference between males and females. But only in Control animals (t_{13} = 3.36, p = 0.00507; Figure 1) with higher levels of *gad1* mRNA signal in males than females. This gender difference disappeared in both asphyxia groups. Illustrative images of the hybridised *gad1* signal, as well as the morphology of the evaluated hippocampal structures, are shown in Figure 2. Comparison of the OD of *gad2*, *gad1* and *slc6a1* mRNA in the CA1 region did not reveal significant differences between groups and genders.

The two-way ANOVA statistical comparison of *gad2* mRNA OD signals in CnA showed a statistical difference between sex, with higher levels in females than males $F_{2,53}$ = 6.9835, p = 0.0107. But the Student´s t-test implemented for animals from all experimental groups separately show significant sex difference only in the H8 group (t_{18} = -2.24, p = 0.037; Figure 3). The sex difference in Control and H12 group of animals was not significant. The mRNA levels of *gad1* and *slc6a1* in this amygdala nucleus were not significantly different between groups, including sex. The two-way ANOVA statistical comparison of OD signals of all tree measured genes in BlnA showed no significant differences in sex, group either in boundaries between sex and group. However, the OD of *slc6a1* mRNA in BlnA had a tendency for sex differences at higher levels in females than males $F_{1,54}$ =3.74, p = 0.058. The Student´s t-test implemented for animals separately by groups showed significant sex difference in H8 animals (t_{18} = -2.13, p = 0.046; Figure 4) with no significant difference between sex in Control and H12 group of animals. The two-way ANOVA comparison of mRNA OD of all three investigated genes in the BmnA showed no signifi-

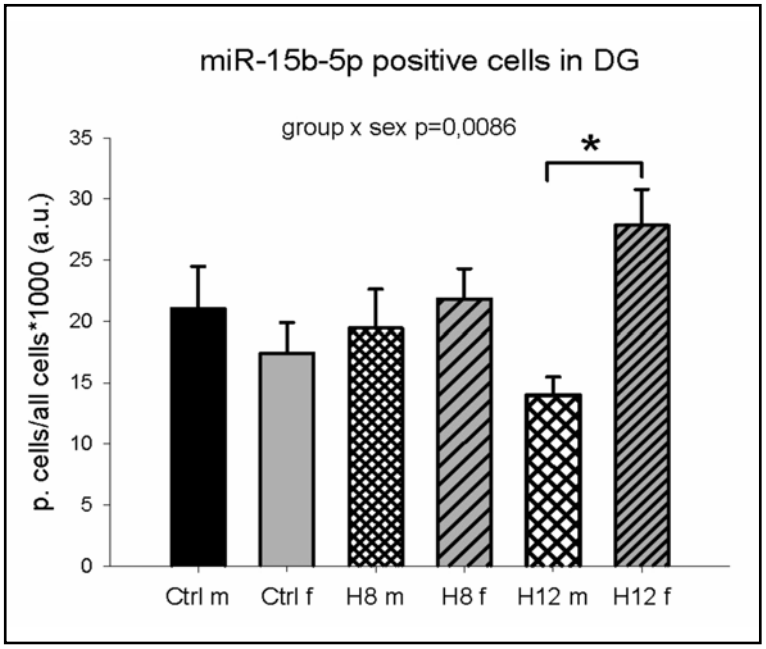

Fig. 8. Relative number of positive miR15b-5p neurons in dentate gyrus (DG) in control rats (Control; males (m), n = 10; females (f), n = 10), prenatal asphyxia-affected rats at E19 and E20 for 8 hours (H8; males (m), n = 10; females (f), n = 9) and prenatal asphyxia-affected rats at E20 for 12 hours (H12; males (m), n = 10; females (f), n = 9). Group and gender factor binding is significant at the level of $F_{2,52}$ = 5.207, p = 0.0086. *represent significance p <0,05 and ***represent significance in binding of sex and asphyxia factors p <0.001.

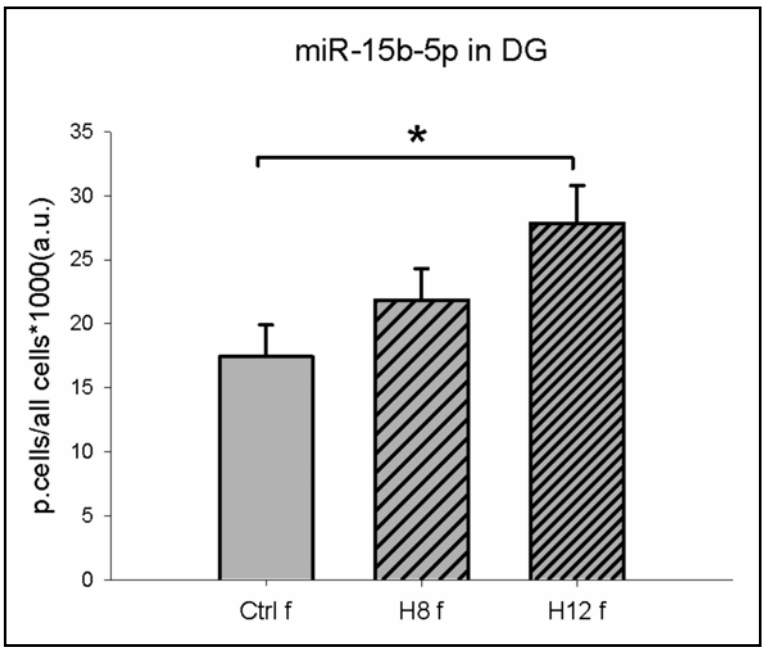

Fig. 9. Relative number of positive miR-15b-5p neurons in dentate gyrus (DG) of females from control group (Control f; n = 10) prenatal asphyxia-affected female rats at E19 and E20 for 8 hours (H8 f; n = 9), and prenatal asphyxia-affected female rats at E20 for 12 hours (H12 f; n = 9) evaluated by one-way ANOVA. The factor of group is significant at the level of $F_{2,25}$ = 4.0049, p = 0.0309. *represent significance p <0,05.

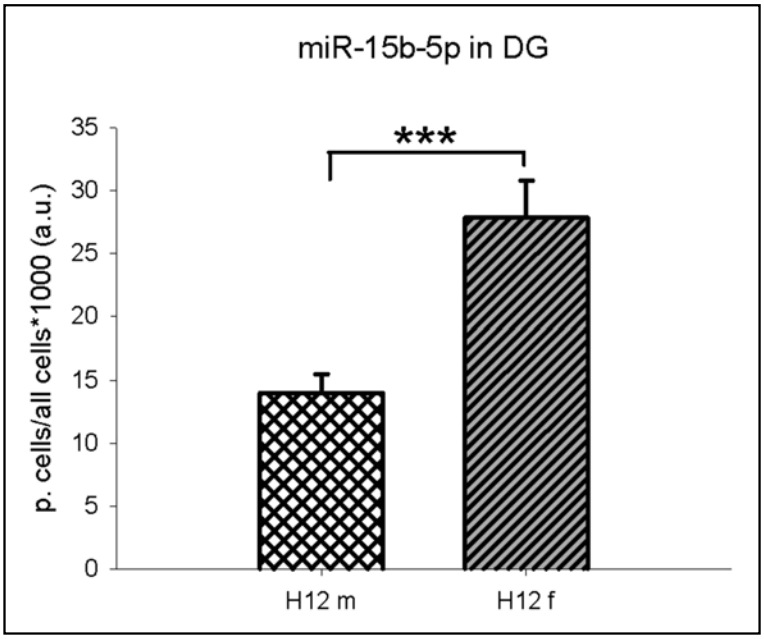

Fig. 10. Relative number of positive miR-15b-5p neurons in dentate gyrus (DG) of prenatal asphyxia-affected rats at E20 for 12 hours (H12; males (m), n = 10; females (f), n = 9) evaluated solely by student t-test, *df* = 17, $p = 0.000453$. ***represent significance $p < 0.001$.

cant differences between sex, group nor boundaries between sex and group. But the *gad1* mRNA OD in this nucleus showed a tendency in the sex and group boundary $F_{2,48} = 2.77$, $p = 0.07$. The Student´s t-test implemented for animals separately by group showed that significant differences between sex was only seen in H12 animals with higher levels of *gad1* mRNA in males than females ($t_{17} = 2.48$, $p = 0.023$; Figure 5). In the other two groups there were no significant differences. The two-way ANOVA statistical comparison of investigated genes in RtnT showed significant sex difference in *slc6a1* mRNA OD ($F_{1,50} = 4.977$, $p = 0.03$), with higher levels in females than males, however, the Student´s t-test showed that for animals by group, the difference was significant in the control group of animals ($t_{18} = -2.31$, $p = 0,032$; Figure 6), but in H8 and H12 animals it disappeared. A schematic picture of the investigated neuronal structures, as well as photographs documenting the observed differences in the optical density of the signals, are shown in Figure 7.

Mir-15b-5p in DG and miR-124-3p in SVZ levels

The correlation of miRs evaluation between three independent evaluators was positive and statistically significant with $p < 0.05$. In the case of miR-124-3p, the data were normalised by its logarithmic function. The two-way ANOVA statistical comparison of positive miR-15b-5p signals in DG showed strong significance in the binding of the sex and asphyxia factor. ($F_{2,52} = 5.2077$, $p = 0.00868$; Figure 8). The one-way ANOVA analysis shows that prenatal asphyxia factor is more significant in females ($F_{2,25} = 4.0049$, $p = 0.0309$; Figure 9). The one-way ANOVA of males showed no significant differences. Comparison of sex differences in each group separately using the Student's t-test further showed that significant sex differences in the amount of miR-15b-5p positive signals were only in the H12 group, which was prenatally exposed to a single 12 h asphyxia ($t_{17} = -4.33$, $p = 0.000453$; Figure 10). The fluorescently labelled miR-15b-5p signal in the DG region is on (Figure 11). We evaluated positive miR-124-3p signals in the SVZ. A two-way ANOVA

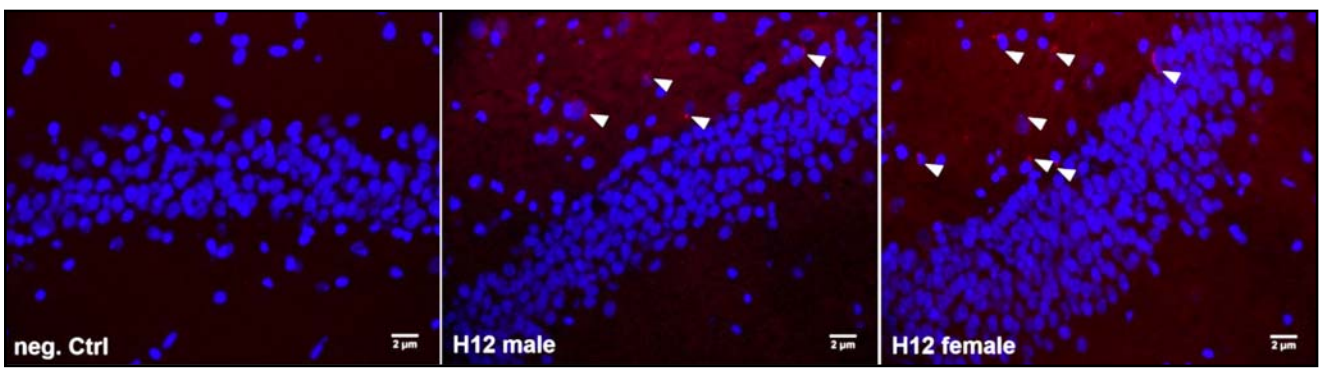

Fig. 11. Representative photos of dentate gyrus (DG) of negative control (neg. Control) without LNA probe and DG of male (H12 male) and female (H12 female) from asphyxia prenatally exposed rats on E20 for 12 hours. White arrows indicate positive miR-15b-5p signals (red).

statistical comparison showed a significant sex difference ($F_{1,46}$ = 8.5076, p = 0.00545) with higher levels in males than females (Figure 12). A one-way ANOVA comparison confirmed that the hypoxia factor did not play a significant role in either sex. However, a separate comparison of gender differences in each group with Student´s t-test showed that the difference in miR-124-3p levels between the sexes in the control group is no significant (t_{14}= 1.38, p = 0.187) in the H8 group is the tendency strong (t_{15} = 2.05, p = 0.052) and in H12 group is the gender difference significant (t_{17} = 2.36, p = 0.030; Figure 13). The fluorescently labelled miR124-3p signal in SVZ is on (Figure 14).

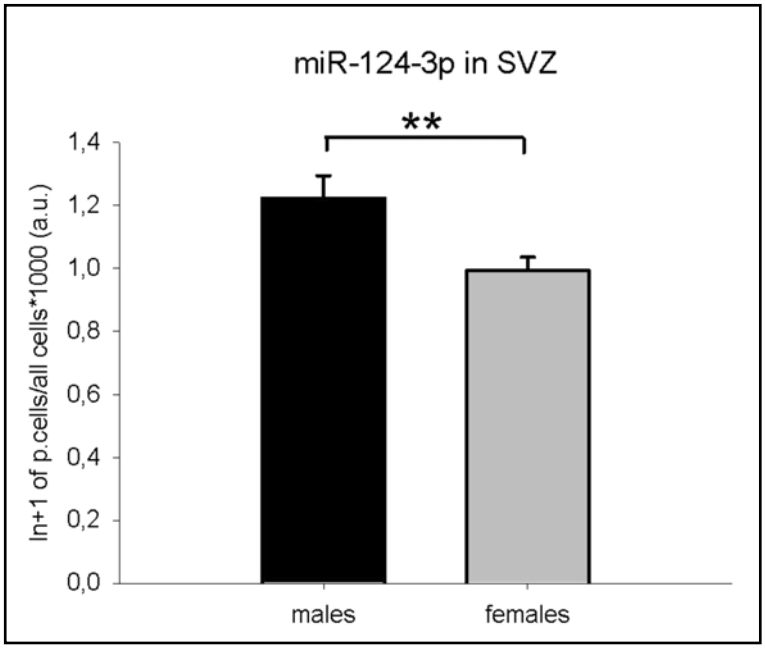

Fig. 12. Logarithm of the relative proportion of miR-124-3p positive cells in the SVZ region in males (n = 23) and females (n = 29). The sex difference is significant at $F_{1.46}$ = 8.50, p = 0.0054. **represent significance p <0.01.

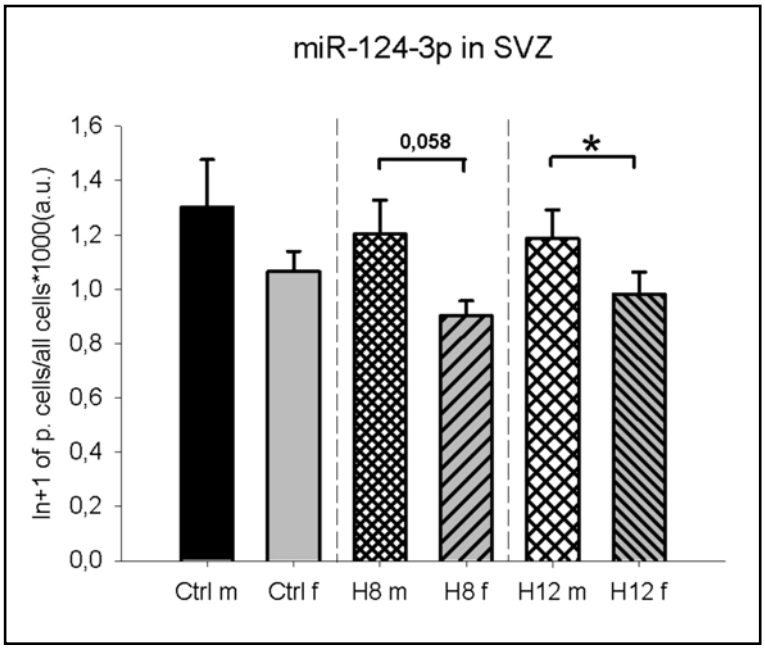

Fig. 13. Logarithm of the relative proportion of miR-124-3p positive cells in the SVZ region of control rats (Control; males (m), n = 6; females (f), n = 10), prenatal asphyxia-affected rats at E19 and E20 for 8 hours (H8; males (m), n = 8; females (f), n = 9) and prenatal asphyxia-affected rats at E20 for 12 hours (H12; males (m), n = 9; females (f), n = 10) evaluated by the student's t-test for each group separately. Only in group of prenatal asphyxia-affected rats at E20 for 12 hours (H12) is the difference significant df = 15, p = 0.0359. * p <0.05.

Discussion

SPA is a complex set of biochemical events that also includes the genetic response of such affected tissue, while several authors agree that the re-oxidation process is at least as similar to the risk factor as asphyxia itself. In the clinical scenario, resuscitation may even imply hyperoxemia, leading to further production of free radicals and oxidative stress and worsening brain injury (Davis *et al.* 2004; Solberg *et al.* 2007; Kapadia *et al.* 2013), therefore, both aspects need to be considered when interpreting the results of our study. It should be emphasised from the outset that the differences in GABAergic system expression observed between control and prenatal asphyxia-affected animals are relatively discrete. In the literature, we can find gender differences in adult rodents at the level of morphology, numbers of granule (Madeira *et al.* 1992; Wimer & Wimer 1985) and pyramidal (Madeira *et al.* 1991) neuronal cells in CA1, but also in the subiculum (Andrade *et al.* 2000). At the level of expression of key molecules for the function of the inhibitory neuro-signal component (GABA), we did not observe gender differences in CA1. But in DG, we observed gender difference in *gad1* signal density of control rats with higher levels in males than females. In DG of rats prenatally exposed to asphyxia, this difference is not present anymore. Higher levels of *gad2* and *gad1* expression in males compared to females were measured in the first postnatal day (P1) in the CA1 of hippocampus, or some structures of the hypothalamus. In the P15 but was the gender difference in the *gad2* and *gad1* gene expression unconfirmed in CA1 but still present in amygdala

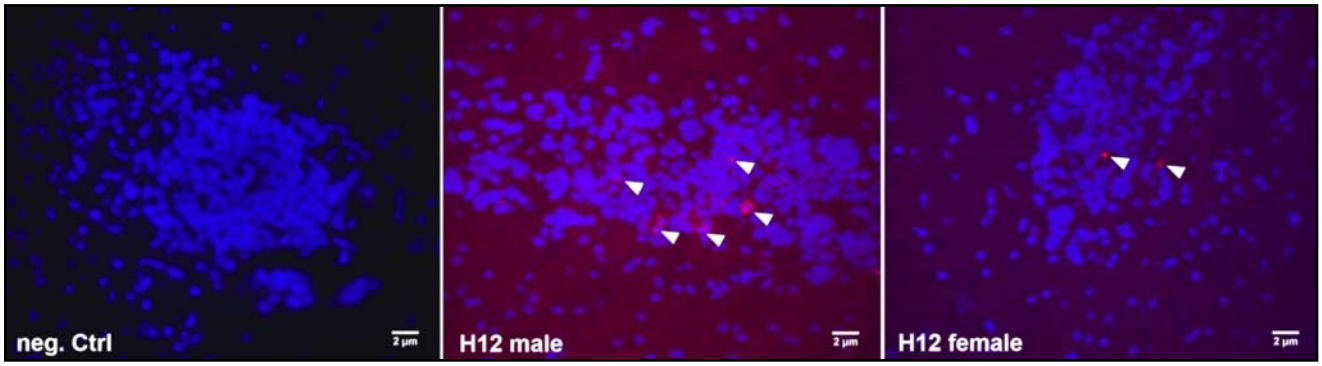

Fig. 14. Representative photos of subventricular zone (SVZ) of negative control (neg. Control) without LNA probe and SVZ of male (H12 male) and female (H12 female) from asphyxia prenatally exposed rats on E20 for 12 hours. White arrows indicate positive miR-124-3p signals (red).

(Davis *et al.* 1996). According to Davis and co-workers (1996), the difference between CA1 and the amygdala, however, is that higher levels are present in females rather than males. We also observed higher mRNA levels of the *gad2* gene and also *slc6a1* gene in adult females compared to males. The *gad2* gene expression was higher in females' CnA region than males, and the *slc6a1* gene expression was higher in females' BlnA region than males. This difference was present in animals exposed to SPA conditions for 8 h on days E19 and E20. At the same time, the organism of these unborn rats was forced to undergo a repeated process of re-oxidation with all the risks associated with it. In contrast, higher mRNA levels of the *gad1* gene were found in males compared to females in the BmnA region of amygdala, but this difference was present only in the group of animals affected by asphyxia for 12 h at E20. To complete the picture, it should be added that the sex difference in the mRNA level of the *slc6a1* gene was also found in the thalamus in its reticular nucleus (RtnT); control females had higher levels compared to males, and in both groups of animals prenatally affected by asphyxia, this difference was not present.

In any case, we would not like to draw far-reaching conclusions from our findings. This is particularly true when it is well known that the GABAergic system plays one of the key roles in the sexual differentiation of the brain (McCarthy *et al.* 2002) and the expression of its components in different areas of the brain changes dynamically during life, depending on gender (Pandya *et al.* 2019). It is known from the literature that lower levels of GAD67 in the prefrontal cortex, but also in the hippocampus are often identified as a concomitant phenomenon of schizophrenia, clinical depression or manic-depressive psychosis (Konradi *et al.* 2011; Wu *et al.* 2014; Guidotti *et al.* 2000). Therefore, we believe that our findings may be helpful in interpreting the increased anxiety (Weitzdoerfer *et al.* 2004) or long-term spatial memory deficits and social behaviour (Herrera-Marschitz *et al.* 2014; Morales *et al.* 2010) that are repeatedly observed in prenatal asphyxia-affected animals.

With some caution, prenatal asphyxia affected the amount of miR-15b-5p in the DG region. However, only if we consider the sex of the animals. Indeed, a significant increase in miR-15b-5p levels was observed only in females prenatally exposed to 12 hours of asphyxia during E20, compared to control females. In contrast, miR-15b-5p levels in DG of males exposed to 12 hours asphyxia during the prenatal period tended ($p = 0.07$) to be lower than in the brain of control males. Therefore, in the final evaluation of our observations, we see a massive gender difference in miR-15b-5p levels in DG in a group of animals prenatally exposed to a single 12 hours asphyxia, as well as a significant differences in sex and asphyxia factor-binding. While miR-15b-5p levels in the male DG prenatally exposed to asphyxia tended to decrease, in the female DG we observed the significant opposite effect.

Based on in silico analysis in (TargetScan.org), miR-15b-5p was the same as miR-16-5p sets on the mRNA of *bcl2-like 2* gene, and in this way could regulate its gene expression. The product of genes from the BCL-2 family has been shown to prolong neuronal survival in a hypoxic environment (Banasiak *et al.* 1999). It is part of a family of genes called B-cell lymphoma and *Bcl2-like 2*, also known as *bcl2-W* is itself is an antiapoptotic gene. According to these authors, the product of this gene prolonged survivor of neuronal cells in hypoxic condition, and products of this gene family are also associated with brain tissue response to cerebral ischemia (Hata *et al.* 1999; Shimazaki *et al.* 2000; Clark *et al.* 1997). An already heterogeneous state with one dysfunctional copy of the Bcl-2 gene significantly reduces the survival of hippocampus knockout mice cells compared to wild type animals after focal cerebral ischemia. And in the homogeneous state, the reaction is even more pronounced (Hata *et al.* 1999). The increased amount of the Bcl-2 gene product by the viral vector noticeably reduces the amount of fragmented DNA resulting from apoptosis following focal cerebral ischemia in the CA1 region of the hippocampus (Shimazaki *et al.* 2000). We believe that a reduction in miR-15b-5p expression, could have a positive effect on neuronal survival in the hippo-

campus. Therefore, decreased levels of miR-15b-5p could potentially indicate an increased production of Bcl2-W in the brain. In our experiment, this hypothetical condition would involve male neuronal tissues prenatally affected by asphyxiation.

Expression of Bcl-2 is unequivocally regulated by steroid receptors and oestrogen, respectively. This demonstrates better neuronal tissue survival in females compared to males, but also ovariectomised female rats exposed to experimental stroke (Alkayed *et al.* 2001). Even the expression of Bcl-2 in rat brain following experimentally induced stroke is significantly higher in females than in males and ovariectomised females. At the same time, Bcl-2 levels are also induced in ovariectomised females undergoing oestrogen substitution treatment. The problem with our interpretation of the observed changes in miR-15b-5p levels in animals prenatally exposed to asphyxia is that they reflect the state of healthy adult animals and not animals that are immediately recovering from an event threatening brain tissue survival. The increased presence of miR-15b-5p in DG females prenatally affected by asphyxia suggests the long-term need to inhibit the *bcl2-like 2* gene expression. In other words, these may be consequences of the physiological and molecular response of the brain tissue to the hypoxic state in the prenatal period, which are detectable even in adulthood.

According to Ma and co-workers (2016) miR-15b-5p is in a group of regulatory molecules whose elevated levels have been associated with the development of depressive-like behaviour in mice. In doing so, these authors have been able to identify a link with GABAergic system in the form of significant inhibition of GABA transporter type 3 gene expression. So at least we seem to have come across a link between the response to prenatal hypoxic conditions and the imbalance in the regulation of the most important inhibitory neurotransmitter in the adult brain, although the strength of this connection appears to be sexually determined. This suggests an important role for steroid hormones in response to asphyxia conditions; the 17β-oestradiol is most frequently mentioned in this context (Saraceno *et al.* 2010; Bourque *et al.* 2009; Suzuki *et al.* 2009).

To analyse the amount of miR-124-3p in the brain of rats prenatally exposed to hypoxic conditions, we focused on the subventricular zone (SVZ). The association with hypoxia is due to the ability of miR-124-3p to inhibit *serp1* gene mRNA (Yamaguchi *et al.* 1999). As with the previous miR-15b-5p, this connection is also proved by in silico analysis in the (TargetScan.org) database. The product of this gene is the stress-associated endoplasmic reticulum protein 1 (Serp1) which stabilises membrane proteins during hypoxia at least in in vitro conditions (Yamaguchi *et al.* 1999).

We observed a significant sex difference in the amount of miR-124-3p, however, males showed higher levels of this miR than females. The effect of prenatal asphyxia did not appear to be decisive, although the most significant gender difference was observed in animals that underwent asphyxia for 12 h on E20. From our point of view, it was also interesting that in animals exposed to asphyxia for 8 h of the E19 and E20, the tendency in the intersexual difference was strong ($p = 0.054$), but the sex difference in the control animals proved to be insignificant. According to this relationship, lower levels of miR-124-3p are associated with higher mRNA levels for *serp1* and thus to higher stability of membrane proteins of cells exposed to hypoxia. We believe that this can also help to higher resistance of female neuronal tissue.

However, this regulatory miR is very widely represented in neuronal tissue, which is not quite ideal in terms of the FISH method. We therefore decided to focus on the area of SVZ, which is well morphologically recognisable from the surrounding neuronal tissue. The SVZ is a known site of neurogenesis and self-renewing neurons in the adult brain (Lim and Alvarez-Buylla 1999), serving as such due to the interacting cell types, extracellular molecules and localised epigenetic regulation promoting cellular proliferation. The animal model of asphyxia used in our experiment overlaps in time with the period when neuroblastoma progenitor cells in the rodent brain are differentiating towards inhibitory neurons and are localised in the marginal and subventricular zone (Le Magueresse *et al.* 2011). Asphyxia conditions could affect their survival or the rate of migration. According to Makeyev *et al.* (2007), this area during the embryonic formation of the brain is abundant with miR-124-3p as well as PTBP2 (polypyrimidine tract binding protein 2). PTBP2 and its non-neuronal homologue PTBP1 are regulators of alternative mRNA splicing. In other words, based on the presence of PTBP1, we can prove with certainty that they are not neuronal cells and vice versa. This pair of regulatory molecules in the brain are involved in the separation of cells that differentiate towards neurons and, conversely, towards astrocytes, or other types of glial cells (Black 2003; Sharma *et al.* 2005; Spellman & Smith 2006; Wagner & Garcia-Blanco 2001). This fact is interesting to us because if we study the potential target molecules of miR-124-3p in detail using the (TargetScan.org) tool, we will find PTBP groups of mRNA´s among them, exactly as Makeyev *et al.* (2007) claims. in addition, where PTBP1 is inhibited, its counterpart PTBP2 is transcribed and potentiates mRNA splicing toward neuron-specific transcripts. If this hypothesis is valid, we can assume that SVZ of males in general and after prenatal asphyxia even more markedly need to increase the mRNA levels of the neuron-specific splice regulator PTBP2, which may be a response to the need to replace damaged neurons to a greater extent.

Conslusion

We were able to point out small, but nevertheless demonstrable, differences in the expression of the three genes of the GABAergic system in rats prena-

tally affected by asphyxia. We identified these altered levels in the limbic system, hippocampus, amygdala and thalamus.

We were able to show that prenatal asphyxia leaves the altered expression of miR-15b-5p in DG into adulthood, however, this change is sex-specific. We also found a significant sex difference in the level of miR-124-3p expression in the SVZ region. This gender difference appears to be dependent on the parameters of prenatal asphyxia to which the offspring were exposed during maternal pregnancy.

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