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Prenatal and perinatal phthalate exposure is associated with sex-dependent changes in hippocampal miR-15b-5p and miR-34a-5p expression and changes in testicular morphology in rat offspring

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MicroRNAs are a large group of non-coding nucleic acids, usually 20–22 nt long, which bind to regulatory sections of messenger RNA (mRNA) and inhibit gene expression. However, genome activity is also regulated by hormones. Endocrine disruptors such as those from the phthalate group imitate or block these hormonal effects, and our previous study showed a long-lasting decrease in plasma testosterone levels in rat offspring exposed to a mixture of three phthalates *in utero* and postnatally. These effects were also observed at the behavioural level. To shed more light on these findings, in this new study we compared testicular tissue morphology between control and phthalate-treated males and investigated possible persistent changes and sex differences in the expression of two hippocampal microRNAs – miR-15b-5p and miR-34a-5p – participating in the transcription of steroidogenic genes. Histologically observed changes in testicular tissue morphology of phthalate-exposed males compared to control support testosterone drop observed in the previous study. At the microRNA level, we observed more significant changes in phthalate-treated females than in males. However, we are unable to relate these effects to the previously observed behavioural changes.

KEY WORDS: *cornus ammonis*; dentate gyrus; di-(2-ethylhexyl)phthalate; di-isononyl phthalate; di-n-butyl phthalate; microRNA

Since the 1930s, the amount and chemical scale of phthalates to which humans are exposed has greatly increased (1), di-(2-ethylhexyl)phthalate (DEHP), di-isononyl phthalate (DINP), and di-n-butyl phthalate (DBP) in particular (2-4).

Phthalates can act as endocrine-disrupting chemicals (EDCs) by exerting strong antiandrogenic (5, 6) and weak oestrogenic effects (7, 8). In rodents, perinatal exposure to DEHP and other phthalates results in major alterations in Sertoli and Leydig cells and consequent disruption of testicular function and androgen-dependent development of male offspring (9–11). In addition, DEHP seems to interfere with oestrogen metabolism by suppressing aromatase [cytochrome P450 19A1 (CYP19A1)], which catalyses the final, rate-limiting step in the conversion of androgens to oestrogens (12–14). In a previous study (15) we measured blood testosterone concentrations in adult rats of both sexes that were prenatally and perinatally exposed to a phthalate mixture and found significantly reduced testosterone compared to controls. Lower testosterone levels and aromatase suppression reflect lower amount of substrate and lower speed of enzymatic reaction and, as a result, reduced oestrogen levels (16).

In their *in vivo* study, Buñay et al. (17) showed that perinatal exposure of mice to phthalates correlated with transcriptional modifications of genes that encode proteins involved in the steroidogenic pathway, such as *Star*, *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, and *Cyp19a1*. More specifically, it changed some microRNAs (miRNAs) in testes (lowered miR-15b-5p and increased miR-34b-5p). We therefore decided to investigate whether exposure to phthalates would also affect miR-15b-5p in the brain. At the same time, we knew from our previous study of brain miR-15b-5p that its response to pathophysiological factors is sex-dependent (18). MiR-34a-5p is also known as a sex-specific regulatory molecule (19). Both miRNAs are oncogenic markers, but we were not primarily interested in this connection and mainly focused on the connection with sex hormones and differentiation.

Many long-term effects of phthalate exposure on development are mediated by changes in the brain. During perinatal development of the central nervous system (CNS) in rodents, local oestrogen production (mediated by aromatase) is critical for the sexual differentiation of neural structures in specific brain regions (e.g. the medial basal hypothalamic preoptic area and limbic regions) (20,

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21). An ontogenetic *in vivo* study (22) reported that the effect of DEHP on aromatase was more pronounced in males at postnatal day 1 (PND1) than in females. At weaning (PND22), however, the effect of DEHP on aromatase activity was higher in females than in males.

The influence of prenatal and perinatal exposure to EDCs on various aspects of behaviour has been studied intensively in laboratory rodents. This is mainly because exposure to gonadal steroid hormones during intrauterine and neonatal life plays a pivotal role in the development of brain circuits, and this differentiation persists, determining behaviour patterns throughout life (23, 24). Oestrogen receptors (ERs), ERα and ERβ, are regulated by oestrogen and expressed in various regions of the CNS, including the hippocampus, and they have direct and indirect influence on higher cognitive functions (25) and anxiety-like behaviour (26). EDCs mimic or block endogenous hormones and may modify the synthesis, transport, metabolism, or elimination of natural hormones (27, 28). These chemicals influence the development and function of the CNS (30, 23, 31). Gonçalves et al. (30) reported short- and long-term memory defects – by using a novel object recognition test and the Moris water maze – in rats prenatally and postnatally treated by low doses of bisphenol A (BPA). They emphasised sex differences, and their findings could be interpreted as gradual de-feminisation or de-masculinisation of the brains of affected animals. Gioiosa et al. (31) and Kubo et al. (22, 29) provided a similar interpretation of the prenatal effect of phthalates on the CNS and its function.

The aim of our study was to elucidate the relationship between prenatal and early postnatal administration of a mixture of three phthalates on developmental changes in rats with a focus on sex differences and phthalate effects on testis morphology and on the expression of two mature miRNA molecules in the hippocampus.

METHODS

Animals

Wistar rats used in the study were obtained from the Department of Toxicology and Laboratory Animal Breeding station of the Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine of the Slovak Academy of Sciences in Dobra Voda (SK CH 20021). The animals were bred in an accredited vivarium of the Department of Animal Physiology and Ethology, Faculty of Science, Comenius University in Bratislava (SK U 09021). During the experiment, they were kept in 57×37×20 cm plastic cages with shaving litter. The room housing the animals had a controlled 12-h photoperiod, with the light phase from 8:00 to 20:00 h, average room temperature of 21±1 °C, and relative humidity of 55±10 %. Standard granulated feed (MP-OŠ-06, Snina, Slovak Republic) and water were available *ad libitum*.

The parental generation consisted of 18 rats (12 female and six male weighing in average 215 g and 350 g, respectively). The animals were quarantined for seven days, marked, and kept in groups of 3–4. To determine excitability, the animals were tested in an open field test and then divided in two balanced groups based on horizontal motor activity. The ovulatory cycle of females was determined from vaginal swabs based on cytology (32). One to three females were paired with one compatible male in terms of activity. The presence of a vaginal plug was marked as day zero of pregnancy. The females were then kept in the same cages, but on gestational day 20, they were placed in separate cages. After giving birth, they remained in the cage with their litter until weaning.

Phthalate-exposed females of the parental generation (Pht; n=6) were receiving 4.5 mg of each DEHP, DINP, and DBP (Sigma-Aldrich, St. Louis, MO, USA) per kg of body weight dissolved in 500 μL of peanut oil (Biopurus, Brno, Czech Republic). The solution was administered orally from gestational day 15 until postnatal day 4 (PND4). Control group females (n=6) were receiving 500 μL of pure peanut oil alone in the same way as the experimental group over the same period.

Offspring

To better monitor growth and development parameters, the litter sizes of phthalate-treated and control female parents were optimised to eight pups per parent (4 males and 4 females) on PND4, and 12 (six male and six female) pups from the phthalate-treated parents were assigned to the Pht group and the same number of pups from control parents to the control group. The weight of Pht pups was significantly lower than control on PND1, but this difference disappeared as the pups developed, as reported in our previous study (15).

Testis histology

On PND118, we terminated the experiment by euthanising rats under isoflurane anaesthesia and collected biological samples. One testis from each male rat (Ctrl=12, Pht=12) was fixed in 30 % sucrose in phosphate-buffered saline (PBS) for 48 h and then embedded in Cryomouth (Histolab AB, Goteborg, Sweden) cryoprotective medium. Cryosections (10 μm thick) were prepared from the testes and then stained with Gill's haematoxylin and eosin (H&E) solution. The stained sections were then photographed under a light microscope (Zeiss Axio Scope A1, Carl Zeiss, Oberkochen, Germany) at 40× magnification. Using Image J software (version 1.50i; National Institutes of Health, Bethesda, MD, USA) we determined the cross-sectional area of the sperm ducts as well as the luminal area in the digital photographs. Each value for an individual animal is an average value of at least 10 individual measurements. We compared the cross-sectional area of the sperm ducts, the luminal area of these ducts, and the luminal area to total area ratio.

FISH

Fluorescence *in situ* hybridization (FISH) was performed on coronal cryosections of the brain (12 μm) containing the hippocampus. Samples were taken in the early light phase (9:00 am). The hybridisation protocol for locked nucleic acid (LNA) probes on the frozen tissue section was modified as described elsewhere (33, 34). Hybridisation temperature and post-hybridisation washes were adjusted to the sequence of the probes (miR-15b-5p: /5'DiGN/TGTAAACCATGATGTGCTGCTA-3' and miR-34a-5p: /5'DiGN/ACAACCAGCTAAGACACTGCCA-3'). Both LNA probes (Qiagen, Dusseldorf, Germany) were modified with digoxin (DiG) at the 5' end. After post-hybridisation washes, the slides were incubated for 1 h with primary anti-DiG antibodies (Thermo Fischer, Waltham, MA, USA) diluted in PBS with 1 % goat serum and 0.01 % saponin in the ratio of 1:300. The slides were then washed and incubated for 30 min with anti-goat secondary antibody (conjugated to Alexa Fluor 488, Sigma-Aldrich) diluted in PBS 1:1000. Nuclei were labelled with DAPI, and then the sections covered with Fluoromont (Sigma-Aldrich). The samples were examined with a fluorescent microscope (Zeiss Axio Scope A1) at 40× magnification. We used approximately six microscopic photos per sample for relative quantification of labelled miRNAs. Images were evaluated using open-source digital pathology software QuPath-0.2.3 (35).

Statistical analysis

The FISH and H&E data were subjected to a normality test (Shapiro-Wilk test). Nonparametric data were logarithmically transformed and then subjected to two-way analysis of variance (ANOVA) with the factors sex and treatment. If there was a significant interaction between the factors, we used Tukey's honestly significant difference (HSD) *post hoc* test. We used an unpaired Student's *t*-test for a more accurate comparison of sex differences between the groups. We also used an unpaired Student's *t*-test to compare luminal areas of sperm ducts in the testes. We employed STATISTICA 10 (StatSoft Inc., Tulsa, OK, USA) for all statistical analysis. Significant differences were set at *p<0.05, **p<0.01, and ***p<0.001.

Ethics statement

The experiments were performed in compliance with the Principles of Laboratory Animal Care issued by the Ethics 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 (approval no. Ro-3726/16-221).

RESULTS

Testis histology

At the time of sacrifice, body and relative testis weights not differ between exposed and control males. The two groups also did not differ significantly in the average sperm duct area. However, Pht males had a significantly larger luminal and inner duct area to total duct area ratio than controls (Table 1). Figure 1 shows an example of pathological changes in the testes.

miR-15b-5p and miR-34a-5p expression in the hippocampus

miR-15b-5p and miR-34a-5p hybridisation signals were evaluated in the hippocampal CA1, CA2, CA3, and DG regions. Figure 2 shows representative images of positive miR-15b-5p signals in the hippocampal CA2 region. In the hippocampal CA1 region, miR-15b-5p expression in Pht females significantly differed from control, whereas no difference was observed between Pht and control males. Two-way ANOVA showed a significant sex × treatment interaction ($F_{1,25}$=8.3501; p=0.007; Figure 3). When we compared the expression of miR-15b-5p in the hippocampal CA1 in control animals only, there was a significant difference between the sexes (t=-2.778; p=0.017). However, this difference between sexes was not present in Pht animals (t=1.374; p=0.193).

Two-way ANOVA of miR-15b-5p expression in the hippocampal CA2 region revealed a significant treatment effect ($F_{1,25=}$4.7352; p=0.039) and sex effect ($F_{1,25=}$4.9969; p=0.035). *Post hoc* analyses confirmed these differences but also indicated that miR-15b-5p expression was significantly higher in the hippocampal CA2 region of controls compared to the other three groups (Figure 4).

Two-way ANOVA of the miR-15b-5p-positive signal in the hippocampal CA3 region showed no differences between the sexes or treatments (Figure 5). However, unpaired Student's *t*-test indicated a significant sex difference in controls (t=-2.758; p=0.017). In addition, a comparison between control and exposed females showed a significant effect of phthalates that persisted into

Table 1 Comparison of sperm-producing duct areas (μm²) between control (n=12) and phthalate-treated (n=12) male rats. Values are presented as means±standard deviations (SD)

Items	Control (Mean±SD)	Phthalates (Mean±SD)	p value
Channel area (μm²)	100292±3768.6	103586±5053.5	0.595936
Inner duct area (μm²)	19419±1769	25151±1433.3	**0.019613**
Inner duct area / channel area (μm²)	188091±1430	241257±1130	**0.008059**

Bolded p values are statistically significant

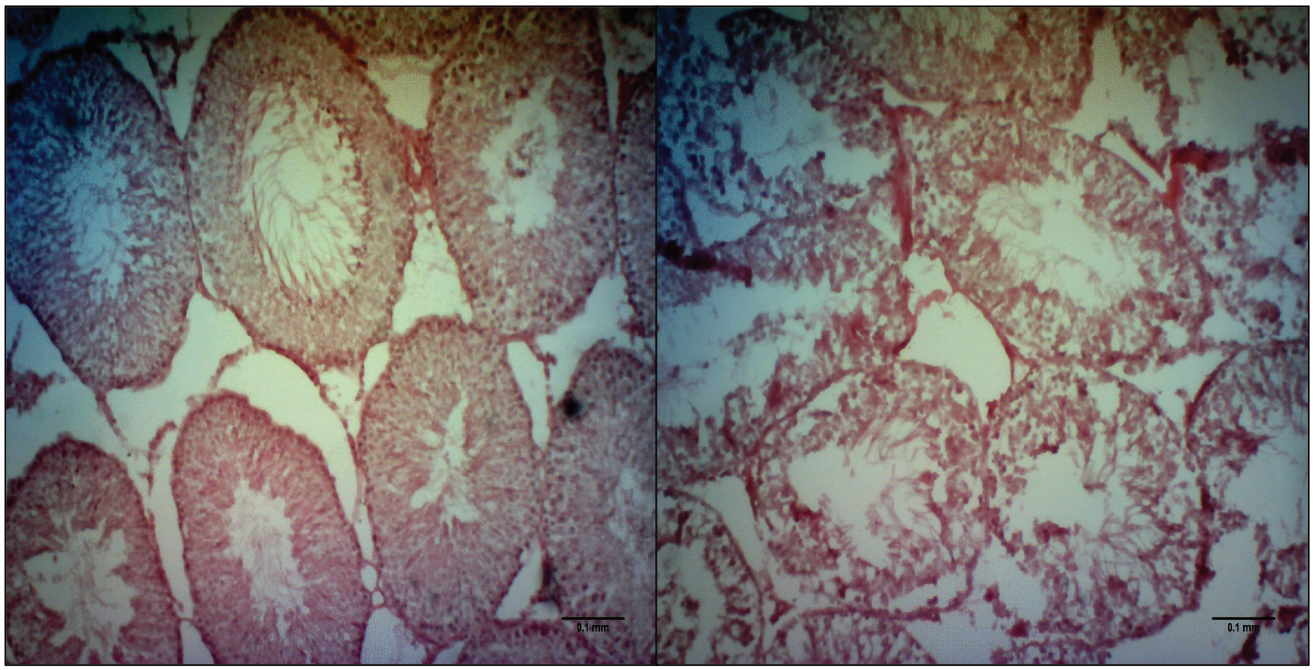

Figure 1 Comparison of luminal area cryosections of testes stained with Gill's haematoxylin and eosin between a control and a phthalate-treated male rat

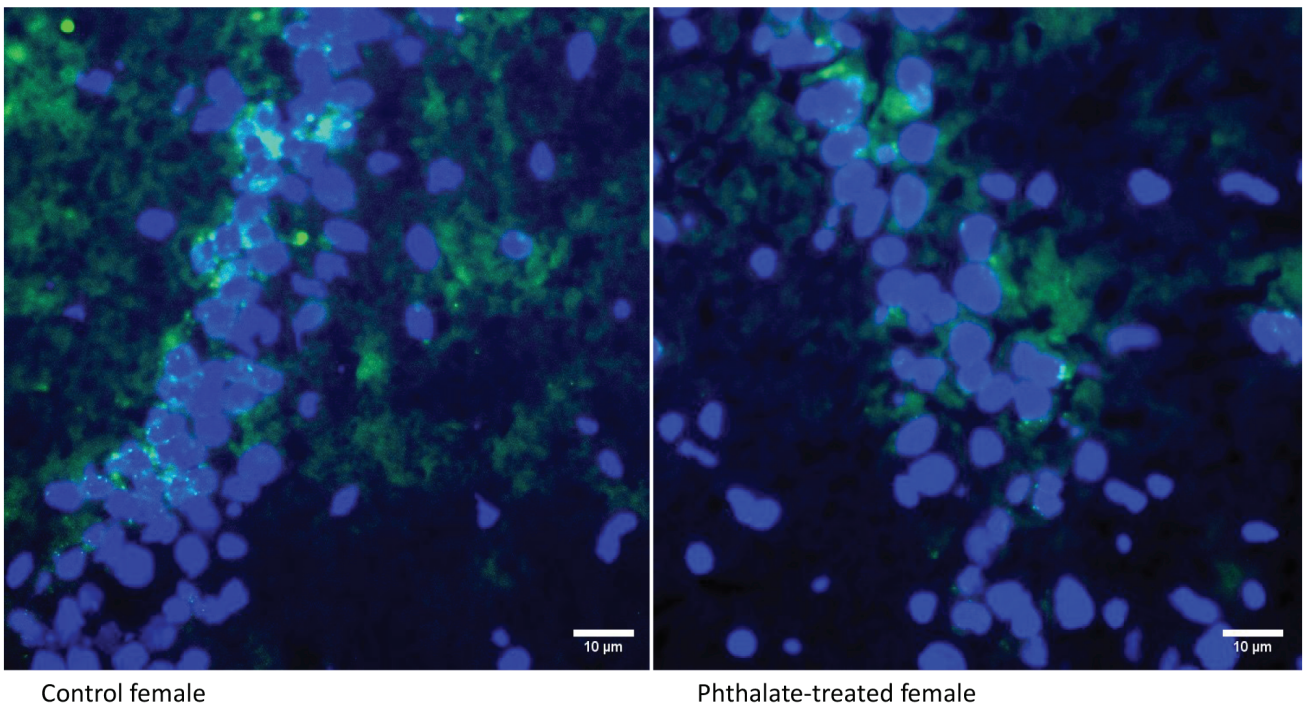

Figure 2 Comparison of representative micrographs of miR-15b-5p positive signals (green) in the hippocampal *cornus ammonis* 2 (CA2) region between a control and a phthalate-treated female rat

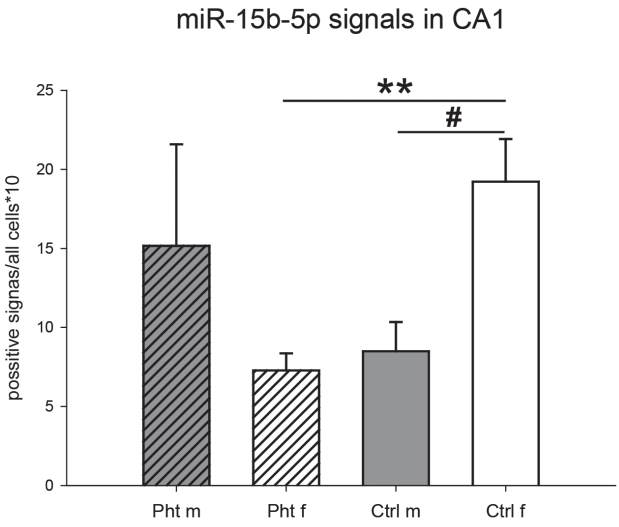

Figure 3 Relative number of miR-15b-5p-positive neurons in the hippocampal *cornus ammonis* 1 (CA1) region of control male rats (Ctrl m; n=5), control female rats (Ctrl f; n=9), phthalate-treated male rats (Pht m; n=5), and phthalate-treated female rats (Pht f; n=10). Sex × treatment interaction is significant (F1,25=8.3501, p=0.0078). ** p<0.01 and # p<0.05 vs respective male and female controls (unpaired Student's *t*-test: t=-2.778, p=0.017)

adulthood (t=-2.529; p=0.022). This effect was observed in females alone.

Two-way ANOVA of data from the DG did not reveal significant differences in miR-15b-5p expression (Figure 6). However, a more detailed analysis with unpaired Student's *t*-test revealed a significant sex difference in controls (t=-3.143; p=0.008). Control and exposed females did not differ significantly (t=-1.792; p=0.091), but the exposed ones showed a trend towards a lower miR-15b-5p expression.

As for miR-34a-5p, its expression in the hippocampal CA1, CA2, CA3, and DG regions was not affected by either sex or phthalate treatment in the hippocampal CA1 (Figure 7) or CA2 region (Figure 8) (two-way ANOVA). However, a more detailed comparison shows a trend towards a lower miR-34a-5p expression in Pht females compared to control (t=-1.927; p=0.076) or Pht males (t=2.041; p=0.072).

The hippocampal CA3 region showed a different picture, though (Figure 9). We found a significant treatment effect ($F_{1,19=}$5.009; p=0.0373) and a significant treatment × sex interaction ($F_{1,19=}$4.39; p=0.049) on miR-34a-5p expression. A separate comparison (Student's *t*-test), showed a significant decrease in miR-34a-5p expression in Pht females compared to control females (t=2.465; p=0.035) and compared to Pht males (t=2.363; p=0.042).

In the DG (Figure 10), two-way ANOVA showed near significant sex ($F_{1,19}$=4.116; p=0.056) and phthalate effect ($F_{1,19}$=3.69; p=0.069) on miR-34a-5p expression. Unpaired Student's *t*-test revealed that phthalate treatment significantly affected females but not males (t=-2.476; p=0.028).

DISCUSSION

The significantly increased luminal space in the sperm ducts in Pht compared to control males supports the assumption of impaired testosterone production (15), which is clearly associated with long-term exposure to the phthalate mixture. Despite some differences in study design, our findings also confirm the connection between disrupted testosterone production and structural and cellular damage of the seminiferous tubules (17, 36–39).

Although, at first glance, the male sex appears to be more affected by prenatal and perinatal exposure to phthalates, it is clear from our additional findings that the picture is much more complex. Sex hormone levels play a key role in the development and differentiation of the brain masculinisation or feminisation (40, 41). A decrease in circulating testosterone levels as well as morphological defects in the testes, such as the effect of prenatal and occasionally postnatal exposure to the phthalate mixture, are also noticeable in adulthood. Therefore, we believe that the adverse effects on the brain during development and adolescence and significant changes in miRNA expression persist into adulthood. The hippocampal expression of both miRNAs analysed in this study supports this hypothesis. However, before interpreting the observed effects of phthalates on miRNA expression in different parts of the hippocampus, it is necessary to comment on the abundance of miRNAs in control animals and the observed sex differences.

We observed sexual dimorphism in the so-called basal miR-15b-5p expression in virtually the entire hippocampus, with significantly higher expression in females. In contrast, the number of miR-34a-

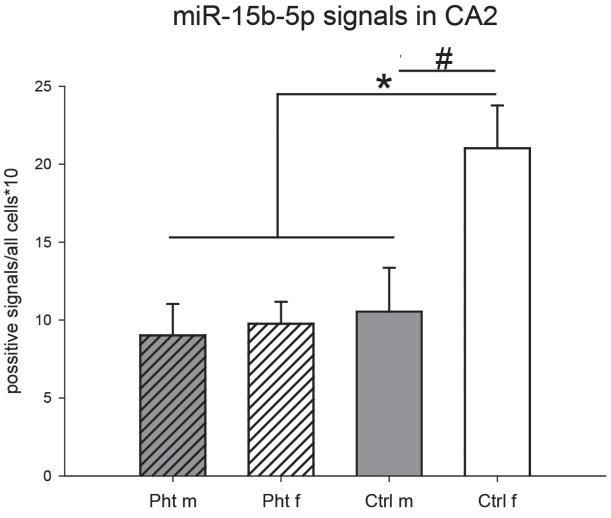

Figure 4 Relative number of miR-15b-5p-positive neurons in the hippocampal *cornus ammonis* 2 (CA2) region of control male rats (Ctrl m; n=5), control female rats (Ctrl f; n=9), phthalate-treated male rats (Pht m; n=5), and phthalate-treated female rats (Pht f; n=10). The sex effect (F1,25=4.9967, p=0.0345) and treatment effect (F1,25=4.7352, p=0.0392) are significant. * p<0.05 and # p<0.05 for the Ctrl m and Ctrl f comparison (unpaired Student's *t*-test: t=-2.459, p=0.03)

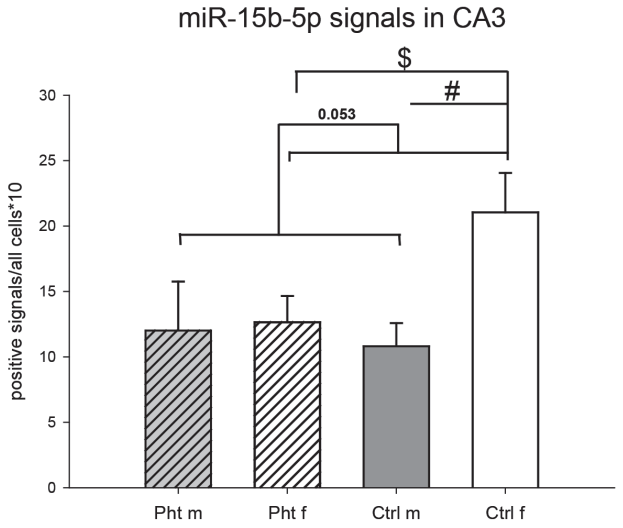

Figure 5 Relative number of miR-15b-5p-positive neurons in the hippocampal *cornus ammonis* 3 (CA3) region of control male rats (Ctrl m; n=5), control female rats (Ctrl f; n=9), phthalate-treated male rats (Pht m; n=5), and phthalate-treated female rats (Pht f; n=10). The sex effect shows a trend towards significance (F1,25=4.0915, p=0.0539). $ p<0.05 for the Ctrl f and Pht f comparison (unpaired Student's *t*-test: t=-2.529, p=0.022); # p<0.05 for the Ctrl m and Ctrl f comparison (unpaired Student's *t*-test: t=-2.758, p=0.017)

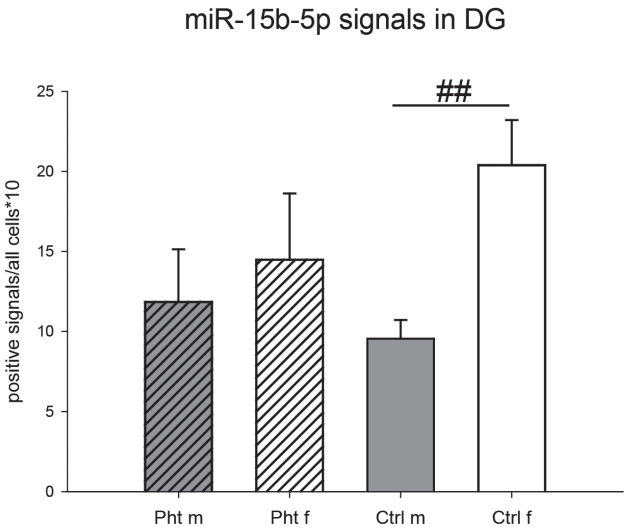

Figure 6 Relative number of miR-15b-5p-positive neurons in the dentate gyrus (DG) of control male rats (Ctrl m; n=5), control female rats (Ctrl f; n=9), phthalate-treated male rats (Pht m; n=5), and phthalate-treated female rats (Pht f; n=10). ## p<0.01 for the Ctrl f and Pht f comparison (unpaired Student's *t*-test: t=-3.143, p=0.008)

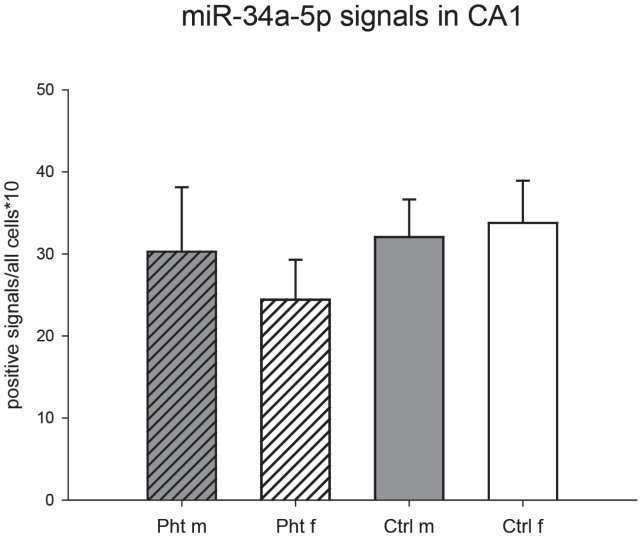

Figure 7 Relative number of miR-34a-5p-positive neurons in the hippocampal *cornus ammonis* 1 (CA1) region of control male rats (Ctrl m; n=4), control female rats (Ctrl f; n=8), phthalate-treated male rats (Pht m; n=4), and phthalate-treated female rats (Pht f; n=7)

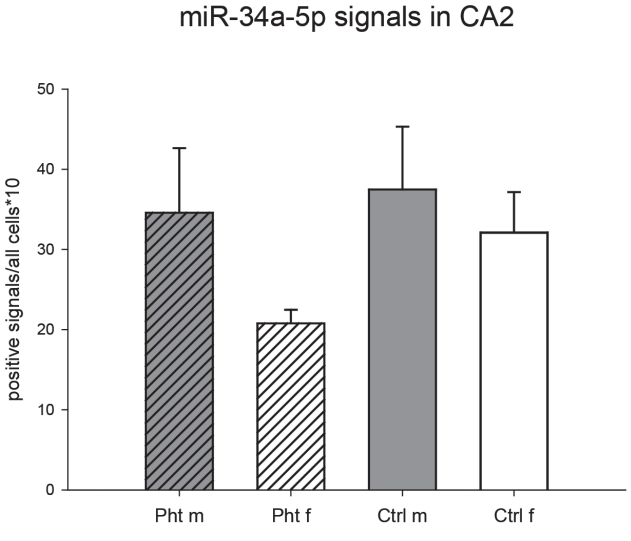

Figure 8 Relative number of miR-34a-5p-positive neurons in the hippocampal *cornus ammonis* 2 (CA2) region of control male rats (Ctrl m; n=4), control female rats (Ctrl f; n=8), phthalate-treated male rats (Pht m; n=4), and phthalate-treated female rats (Pht f; n=7)

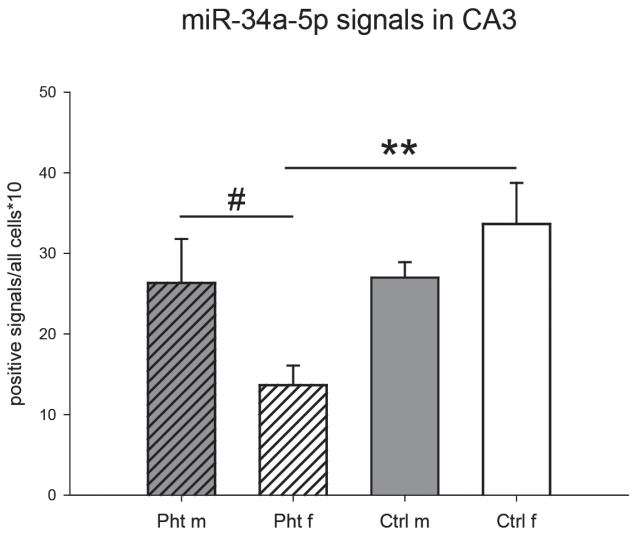

Figure 9 Relative number of miR-34a-5p-positive neurons in the hippocampal *cornus ammonis* 3 (CA3) region of control male rats (Ctrl m; n=4), control female rats (Ctrl f; n=8), phthalate-treated male rats (Pht m; n=4), and phthalate-treated female rats (Pht f; n=7). The treatment effect ($F_{1,19}=5.009$, $p=0.037$) and the sex × treatment interaction ($F_{1,19}=4.39$, $p=0.049$) are significant. ** $p<0.001$ and # $p<0.05$ for the Pht m and Pht f comparison (unpaired Student's *t*-test: $t=2.363$, $p=0.042$)

5p signals in different parts of the hippocampus was more or less the same in control males and females. Koturbash et al. (42) reported similar findings of steady-state sex- and tissue-specific miRNA expression pattern in controls. Our procedure allowed us to focus on the dense layer of pyramidal cells in the hippocampal CA1, CA2, CA3, and DG regions. We calculated positive signals with the QuPath software, which largely eliminated human bias in the evaluation. As sex differences in both basal and induced miRNA expression have been well documented (43, 44), measurements of miR-15b-5p and miR-34a-5p can be considered part of this complex mosaic. Our miR-15b-5p molecule counts in the pyramidal cell layer from CA1 through CA2 and CA3 to DG suggest that the original sexual dimorphism in control animals is eliminated by exposure to phthalate. At the same time, we found a decrease in this regulatory molecule in Pht females. In this respect, the male sex appears to be unaffected by prenatal and perinatal phthalate exposure.

While we observed sexual dimorphism in hippocampal expression of miR-15b-5p in control animals, we observed no such dimorphism for miR-34a-5p. Again, the long-term action of phthalates seems to affect miR-34a-5p levels only in females.

However, male offspring has an organ (testis) that responds significantly to the presence of phthalates in their circulation. We therefore believe that the effect is adverse on testosterone production and probably reduces fertility, but the apparent benefit is that other organs are exposed to lower amounts of phthalates. To some degree our belief is supported by the measurements of phthalate metabolites in urine carried out in human studies, as they show that the metabolism of these substances in the body is sex-dependent (45, 46). Direct measurements of samples taken from experimental animals would shed more light on this issue. Unfortunately, we could not implement them due to technical reasons.

To conclude, we demonstrated that prenatal and perinatal administration of a mixture of three phthalates (DEHP, DINP, and DBP) leads to macroscopic defects in the testes which may be associated with lower circulating testosterone levels reported in our previous study (15). These effects last until adulthood. We also demonstrated that the expression of two miRNAs that interfere with oncogene signalling pathways is altered by phthalate exposure. These changes are long-lasting and observable only in females. Considering that phthalates are being utilised more and more, we believe that their sex-dependent effects in the context of miRNA-mediated regulation of brain development should be evaluated carefully.

Acknowledgments

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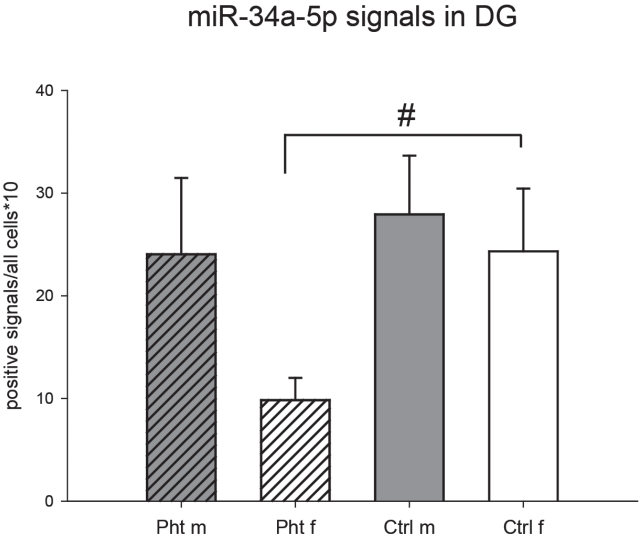

Figure 10 Relative number of miR-34a-5p-positive neurons in the dentate gyrus (DG) of control male rats (Ctrl m; n=4), control female rats (Ctrl f; n=8), phthalate-treated male rats (Pht m; n=4), and phthalate-treated female rats (Pht f; n=7). # $p<0.05$ for the Ctrl f and Pht f comparison (unpaired Student's *t*-test: $t=-2.476$, $p=0.028$)

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Perinatalna izloženost štakora ftalatima povezana je s promjenama ekspresije miR-15b-5p i miR-34a-5p u hipokampusu koje ovise o spolu i o promjenama morfologije tkiva testisa

Mikroribonukleinske kiseline (miRNA) velika su skupina nekodirajućih nukleinskih kiselina, obično dugih 20 – 22 nt, koje se vežu za regulacijske dijelove glasničke RNA (mRNA) i inhibiraju ekspresiju gena. Aktivnost genoma usto reguliraju i hormoni. Endokrini disruptori, poput onih iz skupine ftalata, oponašaju ili sprječavaju djelovanje hormona, a naše je ranije istraživanje upozorilo na dugoročni pad razina testosterona u mladunaca štakora izloženih mješavini triju ftalata *in utero* i nakon poroda. Ti su učinci zamijećeni i na razini ponašanja. Cilj je ovog novog ispitivanja bio rasvijetliti te prijašnje rezultate tako što smo u njemu usporedili morfologiju tkiva testisa kontrolne i skupine izložene ftalatima te istražili moguće stalne promjene i razlike između spolova u ekspresiji dviju miRNA u hipokampusu – miR-15b-5p i miR-34a-5p – koji sudjeluju u transkripciji gena odgovornih za steroidogenezu. Histološki utvrđene promjene u tkivu testisa izloženih mužjaka u odnosu na kontrolu potvrdile su morfološku pozadinu pada razina testosterona iz ranijeg ispitivanja. Na razini miRNA uočene su značajnije promjene u izloženih ženki nego u mužjaka, no ne možemo povezati te učinke s ranije zamijećenim promjenama u ponašanju.

KLJUČNE RIJEČI: *cornus ammonis;* dentatne vijuge; di-(2-etilheksil)ftalat; di-izononil ftalat; di-n-butil ftalat; mikro-RNA