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Injections to pregnant mice produces prenatal stress that affects aggressive behavior in their adult male offspring

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ABSTRACT

Maternal stress could reprogram the developing fetal nervous system. A common target of maternal glucocorticoids is fetal neuro-endocrine axis. In the present study, pregnant mice were exposed to stress by injection and their male offspring were tested for sexual and aggressive behaviors in adult life. Three groups of pregnant mice were exposed to stress by sham syringe injection. The first group was injected on days 13, 14, and 15 *p.c.*, the second group was injected on days 17 and 18 *p.c.*, and the third group was injected daily from days 13 to 18 *p.c.* while control mice were not injected. Male offspring that were exposed to stress on days 13–18 *p.c.* and 17–18 *p.c.* were less aggressive and had lower blood testosterone levels in comparison to the control group. In male sexual behavior, there were no statistically significant differences between the groups. Body weight differed significantly with groups injected on days 13–18 *p.c.* and 13–15 *p.c.* having significantly higher body weight in adult life than the other two groups. After behavioral testing, brains were processed for immunohistochemical staining with antibodies against vasopressin (AVP) and calbindin (CALB). The expression of AVP and CALB in the lateral septum and in the preoptic area, respectively, did not differ between groups, suggesting that these two masculinization markers were not affected by prenatal stress. Present study therefore shows that even presumably mild and short prenatal stress weakens aggressive behavior of adult male mice, possibly due to reduced testosterone levels in blood.

Key words

Mouse, prenatal stress, aggressive behavior, sexual behavior, testosterone, immunohistochemistry, vasopressin, calbindin

SIGNIFICANCE STATEMENT

This study demonstrates that injection of pregnant mice, presumably causing stress, affects aggressive behavior and some other physiological parameters (testosterone levels, body weight) in adult male offspring of stressed mothers. Stress applied to pregnant mice was presumably mild (daily injections) and is similar to what is used in many developmental neurostudies where different substances are applied to pregnant mice. Therefore, this manuscript is very important from methodological point of view as it highlights the importance of taking into account even mild stressors applied to mice in animal studies.

INTRODUCTION

During late prenatal period neuronal system is rapidly developing and is therefore sensitive to potential negative external influences. Stress in pregnant dams could influence developing fetuses as stress hormones (glucocorticoids) could pass through the placenta to the fetus and influence its normal development. Changes in the development can often be discern only in adult period and are connected to different diseases/changes in behavior (Weinstock, 2017).

Numerous studies have shown that pulsatile release of hypothalamic stress hormones during prenatal development affect endocrine control between central and peripheral nervous systems. This affects pituitary gland and production of peripheral hormones, what act as a negative feedback loop to the hypothalamus. These effects of prenatal stress hormones could permanently alter the function of hypothalamus – pituitary – adrenal axis and are considered as organizational effects of steroid hormones. Organizational effects could change the number of responsive cells in the target organs, the number of receptors on target cells or the capacity of endocrine cells for the production of specific hormones. Prenatal stress can therefore change the normal settings of the HPA axis and thus alter permanently neuro-endocrine pathways in the brain, and some effects of stress can be even transgenerational (McEwen, 2018).

Male aggressive behavior is present in most mammalian species. One of the main modulators of aggressive behavior is thought to be sex steroid hormone testosterone as castration reduces aggressive behavior in males, while testosterone injections increases aggression in gonadectomized animals (Primus and Kellogg, 1990). Moreover testosterone during prenatal and early postnatal period is necessary for brain masculinization and expression of aggressive behavior during adulthood (Edwards, 1971; vom Saal, 1979). Interestingly, prenatally stressed animals could have lower testosterone levels (Gerardin et al., 2005) what could potentially influence the aggressive behavior.

In the brain, hypothalamus and parts of the limbic system are thought to be the main regions involved in the regulation of aggressive behavior. Electric stimulation of the anterior and ventromedial hypothalamus can trigger attacks (Kruk, 1991) and lesions in the hippocampus and amygdala eliminate aggression in laboratory animals (Ely et al., 1977). Aggressive behavior is partially influenced by vasopressin (AVP) (Ho et al., 2010), mostly through its receptors in the brain. Lower binding of vasopressin to its receptor 1a (Avpr1a) is connected with lower aggression (Albers, 2012), and knocking out the vasopressin receptor 1b (Avpr1b) significantly reduces aggressive behavior (Caldwell et al., 2008). Effects of the AVP on aggressive behavior are at least partially modulated by gonadal hormones. Castration reduces the binding of AVP to AVP receptors in the ventrolateral hypothalamus, while testosterone treatment of gonadectomized animals can recover this binding. Furthermore, injection of AVP into the ventrolateral hypothalamus stimulates aggressive behavior in gonadally intact males, but does not stimulate aggression in castrated males (Albers, 2012).

Some previous studies have shown that in rodents, prenatal stress could affect aggressive behavior in male and female offspring of stressed mothers, although results from different studies are varied (Kinsley and Svare, 1986; Patin et al., 2005; Velazquez-Moctezuma et al., 1993). It is likely that different forms of stress, different timing of stress and even strain differences in mice and rats influence the effects of prenatal stress on aggressive behavior in adult rodents (Kinsley and Svare, 1987; Velazquez-Moctezuma et al., 1993). However, all previous studies examining possible effects of prenatal stress on aggressive behavior used strong and longer stressors such as continuous light exposure or immobilization/restrain. In our preliminary study (G. Majdic, unpublished), we have observed

reduced aggressive behavior in male mice whose mothers were injected daily from days 13.5 p.c. until 18.5 p.c. In the present study, we therefore examined whether stress in a form of subcutaneous injection could affect the behavior of the male offspring of stressed mothers and if there are differences in stress effects regarding the timing of stress. This is especially important as many developmental studies use injections of different substances to pregnant dams to examine effects of different substances on fetal development, yet it is often disregarded that injection alone could present a stress for a pregnant mice and also for their offspring. Although it is a norm in such studies that control animals receive vehicle injections, it is important to note that observed behavior in both control and treated groups could be influenced by prenatal stress, caused by injections, and could therefore vary from the physiological behavior in animals, not stressed prenatally.

MATERIAL AND METHODS

Mice

C57BL/6J mice were originally obtained from Harlan (Italy) and bred at the University of Ljubljana, Veterinary Faculty in standard conditions with 12-12 hours light/dark cycle (lights on at 4 am and off at 4 pm) and with food (phytoestrogen free diet; Harlan Teklad Diet 2016, Harlan, Milan, Italy) and water *ad libitum*. Tested male mice were weaned at 21 days of age. For aggressive behavior tests, A/J male mice were used as stimulus animals. For sexual behavior tests, adult ovariectomized and hormone primed C57BL/6J female mice were used as stimulus animals. Total number of tested animals was 44.

All animal experiments were approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection of the Republic of Slovenia (U34401-22/2015/13) and were done according to ethical principles, EU directive (2010/63/EU), and NIH guidelines.

Female mice were housed with experienced males and the presence of vaginal plugs was checked daily. The morning when the vaginal plug was detected was considered as day 0.5 *p.c.* (*post coitus*). Pregnant females were isolated into separate cages (36.5 × 20.7 × 14 cm) and divided into 4 different groups, three experimental and one control group. In experimental groups females were fixated by the scruff and stress was caused by piercing of a 26 G syringe needle into the skin fold on the back in the thoracic region. The skin was pierced and immediately removed so that whole procedures lasted only few seconds. Cages with control animals were opened, and animals were handled, but not restrained, grabbed for the scruff or injected. Piercing was done in the light part of the cycle, between 11:00 and 12:00 and after the procedure female was quickly return in to the home cage. The group 1 (group E13-15) was pierced on days 13.5, 14.5 and 15.5 *p.c.*, group 2 (group E17-18) was pierced on days 17.5 and 18.5 *p.c.*, group 3 (group E13-18) was pierced daily from 13.5 to 18.5 *p.c.*, and group 4 was a control group that was not pierced. Cotton nest material was added and bedding was changed once per week until 16.5 *p.c.* To ensure calm early *postpartum* environment bedding was not changed from 16.5 *p.c.* until the postpartum day 7. All pups were weaned on 21. postnatal day when 3-5 males from the same groups

were placed together and left until they reached adulthood. During the entire breeding, handling was reduced to minimum to avoid any additional stress.

Male mice tested in adult life derived from 5 litters in all three experimental (stressed) groups, and 8 litters in control group. Only one or two pups from the same litter were used in experiments with the exception of groups E13-18 and control group where 3 pups were taken from the same litter once. From all other litters only 1 or 2 pups from the same litter were included in the study in all four groups. As litter size could affect different physiological parameters and especially body weight, we followed the size of the litters from stressed and controlled dams and there was no significant difference between the groups. In control group, average number of pups was 7.7 ± 0.6 ($n = 8$, minimum 5, maximum 10), in group E13-15 7.6 ± 0.9 ($n = 5$, minimum 5, maximum 9), in group E17-18 7.8 ± 0.4 ($n = 5$, minimum 7, maximum 9) and in group E13-18 8.0 ± 0.5 ($n = 5$, minimum 6, maximum 9; mean $\pm$ S.E.M.).

Preparation of stimulus females

All female mice used as stimulus mice in behavioral analyses were ovariectomized bilaterally at 60 days of age (after puberty) to eliminate endogenous gonadal steroids. Mice were anesthetized with the mixture of ketamine (Vetoquinol Biowet, Gorzowie, Poland; 100 μ g/g BW), acepromazine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μ g/g BW) and xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 μ g/g BW) and ovaries were excised through a single incision. Mice received two injections of butorfanol (Turbogestic, Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μ g/g BW) after ovariectomy to alleviate potential pain.

To regulate circulating estradiol levels during the testing of sexual behavior, female stimulus mice received subcutaneous implants containing 17 β -estradiol 3-benzoate (EB; Sigma, Steinheim, Germany). Silastic implants (1.02 mm inner diameter, 2.16 mm outer diameter) were filled 5 mm in length with crystalline EB diluted 1:1 with cholesterol (Sigma) (Wersinger et al., 1999) and closed on both ends by medical silastic adhesive (Dow Corning, MI, USA). Implants were inserted subcutaneously in the midscapular region under general anaesthesia as described above. These implants yield plasma estradiol

levels close to the physiological range normally observed during oestrus (Wersinger et al., 1999). Sexual behavior tests were performed at least 10 days after implantation to allow the mice to fully recover from the surgery. Approximately 3 to 5 hours before each behavioral test, females were injected subcutaneously with 0.8 mg of progesterone (P; Sigma) dissolved in 50 μ l of corn oil (Sigma).

Behavioral tests

All behavioral tests were performed by experienced observer, blind in regard to the status of the animal.

Male sexual behavior

Male sexual behavior was tested after 60. days of age. One day before the observation, tested animals were isolated in the glass aquarium with 1/3 of its old bedding and 2/3 of new bedding and let to habituate until the next day. The observations were performed on three successive days, 2-3 hours after the beginning of the dark cycle. Immediately before the experiment, all the bedding was removed from the aquarium and a mirror was placed under the aquarium, with an angle to see the whole bottom side of aquarium for easier observation of penile penetration. The test begun with the placement of stimulus female (prechecked for receptivity, only receptive females were used) into the cage and lasted for 30 minutes or until the ejaculation. For data recording a computer program stopwatch+ (Center for Behavioral Neuroscience, Atlanta, GA, USA) was used for monitoring the following parameters: latency to and number of complete mounts, latency to and number of incomplete mounts, latency to and number of intromissions, latency to and number of pelvic thrusts, latency to ejaculation, latency to, number and duration of anogenital sniffing, latency to and number of male aggressive events and latency to and number of female aggressive events. As test duration differed due to earlier ejaculations, frequencies of events per 1 minute were calculated by dividing total number of events recorded with the duration of test in minutes. After the test, same bedding was returned to the aquarium.

Aggressive behavior

Observation of aggressive behavior was performed 4 days after completing the sexual behavior tests. For observing aggressive behavior resident-intruder test was used, where the tested animal was the resident and the stimulus animal was an intruder. Mice were placed into the glass aquarium with all of its old bedding transferred from the cage where they were housed between sexual and aggressive behavior tests. The bedding was not changed for at least 4 days before the testing and throughout the duration of aggressive behavior testing. The testing was performed 2–3 hours after the beginning of the dark cycle. For stimulus animal an unaggressive strain A/J mice were used. Stimulus male mouse was placed in the aquarium and test lasted for 15 min, unless the biting started, in such case the observation ended 2 minutes after the first bite to prevent excessive stress of mice. The tests were performed 3-times in 3 successive days. The program stopwatch+ was used to record the following parameters: latency to and number of bites, latency to and number of tail rattling, latency to and number of mounts, latency to, number and duration of aggressive grooming, latency to, number and duration of anogenital sniffing. As some tests were terminated before 15 minutes due to excessive aggression, frequencies of events per 1 minute were calculated by dividing total number of events recorded with the duration of test in minutes.

Euthanasia and brain recovery

Animals were euthanised 7–8 days after conclusion of aggressive behavior observations. Mice were anaesthetized as described above and weighed. Blood was collected from the left ventricle of the heart with the syringe washed with heparin and centrifuged for 5 minutes at 3000 rpm on 4°C to obtain plasma. Testes and seminal vesicles were dissected and their wet weight was recorded. Afterward the brains were perfused through upper blood-vascular system. Firstly, blood vessels were flushed with 20 ml 0,1 M PBS (phosphate buffered saline) and then fixed with 25 ml of 4 % paraformaldehyde. The flow rate of the fixative was continuously set to 5 ml/min through the entire process. Brains were then removed and postfixed overnight in 4% paraformaldehyde. Afterwards, brains were stored in 0,1 PB buffer at 4 °C until used.

Determination of plasma testosterone concentration

Plasma testosterone concentration was determined with commercial EIA kit (catalog number DE1559; Demeditec, Kiel-Wellsee, Germany) following the user's manual. The range of the assay was between 0.083 and 16 ng/mL. The precision of the assay was determined with intra- and inter- coefficients of variations, which were 8.77 % and 9.80 % for high (3.68 ng/mL) and 4.15 % and 9.92 % for low (0.22 ng/mL) testosterone values, respectively.

Immunohistochemistry

Perfused mouse brain were embedded in 5 % agarose and then coronally cut with the vibratome (Leica) to 50 µm thick slices starting 0.5 mm rostrally from bregma to -2,54 mm caudally from bregma. Sections were incubated for 30 minutes in 0,1 M glycine and washed 3 times for 5 minutes in 0.05 M PBS. This was followed by incubation for 15 minutes in 0,5 % Sodium borohydride (Sigma) and washing 4 times for 5 minutes in 0.05 M PBS. Afterwards, sections were blocked for 30 minutes in the blocking solution (5 % NGS (normal goat serum), 0.5 % Tryton and 1% H₂O₂, in 0.05M PBS), Primary rabbit antibodies against vasopressin (1:10 000; Immunostar, WI, USA), and mouse antibodies against calbindin (1:20 000, Sigma), were diluted in 0.05 PBS containing 1 % bovine serum albumin and 0.5 % Triton X-100 and sections were left in the solution of primary antibodies for 2 days with shaking at 4 °C. All further steps were done at the room temperature. In the next step sections were washed 4 times for 15 minutes with 0,05 M PBS, 0,02 % Triton, and 1 % NGS. Biotinylated secondary antibodies (Jackson ImmunoResearch) against primary rabbit antiserum were diluted 1:500 in 0.05 M PBS containing 1 % normal goat serum and 0.5 % Triton X-100. Sections were incubated with biotinylated secondary antibodies for 2 h, followed by 4 washes for 15 min in 0.05 M PBS buffer containing 0.02 % Triton X-100. Streptavidin conjugated horseradish peroxidase (S-HRP) (Jackson ImmunoResearch) was diluted 1:2500 in 0.05 M PBS solution containing 0.5 % Triton X-100. Sections were incubated with S-HRP for 1 h at room temperature and then washed in Tris-buffered saline (0.05 M Tris-HCl/ 0.9 % NaCl; pH 7.5; Sigma) for 1 h (four times for 15 min) at room temperature. Antigen-antibody complexes were

visualized as a black reaction product by incubating sections in 0.025 % 3',3'-diaminobenzidine/ 0.2% ammonium nickel (II) sulphate substrate (Sigma) in Tris-buffered saline containing 0.02 % H₂O₂ for 5 min at room temperature. Sections were finally washed in Tris-buffered saline 3 times every 10 min. After mounting on silane-coated slides, sections were dried and coverslipped using hydrophobic medium (Pertex; Burgdorf, Germany). Immunohistochemical controls were based on omitting primary antibodies from control sections to control for unspecific binding of the secondary antibodies.

Data collection and cell quantification

Digital images of brain regions of interest were obtained using a Nikon Eclipse 80i microscope with Nikon DS-Fi1 camera. Digital images were taken under 40× magnification. One section from each brain was analyzed and section with the most cells or the highest density of fibers was chosen. The third ventricle and the base of the brain served as reference boundaries for the medial preoptic area (MPOA). The number of calbindin-immunoreactive cells was quantified (counted manually with the help of Cell counter ImageJ plugin) bilaterally in the MPOA in coronal sections approximately 0.1 mm caudally from bregma according to stereotaxic coordinates (Franklin and Paxinos, 2008). The amount of immunoreactive AVP fibres was quantified in the lateral septum (approximately 0.2 mm rostrally from bregma) area using Particle analysis tool of ImageJ (NIH, Bethesda, MD). Top and lateral borders of the lateral septums were used as reference boundaries. All images were taken under the same illumination, converted to grayscale to standardize the collection of immunoreactive area data and then subjected to threshold conversion to selectively identify immunoreactive elements using Photoshop software 8.0. All image analyses were performed by observer blind to the group that animal was from.

Statistical analyses

Results from behavioral testings were analyzed with repeated measures ANOVA with treatments as independent variable and trial as within factor, followed by a posthoc Fisher LDS test. Differences between the groups in body mass, testes weight, seminal vesicles weights, number of calbindin positive cells in MPOA and density of AVP fibers in lateral septum were analyzed by ANOVA with prenatal

263 stress as independent variable, followed by Tukey-Kramer posthoc test. Differences in testosterone
264 blood levels were determined with Kruskal – Wallis test. Differences in number of animals displaying
265 aggressive behavior were analysed by X^2 test. Results were considered statistically significant with $p <$
266 0.05.

RESULTS

Aggressive behavior

Tested mice from group E17-18 (N = 9), and group E13-18 (N = 11), showed reduced aggressive behavior in comparison to control mice (N = 17) and mice from group E13-15 (N = 7). This was evident as lower frequency of bites ($F(3,40) = 4.39$; $p < 0.05$, Fig. 1a), longer latency to the first bite ($F(3,40) = 4.13$; $p < 0.01$, Fig. 1b), lower frequency of all aggressive events (bite, tail rattling, aggressive grooming; $F(3,40) = 3.67$; $p < 0.01$, Fig. 1c) and longer latency to the first aggressive event ($F(3,40) = 5.23$; $p < 0.01$, Fig. 1d). Posthoc tests for all four parameters showed that groups E17-18 and E13-18 were different from control group and E13-15 group.

Total number of tests when aggressive behavior was observed was also compared using X^2 test, which revealed statistically significant difference between groups with groups E17-18 and E13-18 showing significantly less aggressive events than control group and group E13-15 ($p < 0.001$; Fig. 2).

Sexual behavior

In tests of sexual behavior, there were no statistically significant differences in any of the observed parameters analyzed by repeated measures ANOVA. Similarly, there was no statistically significant difference in the number of ejaculating males between groups as tested by X^2 test (table 1). There was very low frequency of aggressive events in all four groups of mice and there were no statistical differences in the number of aggressive events between groups.

Body mass and sexual organ mass

Body mass at the time of euthanasia was significantly different between groups with males from the groups E13-15 (N = 7) and E13-18 (N = 11) being significantly heavier than males from control group (N = 17) and group E17-18 ($F(3,41) = 5.26$; $p < 0.01$; N = 9; Fig. 3a). Absolute and relative testis weights did not differ between groups (Fig. 3b, absolute testis weights).

292

293 Testosterone

294 Testosterone levels were significantly lower in groups E17-18 (N = 6) and E13-18 (N = 6) in comparison
295 to control group (N = 12), but not in comparison to group E13-15 (N = 6), which was also not different
296 from control group ($F(3,23) = 3.36$; $p < 0.05$, Fig. 4).

297

298 Calbindin and vasopressin immunoexpression

299 Image analyses of calbindin and vasopressin expression did not reveal any statistical difference either
300 in the number of calbindin-immunopositive cells in the medial preoptic area (Figs. 5, 7a) or in the
301 amount of vasopressin immunopositive fibers in the lateral septum (N = 6 for all experimental groups
302 and 12 for control group; Figs. 6, 7b).

303

DISCUSSION

In the present study we show that prenatal stress caused by injection of pregnant mice influences both body weight and aggressive behavior in adult male offspring of stressed mothers. Interestingly, the timing of injection stress seems to differentially affect these two parameters as males whose mothers were stressed from days 13.5 to 15.5 *p.c.* had statistically significant increase in body weight, while males whose mothers were stressed on days 17.5 and 18.5 *p.c.* showed reduced aggressive behavior. This suggests that injection stress have different effects regarding the time of exposure. In earlier prenatal period (days 13.5, 14.5 and 15.5 *p.c.*) stress apparently has stronger effect on the development of metabolic pathways resulting in increase in body mass in adult life, while stress in the later prenatal period (days 17.5 and 18.5 *p.c.*) more severely affect brain circuits which control aggressive behavior and this effect could be the result of long-term reduction of testosterone, as testosterone levels in these mice were also reduced in comparison to control mice not exposed to stress. Although in previously published studies all three parameters; aggressive behavior (Kinsley and Svare, 1986), testosterone levels (Pallares et al., 2013) and body weight regulation (Mueller and Bale, 2006) were shown to be affected by prenatal stress, stress used in those studies was much more severe and usually longer (for example, 1 or 2 hour restraint, continuous light exposure etc.) than in the present study. This is especially important as injections of pregnant dams are often used as a tool in research yet possible stress that such injections could cause to pregnant mice and especially to their offspring, is usually disregarded.

Our results regarding aggressive behavior are somewhat similar to previous studies, although all of these previous studies use more severe stressors and especially longer (Kinsley and Svare, 1986; Kiryanova et al., 2016; Politch and Herrenkohl, 1979). A study by Patin et al. (Patin et al., 2005) in rats reported that number of attacks and duration of attacks were lower in rats whose parents were exposed to severe stress (presence of a predator, cat, on days 10, 14 and 19 *p.c.*). Interestingly, the duration of attacks in this study was significantly lower in comparison to control group only in the group exposed to predator on day 19 *p.c.*, what is in agreement with our results suggesting that injection stress preferentially affects aggressive behavior if mothers are exposed to it during late period of pregnancy.

Some previous studies also reported an impairment of male sexual behavior as a consequence of prenatal stress. In rats and mice, several studies reported reductions in the number of intromissions and ejaculations following different forms of prenatal stress such as restraint stress in rats for 1h/day from 18.5 to 22.5 *p.c.* (Gerardin et al., 2005), nutritional stress, environmental stress (immobilization-illumination-heat), or injection of ACTH (adrenocorticotrophic hormone) in rats from 14.5 to 21.5 *p.c.* (Rhees and Fleming, 1981), immobilization stress in rats once per day for 2h from 14.5 to 21.5 *p.c.* (Hernandez-Arteaga et al., 2016), various stress (light, heat, noise, handling) during the last week of gestation in mice (Meek et al., 2006) or overcrowding from days 12.5–17.5 *p.c.* in mice (Harvey and Chevins, 1984). In our study there were no significant differences in any of the parameters measured during sexual behavior tests, although in some parameters, there were insignificant trends toward lower sexual behavior in male mice prenatally stressed on days 17.5 and 18.5 *p.c.* and from 13.5 until 18.5 *p.c.* These differences between studies most likely suggest differences in the form of stress. All of the studies listed above used stronger stressors in comparison to our study and these differences strongly suggest that different stressors, especially the magnitude of the stressor, have different effects on behavior of adult, prenatally stressed male mice. The difference between stressors and their effects on male sexual behavior in adult offspring of stressed mothers was reported before, with sleep deprivation and immobilization having strong effect on male sexual behavior while foot electric shock had mild effect, and water immersion (likely the least severe stressor) did not affect male sexual behavior at all (Velazquez-Moctezuma et al., 1993), similar to our study. Interestingly, in one older study, even stronger stress, heat-restraint stress, did not affect male sexual behavior in adult offspring (Politch and Herrenkohl, 1984).

The mechanisms behind reduced aggressive behavior after prenatal exposure to stress are not known. Aggressive behavior is regulated by numerous brain regions and numerous neurotransmitters (reviewed in (Takahashi and Miczek, 2014), and it is not yet understood how all these different pathways interact together to regulate aggressive behavior. Beside hypothalamus, serotonin released from the dorsal raphe, dopamine in the nucleus accumbens and prefrontal cortex have been shown to have important roles in the regulation of aggressive behavior (Miczek et al., 2001), and all these areas could be affected

by prenatal stress (Muhammad et al., 2012; Oerlemans et al., 2016; Soztutar et al., 2016). Therefore, further studies will be needed to clarify the mechanistic connections between prenatal stress and aggressive behavior in adult animals. Especially intriguing result of our study is that prenatal stress, caused by injection, only affected aggressive behavior, but not male sexual behavior. Previous studies have shown that regions involved in the regulation of aggressive behavior such as mesolimbic dopamine pathway, serotonergic pathways and prefrontal cortex, also influence male sexual behavior (Agmo et al., 1995; Hull et al., 2004). Thus it is difficult to speculate which regions might be affected by stress to specifically influence the capacity to display aggressive behavior, as brain circuits regulating both aggressive and male sexual behaviors seem to be closely intertwined.

Prenatal stress during the critical periods can reduce testosterone levels in adult offspring of stress-exposed mothers (Pallares et al., 2013; Ward, 1972). Similar to these studies, we have found significantly reduced levels of testosterone in blood in mice, exposed to prenatal stress on days 17.5 and 18.5 *p.c.* and from days 13.5 to 18.5 *p.c.* This reduced testosterone levels could explain reduced aggressive behavior, although it is interesting that sexual behavior in stressed males was not affected by prenatal stress, despite lower testosterone levels. This could suggest that different levels of testosterone are needed to induce sexual and aggressive behaviors in adult life, although it is also possible that aggressive behavior was not diminished due to lower testosterone levels in adult mice but rather due to unknown disturbances in the development of aggression regulating circuits in the brain. Mass of sexual organs is partially linked to the amount of testosterone during development of these organs and disturbances in testosterone secretion during development could affect mass of sexual organs. In our study both absolute and relative testis weights and seminal vesicle weights did not differ between groups. This is consistent with the study by Gerardin et al. (Gerardin et al., 2005), where maternal restraint stress also did not affect the weights of sexual organs despite reduction in testosterone levels and reduced male sexual behavior. This suggest that also during development, during critical periods of Sertoli cell proliferations, prenatal stress is not affecting testosterone levels so strongly to affect adult testis size, or perhaps the effect of stress during this period is only transient and testis growth is compensated after conclusion of stress.

In the brain, two sexually dimorphic regions, sexually dimorphic nucleus (SDN) in the medial preoptic area and vasopressin fibers in the lateral septum, are thought to be involved in the regulation of male specific behaviors (De Vries et al., 1983; Sakuma, 2009). The SDN in rats and its equivalent group of calbindin positive cells in mice is much larger in male brain (Robinson et al., 1985), and vasopressin fibers in the lateral septum are more dense in male brain (de Vries, 2008). Testosterone influences the development of sexual dimorphism in both areas (De Vries et al., 1983; Lephart et al., 1998) and some studies suggest that testosterone effects on aggression are through the regulation of AVP binding to its receptors in the hypothalamus (reviewed in (Albers, 2012)). Some studies also suggested that prenatal stress could influence the development of SDN and thus affect sexual differentiation of the brain in males (Anderson et al., 1985; Rhees et al., 1999), and expression of vasopressin and its receptor 1a in the brain, although the vasopressin was only studied in the paraventricular and supraoptic nuclei and not in the lateral septum (Bosch et al., 2006; de Souza et al., 2013; Grundwald et al., 2016).

In the present study we did not detect reduction in the number of calbindin positive cells in the preoptic area, or in the density of vasopressin immunopositive fibers in the lateral septum, despite the reduction in testosterone level in adult mice. This suggests that prenatal stress in our experiment did not affect the development of these two sexually dimorphic areas, possibly implying that reduction in testosterone levels during development were low, if present at all, and not sufficient to influence the masculinization of these two areas. Lack of difference in the number of calbindin positive cells could possibly explain no effect on male sexual behavior in our study, as some previous studies have shown that SDN is involved in the regulation of male sexual behavior (De Jonge et al., 1989). However, this is not generally accepted as some other studies failed to find a significant role of SDN in the regulation of male sexual behavior (Arendash and Gorski, 1983; Auger et al., 2002).

In conclusion, our present study shows that even presumably mild and very short maternal stress during critical developmental period could have negative consequences on physiological and behavioral parameters in adult mice. Injections are often applied to pregnant mice during different developmental studies, but the stress caused by such injections is often not taken into the account, although our results clearly shows that such injections could affect both aggressive behavior and some physiological

411 parameters (body weight, testosterone levels) in adult mice exposed to injection stress prenatally.
412 Therefore, in the future studies, such effects should be considered when planning developmental studies
413 and with the interpretation of the results.

414

415 **Compliance with Ethical Standards**

416 **Conflict of Interest:** The authors declare that they have no conflict of interest.

417 **Ethical approval:** All applicable international, national, and/or institutional guidelines for the care and
418 use of animals were followed. All animal experiments were approved by the Administration of the
419 Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection of the Republic of Slovenia
420 (U34401-22/2015/13).

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531

532 **Tabel 1:** Results of sexual behavior tests revealed no difference between control and prenatally stressed male mice

Group	Trial	Number of mounts (mean $\pm$ S.E.M.)	Latency to mount (mean $\pm$ S.E.M.)	Number of intromissions (mean $\pm$ S.E.M.)	Latency to intromission (mean $\pm$ S.E.M.)	Duration of anogenital sniffing (mean $\pm$ S.E.M.)
Control (n = 17)	1	3.6 $\pm$ 1.0	1280.7 $\pm$ 126.1	4.0 $\pm$ 1.1	1350.4 $\pm$ 122.1	48.6 $\pm$ 7.7
	2	4.3 $\pm$ 2.2	1308.7 $\pm$ 153.1	2.8 $\pm$ 1.7	1426.5 $\pm$ 137.7	39.5 $\pm$ 5.9
	3	6.4 $\pm$ 2.0	1140.3 $\pm$ 165.7	6.7 $\pm$ 2.3	1259.6 $\pm$ 152.5	36.4 $\pm$ 6.3
E13-15 (n = 7)	1	3.25 $\pm$ 2.1	1493.1 $\pm$ 198.3	3.0 $\pm$ 2.3	1493.2 $\pm$ 198.2	37.2 $\pm$ 10.0
	2	5.4 $\pm$ 5.1	1324.9 $\pm$ 306.7	4.4 $\pm$ 4.1	1325.5 $\pm$ 306.4	27.2 $\pm$ 10.2
	3	4.1 $\pm$ 2.8	1137.5 $\pm$ 323.3	2.6 $\pm$ 1.8	1181.5 $\pm$ 305.5	40.1 $\pm$ 13.1
E17-18 (n = 9)	1	0.5 $\pm$ 0.2	1657.9 $\pm$ 91.5	0.3 $\pm$ 0.2	1698.1 $\pm$ 91.3	68.9 $\pm$ 13.8
	2	1.5 $\pm$ 0.8	1439.2 $\pm$ 211.2	1.3 $\pm$ 0.7	1464.5 $\pm$ 193.4	24.4 $\pm$ 5.4
	3	4.5 $\pm$ 2.3	1163 $\pm$ 260.5	3 $\pm$ 1.4	1213.2 $\pm$ 240.2	36.4 $\pm$ 8.6
E13-18 (n = 11)	1	4.0 $\pm$ 2.2	1444.6 $\pm$ 158.7	4.25 $\pm$ 2.4	1464.9 $\pm$ 155.2	45.0 $\pm$ 8.3
	2	5.1 $\pm$ 2.8	1150.3 $\pm$ 266.3	4.5 $\pm$ 2.9	1315.7 $\pm$ 247.9	68.1 $\pm$ 13.8
	3	6.6 $\pm$ 2.2	903.5 $\pm$ 234.3	8.1 $\pm$ 3.4	1065.2 $\pm$ 226.3	63.8 $\pm$ 9.5

Figure legends

Fig. 1 Number of bites (a), number of aggressive events (bites, attacks, tail rattling (c), latency to the first bite (b) and latency to the first aggressive event (d) were all reduced in males from groups E17-18 (N = 9) and E13-18 (N = 11) in comparison to control group (N = 17) and group E13-15 (N = 7); * $p < 0.05$, ** $p < 0.01$; mean $\pm$ S.E.M.)

Fig. 2 Majority of male mice that were not exposed to prenatal stress or were exposed on days 13.5 to 15.5 *p.c.* showed aggressive behavior in most tests while in the groups exposed to stress on days 17.5 and 18.5 *p.c.* or daily from day 13.5 to 18.5 *p.c.* aggressive events were noted only in approximately one half of the tests (X^2 test $p < 0.001$)

Fig. 3 Body weight at the time of euthanasia (a) was significantly increased in groups of male mice exposed to stress prenatally on days 13.5 to 15.5 *p.c.* (N = 7) and daily from day 13.5 to day 18.5 *p.c.* (N = 11) in comparison to control group (N = 17) and group exposed to prenatal stress only on days 17.5 and 18.5 *p.c.* (N = 9; ** $p < 0.01$). There was no significant difference in testis weight (b, absolute testis weight) between all four tested groups (mean $\pm$ S.E.M.)

Fig. 4 Testosterone levels were significantly reduced in adult male mice, exposed to stress prenatally on days 17.5 and 18.5 *p.c.* (N = 6), and daily from days 13.5 until day 18.5 *p.c.* (N = 6), in comparison to control group (N = 12) and group exposed to injection stress on days 13.5 to 15.5 *p.c.* (N = 6; * $p < 0.05$; mean $\pm$ S.E.M.)

Fig. 5 Representative images of calbindin-immunopositive cells in the medial preoptic area in all four groups of mice (a - E13–E15, b - E17–E18, c - E13–E18, d - control; 3V – third ventricle, OC – optic chiasm, AC – anterior commissure, arrow – male specific group of calbindin positive cells; bar 100 μ m)

Fig. 6 Representative images of vasopressin fibers in the lateral septum detected by immunohistochemistry (a - E13–E15, b - E17–E18, c - E13–E18, d - control; LV – lateral ventricle, AC – anterior commissure; bar 100 μ m)

Fig. 7 Number of calbindin positive cells in the medial preoptic area (a) and density of vasopressin immunopositive fibers in the lateral septum (b) did not differ between all four groups (N = 6 for all experimental groups and N = 12 for control group), suggesting that injection prenatal stress did not affect masculinization of the brain in the expression of these two genes (mean $\pm$ S.E.M.).