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Injections to pregnant mice produce 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.

Significance statement

This study demonstrates that injecting 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.

1. 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

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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.

2. Material and methods

2.1. Mice

C57BL/6 J mice were originally obtained from Harlan (Italy) and bred at the University of Ljubljana, Veterinary Faculty in standard conditions with 12–12 h 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/6 J 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 returned 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 breading, 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 ± S.E.M.).

2.2. 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 μg/g BW), acepromazine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) and xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 μg/g BW) and ovaries were excised through a single incision. Mice received two injections of butorfanol (Turbogesic, Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/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 17β-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 h before each behavioral test, females