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Prenatal stress changes courtship vocalizations and bone mineral density in mice

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ABSTRACT

Stress during the prenatal period has various effects on social and sexual behavior in both human and animal offspring. The present study examines the effects of chronic restraint stress in the second vs third trimester in pregnancy and glucocorticoid receptor (GR) heterozygous mutation on C57BL/6N male offspring's vocal courtship behavior in adulthood by applying a novel analyzing method. Finally, corticosterone and testosterone levels as well as bone mineral density were measured.

Prenatal stress in the third, but not in the second trimester caused a significant qualitative change in males' courtship vocalizations, independent of their GR genotype. Bone mineral density was decreased also by prenatal stress exclusively in the third trimester in GR mutant and wildtype mice and – in contrast to corticosterone and testosterone – highly correlated with courtship vocalizations. In $Gr^{+/-}$ males corticosterone serum levels were significantly increased in animals that had experienced prenatal stress in the third trimester. Testosterone serum levels were overall increased in $Gr^{+/-}$ males in comparison to wildtypes as a tendency – whereas prenatal stress had no influence.

Prenatal stress alters adult males' courtship vocalizations exclusively when applied in the third trimester, with closely related changes in bone mineral density. Bone mineral density seems to reflect best the complex neuroendocrine mechanisms underlying the production of courtship vocalizations.

Besides, we demonstrated for the first time elevated basal corticosterone levels in $Gr^{+/-}$ males after prenatal stress which suggests that the $Gr^{+/-}$ mouse model of depression might also serve as a model of prenatal stress in male offspring.

1. Introduction

Prenatal stress (PNS) is known to have a large variety of effects on social interaction and emotions – including sexual behavior (Weinstock, 2008). Amongst others, it leads to "demasculized" behavior in male rats, reduced initiation of copulation, lordotic behavior, as well as reduced amount of ejaculation and decrease of ano-genital distance (Weinstock, 2001). Also, less sniffing, grooming, and boxing/wrestling is observed in adult animals after experiencing prenatal stress, which can be interpreted as a diminished quality of social interaction (Lee et al., 2007).

The point when PNS in fetal development has the strongest influence on offspring's behavior in adulthood is inconsistent in literature: Mueller and Bale (2007) demonstrated that male mice have deficits regarding coping strategies and learning performance when stressed early (embryonic days (E) 1–7) compared to mid (E 8–14) or late (E 15–21) in gestation. In rats, mid PNS has a significant impact on neurogenesis and GR density only in the female hippocampus (Pawluski et al., 2015). Finally, van den Hove et al. (2011) showed increased depressive-like behavior in mice after prenatal stress during the third trimester.

The hypothalamic-pituitary-gonadal (HPG) axis, and principally perinatal testosterone (T) spikes, determine – beside other androgens like dihydrotestosterone, androstenedione and dehydroepiandrostenedione, estrogens and progesterone – the adult male rat's sexual behavior: T spikes occur on gestation days 17–19 and within a few hours after birth. These T spikes are considered to organize the development of neural networks which, when stimu-

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lated by androgens in mature animals, are able to react in a typical male pattern (Colciago et al., 2005).

In human, rats and non-rodent mammals, high levels of glucocorticoids produced by the hypothalamic-pituitary-adrenal (HPA) axis generally disrupt all aspects of HPG function, including reproductive behavior, plasma luteinizing hormone (LH) secretion, LH releasing hormone expression, and sex steroid synthesis and release (Rivier et al., 1986). On the other hand, testosterone levels have been shown to have an inhibitory influence on the HPA axis. That is why males typically show lower levels of ACTH and glucocorticoids than females both in basal and in stressful conditions (Handa et al., 1994). Several testosterone-sensitive afferents to the HPA axis mediate the close interaction between HPA and HPG, including the central and medial amygdala, medial preoptic area of the hypothalamus and bed nuclei of the stria terminalis (Morris et al., 2004). Most interestingly, neurons in the paraventricular nucleus – being closely connected to the medial preoptic area – express GR to a great extent (Ceccatelli et al., 1989). Long term changes of serum steroid hormone levels also affect bone metabolism and thus bone strength whereas testosterone increases and glucocorticoids lower bone mineral density. In addition, also stress-induced changes of serotonin serum levels have a direct impact on bone cell metabolism (Warden et al., 2010).

HPA and its inhibitory effect on HPG is activated via stress: Xu et al. demonstrated that not only chronic or acute stress, but also restraint stress of the mother during pregnancy led to hyperresponsiveness of the HPA axis as well as demethylation of the CRH promoter and increased anxiety-like behaviors in adult offspring (Xu et al., 2014). Ward and Weisz (1984) proved that prenatal restraint stress thrice daily in the third trimester leads to an increase of corticosterone in the serum of the pregnant rat dam as well as her male and female fetuses. Furthermore, chronic immobilization stress during gestational day 7–21 caused a clear reduction of testosterone levels in plasma of male offspring at the age of 70 days (Rodríguez et al., 2007).

The glucocorticoid receptor's (GR) role for the etiology and the development of stress-related psychiatric disorders, such as depression, was demonstrated in many studies (Gass et al., 2001; Urani et al., 2005). $Gr^{+/-}$ mice possess an increased vulnerability to stress and dysregulation of the HPA axis that manifest mainly when animals are exposed to challenging situations through a specific external stressor thus representing a gene-environment model of depression (Ridder et al., 2005). Prenatal stress is known to be one crucial factor to develop psychiatric disorders like depression later in life: its association with $Gr^{+/-}$ heterozygosity has never been examined before.

Ultrasonic vocalizations (USVs) in animal models represent a substantial phenotypic dimension and provide an innovative insight into social and sexual communication. Some of the currently available studies indicate that USVs of mice are for the most part innate and strain-specific (Kikusui et al., 2011), but enclose a limited part of vocal learning (Arriaga and Jarvis, 2013).

Mice also emit a complex repertoire of ultrasonic and audible vocalizations in several contexts: pup-dam-interaction, courtship, same sex interactions, isolation and under restraint stress (Grimsley et al., 2011, 2016; Hammerschmidt et al., 2015a; Hofer et al., 2002; Liu et al., 2006; Scattoni et al., 2009). Here we focus on the ultrasonic vocalizations emitted during courtship. These ultrasonic vocalizations are typically emitted by male mice in bouts which are called mating songs or courtship vocalizations (Holy and Guo, 2005). Nunez and Tan (1984) showed a marked decrease in courtship vocalization emission after castration in mice but increased emission after daily treatment with either testosterone, DHT or estradiol. Amygdala lesions in C57/BL6 mice lead to altered courtship vocal behavior with a reduced mean syllable duration (from about 50 to 25 ms) that was due to a decrease of long syllable rates and an increase of short syllable rates. Both decreased instances of mounting behavior that usually co-occur with long syllables in courtship as well as a lack of emotionality due to disintegration of the amygdala might be the cause for the changed ultrasonic vocalizations (Matsumoto et al., 2012).

In the present study we recorded ultrasonic courtship vocalizations in male adult $Gr^{+/+}$ and $Gr^{+/-}$ offspring that had either experienced no prenatal restraint stress or stress during the 2nd or 3rd trimester, respectively. In addition, basal testosterone and corticosterone serum levels were determined.

2. Materials and methods

2.1. Animals

Acclimatized female $Gr^{+/+}$ and male $Gr^{+/-}$ mice in a C57BL/6N background were used for breeding. All animals were housed individually in a conventional type II macrolon cages, maintained at a constant temperature (22 ± 1 °C) on a 12 h light–dark cycle (lights on from 0700 h) with food and water ad libitum. Vaginal plugs were checked on a daily basis and defined as embryonic day 0 (E0) when positive. In the last trimester from E13 to E17, maternal restraint stress was applied by putting the dams into a transparent 250 ml glass cylinder filled up to a height of 5 mm with cold tap water whilst being exposed to bright light (6000 lx) three times a day (between 0800 h and 1000 h, 1200 h and 1400 h, as well as 1600 h and 1800 h) for 45 min per session (van den Hove et al., 2011). Control dams were left undisturbed in their cages.

Pregnant females delivered by caesarean sections on E18. The procedure was completed in 10–20 min (11.5 min in average) using xenon gas and isoflurane anesthesia in order to protect the pups' brain and heart from hypoxia-induced damage (Ruder et al., 2014).

After caesarean sections, dams were sacrificed as they would not be able to nurture the pups in a normal way after the surgery and the pups were tattooed for further identification within 10 min after birth. Experienced wildtype C57BL/6N foster dams whose own litter was aged between PND (postnatal day) 1–PND 4 were used to raise the pups. The transfer into the nest of the lactating foster dam took place within 15 min after birth. The original pups were mixed with litter and urine of the foster dam to pick up her scent and so improving acceptance by the foster dam. We chose foster litters that had approximately the same number of pups (±2 pups in most cases) as the original litters so that no culling was necessary. The average number of pups per litter was 8 and in total, the offspring was raised by 34 different foster dams.

The biological litters of the foster dams were completely removed from the nest. As the original litter was transferred as a whole to one foster dam it contained a natural mix of $GR^{+/-}$ and $GR^{+/+}$ pups and all pups had experienced the same prenatal stress paradigm.

After weaning at four weeks of age, only one heterozygous or wildtype male was chosen per litter for the behavioral experiments to prevent litter effects (Chapman and Stern, 1979) which resulted in 26 offspring male $Gr^{+/-}$ mice and 32 of their wild-type littermates on C57BL/6N –background. They were housed individually in macrolon type II cages with nesting material under a reversed day-night cycle (lights on from 1900 to 0700 h with 12 h dark and 12 h light phase) with an adaptation period of at least 14 days before the start of experiments and supplied with food and water ad libitum. $Gr^{+/-}$ animals were generated as described previously (Ridder et al., 2005; Tronche et al., 1999) and were genotyped with PCR as recommended by the Jackson Labs (Bar Harbour, Maine, USA). Social experience with the other sex was found to be a prior condition to elicit adult males' courtship vocalizations (Nyby et al., 1983, 1978; Sipos et al., 1992). To standardize sexual and social expe-