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Living in a dangerous world decreases maternal care: A study in serotonin transporter knockout mice

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ABSTRACT

Adverse early experiences can profoundly influence the adult behavioral profile. When pregnant and lactating mice are confronted with soiled bedding of unfamiliar males (UMB), these stimuli signal the danger of infanticide and thus simulate a "dangerous world". In a previous study, offspring of UMB treated mothers were shown to display increased anxiety-like behavior and reduced exploratory locomotion as adults, compared to mice treated with neutral bedding (NB, "safe environment"). The aim of this study was to elucidate the mechanisms conveying these effects of living in a "dangerous world" to offspring behavior. We hypothesized the mother to be the major link and focused on the influence of UMB on maternal stress hormones and behavior. Thus, we investigated fecal corticosterone metabolites (CM) and maternal care of pregnant and lactating mice either treated with NB or UMB. The offspring were subsequently tested for their anxiety-like and exploratory behavior. Mothers treated with UMB showed a significantly higher increase of fecal CM following the initial treatment, than NB treated mothers, indicating that the odor cues of potentially infanticidal males represented an ethologically relevant stimulus. Whereas the hormonal stress response habituated, living in a "dangerous world" led to a distinct and consistent reduction of maternal care behavior, particularly concerning the duration of licking and grooming the pups. Surprisingly, we could not confirm our former findings of altered phenotypes in the offspring of UMB treated mothers. In summary, we hypothesize that the frequently described effects of early life adversity on the offspring's behavioral profile are mediated primarily by maternal care in altricial rodents.

Introduction

Early life experiences can have long-lasting effects on adult behavior in animals and humans (Denenberg, 1975; Gross and Hen, 2004; Lesch, 2011; Sachser et al., 2011; Weinstock, 2008). The organism seems to be most susceptible to external influences during early development, that is, the prenatal and early postnatal phase, when brain circuits are highly plastic (Champagne and Curley, 2005; Kaiser and Sachser, 2005). Studies in humans clearly show that severe environmental adversity in early life, such as prenatal stress or child abuse and neglect, can have detrimental effects on development and lead to multiple forms of psychopathology (Anda et al., 2006; Briere and Jordan, 2009; Cutajar et al., 2010; Kessler et al., 2010; Koenig et al., 2002; Seckl, 2004; Seckl and Holmes, 2007; Weich et al., 2009). On the other hand, high levels of maternal care and a secure parent–child attachment are related to elevated self-esteem and self-confidence, reduced trait anxiety, increased capacity for emotional regulation and higher social competence in later life (Carter et al., 2005; Champagne, 2008; Pruessner et al., 2004; Sroufe, 2005).

In animals, the effects of early life experience on later behavior and physiology have been extensively studied in mice and rats. For example, adversity during the prenatal phase is modeled by exposing pregnant females to stressors, such as daily handling, repeated saline injections, light and/or noise, forced swimming, and repeated restraint (Archer and Blackman, 1971; Chung et al., 2005; Kofman, 2002; Maccari et al., 2003; Richardson et al., 2006; Vallée et al., 1997; Ward et al., 2000; Weinstock, 2008). Whereas the treatment does not seem to affect the offspring in a consistent way in mice, in rats these procedures mostly cause increased anxiety-like behavior, decreased locomotion and altered hypothalamic–pituitary–adrenal (HPA) axis regulation in the young. Since during gestation the mother is the only link between her offspring and the current environment, it is likely that such effects are mediated by intra-uterine mechanisms during gestation (e.g., Kaiser and Sachser, 2005). One possible mechanism is the activation of the maternal HPA axis, since

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prenatal exposure to glucocorticoids and inhibition (or knockout) of placental 11β-hydroxysteroid dehydrogenase type 2 – the fetoplacental barrier to maternal glucocorticoids – cause increases in HPA axis activity and anxiety-related behaviors in the offspring (Harris and Seckl, 2011; Seckl, 2004; Seckl and Holmes, 2007).

During the early postnatal phase, i.e. lactation, adverse experience in mice and rats is frequently created by experimentally inducing maternal separation (Huot et al., 2001; Millstein and Holmes, 2007; Parfitt et al., 2007; Pryce and Feldon, 2003; Romeo et al., 2003; Veenema et al., 2007), which often, but not always, causes increased anxiety-like behavior in the offspring. A possible mechanism mediating these effects might be maternal care, which is a crucial factor influencing offspring development in mice and rats. Indeed, variation in the frequency and intensity of maternal care, in particular maternal licking and grooming (LG) of pups, is a major source of inter-individual differences in behavioral profile and stress responsiveness in adulthood (Kaufman et al., 2004; McCormack et al., 2009; Meaney, 2001). Offspring experiencing high levels of maternal LG are behaviorally less fearful as adults and show more moderate HPA responses to acute stress than offspring subjected to less maternal LG in rats (Caldji et al., 1998; Champagne, 2010; Francis et al., 1999; Liu et al., 1997; Meaney, 2001) and mice (Calatayud et al., 2004; Carola et al., 2006; Wei et al., 2010).

Depending on genetic factors, individuals seem to be differentially susceptible to effects of maternal care. For instance, when mice with a heterozygous disruption of the serotonin transporter (5-HTT) gene, and thus a reduced number of 5-HTT proteins (5-HTT +/− mice, Bengel et al., 1998), are reared by mothers providing either low or high maternal care, they show a more pronounced increase of anxiety-like behavior than wildtype littermates, indicating a higher susceptibility to variations in maternal care behavior (Carola et al., 2008). These results are in accordance with findings in humans, showing that individuals carrying one low expressing copy of the 5-HTT gene (Lesch et al., 1996) suffer an increased risk of depression after the experience of childhood maltreatment, whereas carriers of two high expressing alleles are not affected (Caspi et al., 2003; but also see Homberg and Lesch, 2011). Other studies in humans (Kaufman et al., 2004; Stein et al., 2008) and animals (McCormack et al., 2009; Spinelli et al., 2007) find similar effects, indicating that individuals with a rather low expression of the 5-HTT gene seem to be particularly prone to the effects of adverse early life experience.

In a previous study we confronted 5-HTT knockout (+/− and −/−) and wildtype mice with a "dangerous world" during early life by inserting male-soiled bedding into the cages of pregnant and lactating 5-HTT +/− females (Heiming et al., 2009). These stimuli signal the presence of unfamiliar males in the mother's habitat and thus indicate the danger of infant killing (Elwood and Kennedy, 1991; Perrigo et al., 1993; vom Saal and Howard, 1982; Weber and Olsson, 2008). Strikingly, offspring growing up in such a "dangerous" environment showed increased anxiety-like behavior and reduced exploratory locomotion in comparison to mice treated with neutral bedding (NB, "safe environment"). The effects of treatment with unfamiliar male bedding (UMB) were most pronounced in mice completely lacking 5-HTT expression (5-HTT −/− mice), as compared to 5-HTT +/− and wildtype mice (Heiming et al., 2009).

The purpose of the present study was to elucidate the mechanisms conveying the effects of living in a "dangerous world" on offspring behavior. In particular we investigated the influence of treatment with UMB on maternal stress hormones and maternal care behavior as two possible mediators. Therefore, pregnant and lactating 5-HTT +/− mice treated with either NB or UMB were monitored regarding their HPA axis response, hypothesizing that UMB mothers would show a higher stress response after the treatments. Furthermore, we observed maternal care, which was hypothesized to differ between NB and UMB mothers following the treatment. Finally, the offspring were tested in a range of behavioral tests in order to find out, whether the previously described effects of UMB treatment and 5-HTT genotype on offspring behavioral profile could be confirmed.

Methods

Animals and general housing conditions

5-HTT +/+, 5-HTT +/− and 5-HTT −/− mice (Bengel et al., 1998), backcrossed into a C57BL/6J genetic background for >10 generations, originated from the internal stock of the Department of Behavioural Biology, University of Muenster, Germany. The original breeding stock was obtained from the Department of Psychiatry, University of Wuerzburg, Germany. Breeding pairs each consisted of a 5-HTT +/− male and female, resulting in homozygous (−/−) and heterozygous (+/−) 5-HTT knockout offspring, as well as their wildtype littermates (+/+). Genotyping was accomplished using ear tissue to extract genomic DNA, amplified by PCR. Genotypes were identified by agarose gel electrophoresis with DNA-fragments of either 225 bp (5-HTT +/+), 272 bp (5-HTT −/−) or both (5-HTT +/−).

All mice were housed in transparent standard Macrolon cages type III (42.5 × 26.6 × 15.5 cm) with sawdust as bedding material (Allspan, Höveler GmbH & Co.KG, Langenfeld, Germany), a paper towel as nesting material, and food (1324 for experimental animals and 1314 for breeding females, Altromin GmbH, Lage, Germany) and water provided *ad libitum*. Breeding females were additionally fed oat flakes (Fortin GmbH & Co.KG, Duesseldorf, Germany) every workday and their paper towel was cut into small pieces in order to ensure good visibility during the behavior observations. The housing room was maintained at a 12 h light/dark cycle (lights on at 8:00 h) and a temperature of 22 ± 3 °C.

The present work complies with current regulations covering animal experimentation in Germany and the EU (European Communities Council Directive 86/609/EEC). All experiments were approved by the 'Animal Welfare Officer' of the University of Muenster (reference number: 8.87-50.10.46.08.140).

Treatment of mothers

Adult virgin 5-HTT +/− females ($n = 37$) were housed singly for three days (days 1–3) and subsequently mated with an adult and sexually experienced 5-HTT +/− male ($n = 19$) for five days (days 4–8, Fig. 1). On day 1 each female received a small amount of soiled bedding stemming from the prospective mating partner, in order to prepare them for mating. On day 8 the females were housed singly again until the birth of the pups (between days 23 and 26).

During pregnancy and lactation mothers experienced either a simulated "safe" or "dangerous" environment, in accordance to the paradigm established and described by Heiming et al. (2009). 17 of the mothers were treated with fresh sawdust (neutral bedding, NB), which does not signal any specific danger ("safe" environment). In contrast, 20 of the mothers were treated with soiled bedding stemming from the cages of unfamiliar adult males (unfamiliar male bedding, UMB), which indicates the threat of infant killing ("dangerous" environment). The unfamiliar males were of the strains NMRI, MF1 and C3H ($n = 4$ per strain). They were housed in pairs and the bedding of the six cages was used for the treatments alternately. For each treatment approximately 220 ml of the respective bedding material was inserted into the cage of the mother in the corner opposite of the nest and left there until the next cage change. Cages were cleaned once a week, except during the late prenatal and early postnatal phase (days 21–33). The mothers were treated four times during pregnancy (days 13, 15, 18 and 20) and six times during lactation (days 29, 32, 34, 36, 39 and 41) between 8:00 and 8:15 h (mothers whose pups were born on day 23 received the first postnatal treatment already on day 28).

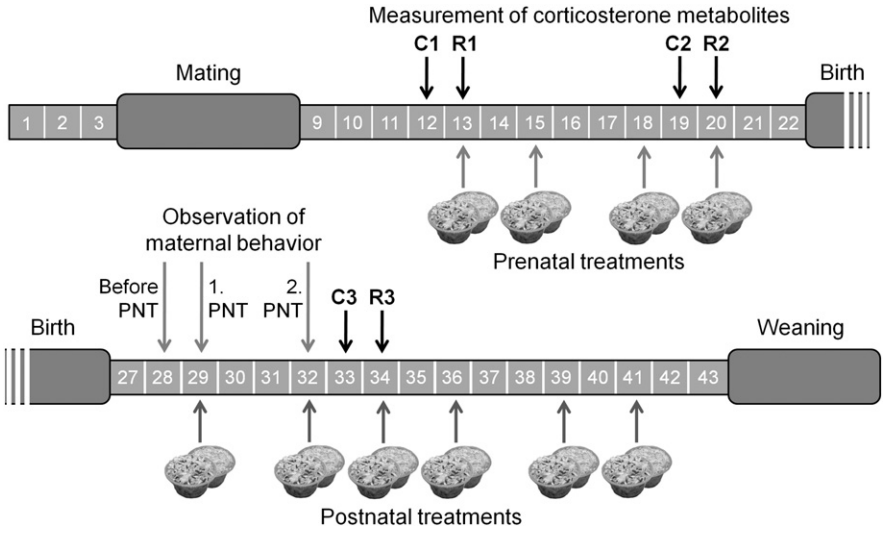

Fig. 1. Experimental design: Mothers were treated four times during pregnancy and six times during lactation with either neutral bedding or unfamiliar male bedding. Maternal fecal corticosterone metabolites were measured before (control: C), and after (reaction: R) the first prenatal treatment, the last prenatal treatment, and the third postnatal treatment. Maternal care behavior was observed before any postnatal treatments (before PNT), after the first postnatal treatment (1. PNT), and after the second postnatal treatment (2. PNT). For mothers whose pups were born on day 23, the first postnatal treatment and the first two observations were performed one day earlier than shown in the figure.

Maternal stress hormones

The adrenocortical activity of the mothers, and thus the stress reaction after the treatment, was monitored non-invasively by measuring concentrations of corticosterone metabolites (CM) in the feces (Lepschy et al., 2010; Touma et al., 2003; Touma et al., 2004). In order to assess the increase of the CM concentration following the treatment, a control CM value (C, taken the day before the treatment) and a reaction CM value (R, taken on the day of the treatment) were analyzed for each measuring time point. The CM increase was calculated by subtracting CM control values from CM reaction values. The stress response was measured after the first prenatal treatment (C1/R1, days 12/13), after the last prenatal treatment (C2/R2, days 19/20), and after the third postnatal treatment (C3/R3, days 33/34, Fig. 1). Since Touma et al. (2003) showed that a peak of CM can be found in the feces 8–12 h after the exposure to a stressor, all fecal samples were collected between 16:00 and 20:00 h. For the sample collection the mothers were placed in standard Macrolon cages type II ($26.7 \times 20.7 \times 14$ cm) equipped with three paper towels and food and water provided *ad libitum*. When sampling occurred during the lactation period, the mothers' home cages containing the litters were meanwhile placed underneath a heat lamp, to avoid hypothermia of the pups. All feces defecated by the mothers during the 4 h sampling period were collected and frozen at −20 °C.

The fecal samples were homogenized and aliquots of 0.05 g were extracted with 1 ml of 80% methanol. Subsequently, the samples were analyzed with a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay, previously established and successfully validated to measure CM in mice (for details see Touma et al., 2003; Touma et al., 2004). The intra- and inter-assay coefficients of variation for a low and high concentration pool sample ($n = 16$ each) were between 5.8% and 9.1%.

Maternal behavior

Maternal care behavior was observed at three time points, namely before any postnatal treatments (before PNT, day 28), after the first postnatal treatment (1. PNT, day 29), and after the second postnatal treatment (2. PNT, day 32, Fig. 1). For mothers whose pups were born on day 23, the first two observations were performed one day earlier (days 27 and 28). Thus, the age of the pups was 3–5 days (before PNT), 4–6 days (1. PNT) and 7–10 days (2. PNT), respectively. All behavior observations were performed by an experienced observer (C.B.) using focal animal sampling and continuous recording. Data was collected by means of the software "The Observer XT", version 7 (Noldus Information Technology). The observations were conducted between 8:30 and 12:30 h and at each time point each female was observed three times for 10 min within this period, the observation sessions being separated by at least 57 min. The maternal behaviors recorded were arched-back nursing (ABN), blanket nursing (BN), passive nursing (PN), licking and grooming the pups (LG), and nest building (NEB). To measure the total duration of nursing (NUR) the time the mothers spent ABN, BN and PN was summed up. Furthermore, the time the mother spent on the nest (MON) was measured. For a description of the behavior patterns see Table 1.

Offspring behavior

After weaning at the age of 22 days, offspring of NB and UMB mothers were placed in unisex sibling groups of two to five animals. In rare cases individuals without littermates of the same gender were housed together with mice of the same age, sex and treatment group (this occurred in each treatment group). Altogether 34 NB males (12 +/+, 12 +/−, 10 −/−), 36 NB females (12 +/+, 12 +/−, 12 −/−), 35 UMB males (12 +/+, 12 +/−, 11 −/−) and 33 UMB females (9 +/+,

Table 1
Description of maternal behavior patterns.

Behavior	Definition
Nest building (NEB)	The mother moves bedding with her snout or her paws while being in a distance to her pups that is smaller than a body length (may occur simultaneously with nursing).
Licking and grooming (LG)	The mother moves her snout or her paws over the pup, touching it, while her head nods (may occur simultaneously with nursing).
Arched-back nursing (ABN)	The mother is arched over the pups with her legs splayed (Myers et al., 1989).
Blanket nursing (BN)	The mother is over the litter, but has no arch in her back and there is no obvious extension of her legs (Myers et al., 1989).
Passive nursing (PN)	The mother is lying on her side with one or more pups attached or, more generally, off to the side of the pile of pups (Myers et al., 1989).
Mother on nest (MON)	Every body part of the mother is positioned on top of the nest, or the distance between the mother (disregarding the tail) and her pups measures less than a head length. The nest is defined as the area where most of the pups are lying.

12 +/−, 12 −/−) were weighed on postnatal day 28–32 and subsequently tested for their anxiety-like and exploratory behavior in the Dark Light test (DL), the Open Field test (OF), the Elevated Plus Maze test (EPM), and the Free Exploration test (FE). The order of tests was the same for each animal and varying sample sizes in some of the parameters were due to technical reasons.

All testing procedures were carried out in a separate testing room. The animals' movements in the respective test apparatuses were recorded by a camera and analyzed by use of the computer programs Optimas 6.5N (Media Cybernetics) and Tracking Analysis 1.1.1 (Lars Lewejohann, http://www.phenotyping.com). After each test session the apparatuses were cleaned with 70% ethanol.

The DL (Crawley and Goodwin, 1980; Crawley, 1981) was performed at the age of 28–32 days and the testing procedure was conducted between 10:00 and 11:30 h. The DL test apparatus consisted of a light compartment (28×26.6×15.5 cm; 550 lx) and a dark compartment (17×26.6×15.5 cm; covered by a lid), which were connected via a small sliding door. Each mouse was placed inside the dark compartment with lid and sliding door closed. The individual remained there for 1 min before the sliding door was opened and the mouse could freely explore the DL apparatus for 5 min. The parameters analyzed for each animal were the latency to enter the light compartment, the number of entries into the light compartment and the percentage of time spent in the light compartment.

The mice were tested in the OF (Holmes, 2001; Treit and Fundytus, 1988) at the age of 30–35 days. The OF was performed between 8:00 and 11:00 h by means of a white square arena (80×80×42 cm), illuminated by an overhead bulb (550 lx). The mouse was placed inside a cylinder (11 cm diameter, 20 cm high) standing in one corner of the OF apparatus. After 1 min the cylinder was lifted and the mouse was allowed to freely explore the arena for 5 min. The parameters measured were the path length the mice traveled and the percentage of time they spent in the center of the arena (defined as the area of the open field being located at least 20 cm distant from the walls).

At the age of 35–39 days the mice were tested in the EPM (Hogg, 1996; Lister, 1990). The testing procedure was conducted between 8:00 and 10:00 h by means of a plus shaped apparatus, which was elevated 50 cm above the ground and illuminated by an overhead bulb (100 lx). The four arms (30×5 cm) extended from a central platform (5×5 cm) and two of them were enclosed with 20 cm high walls (closed arms), while the other two (open arms) had only 4 mm high walls to prevent the mice from falling off. After spending 1 min in an empty cage, each mouse was placed on the central platform of the EPM, facing a closed arm, and was allowed to freely explore the apparatus for 5 min. The parameters measured were the percentage of time spent on the open arms, the percentage of entries into the open arms and the sum of entries into the open and closed arms.

In all behavior tests described so far the mice have been forced into an aversive novel situation, thus these procedures measure mainly the so-called “state anxiety”, the anxiety a subject experiences at a particular moment in time (Lister, 1990). By contrast, the “trait anxiety”, a permanent characteristic of an individual which relates to an animal's anxiety proneness and does not vary from moment to moment (Lister, 1990), can be measured in the FE, where the mouse can choose by itself when or if at all to enter the unfamiliar environment (Belzung and Berton, 1997; Griebel et al., 1993). The FE was performed at the age of 37–42 days and the testing procedure was conducted between 8:00 and 11:00 h. The FE test apparatus consisted of a white square arena (60×60×36 cm), illuminated by an overhead bulb (190 lx). Via a tunnel (23.5×15×9.5 cm) the arena could be connected to the home cage of the mice, which was equipped with a transparent sliding door (15×9.5 cm). Before the testing procedure, the cage mates of the test subject were taken out of the home cage and placed in another cage containing a small amount of bedding material from the home cage, a paper towel and food and water provided *ad libitum*. Subsequently, the test subject was carried into the testing room in its home cage and the cage connected to the arena. The mouse was placed in an empty cage for 1 min and afterwards transferred back into the home cage. The sliding door was opened and the animal was allowed to freely explore the whole apparatus for 15 min. The parameters measured were the latency to enter the arena, the number of excursions into the arena and the percentage of time spent in the arena. After the testing procedure all cage mates were placed back into the home cage and the cage was left alone for at least 1 h before another testing session with the next mouse in this cage. Whenever possible, individuals from the same cage were tested on different days.

Statistical analysis

The data was first tested for deviation from the normal distribution by means of the Kolmogorov–Smirnov test with Lilliefors correction, as well as by visual inspection of histograms and q–q plots. Whenever data did not deviate from a normal distribution or could be adequately transformed, parametric statistics were applied: univariate analysis of variance (ANOVA) was used to evaluate differences between more than two independent samples. More than two dependent samples were compared using a Repeated Measures ANOVA, with Bonferroni post hoc testing. Pair wise comparisons between independent samples were performed using 2-tailed Independent Samples *t*-tests. Parametric statistics were applied for the analyses of maternal control and reaction CM values (logarithmically transformed), litter size, sex ratio, and for the offspring's path length in the OF.

Since the remaining data were not normally distributed and could not be adequately transformed, they were analyzed with non-parametric statistics: in case of maternal CM increase values and maternal care behavior, comparisons between the treatment groups were performed by means of the Mann–Whitney *U* test (2-tailed). Values of different sample points within the treatment groups were analyzed with the Friedman test, and in case of significance, pair wise comparisons were subsequently performed using the Wilcoxon–Wilcox test (2-tailed).

Concerning offspring weight and behavior, males and females differed from each other in seven out of twelve parameters and were thus evaluated separately (see also Crusio et al., 2008). Differences between the three genotypes were analyzed separately for NB and UMB mice using the Kruskal–Wallis test. In case of significance, pair wise comparisons between genotypes were subsequently performed with the Mann–Whitney *U* test (1-tailed, see Introduction; Bonferroni corrected). Comparisons between the treatment groups were performed separately for mice of different genotypes by means of the Mann–Whitney *U* test (1-tailed, see Introduction).

Statistical significance was set at $p<0.05$. All tests were calculated using the software package SPSS (IBM SPSS Statistics for Windows, Release 19.0.0, 2010), with the exception of the Wilcoxon–Wilcox test, which was calculated as described by Sachs (1999). Graphs were created using the software SigmaPlot (SigmaPlot for Windows Version 11.0, 2008).

Results

Effects of treatment on maternal stress hormones

Mothers were either treated with unfamiliar male bedding (UMB) during pregnancy and lactation, or they received a control treatment with neutral bedding (NB). Their fecal corticosterone metabolites (CM) were measured before (control = C) and after (reaction = R) the first prenatal treatment (C1/R1), the last prenatal treatment (C2/R2) and the third postnatal treatment (C3/R3). Reaction CM values were significantly higher than control CM values at all time points for both, NB and UMB mothers ($F_{(1,\ 35)}=172.455$, $p=0.0001$; Fig. 2). During pregnancy, the control and reaction CM values of NB and UMB

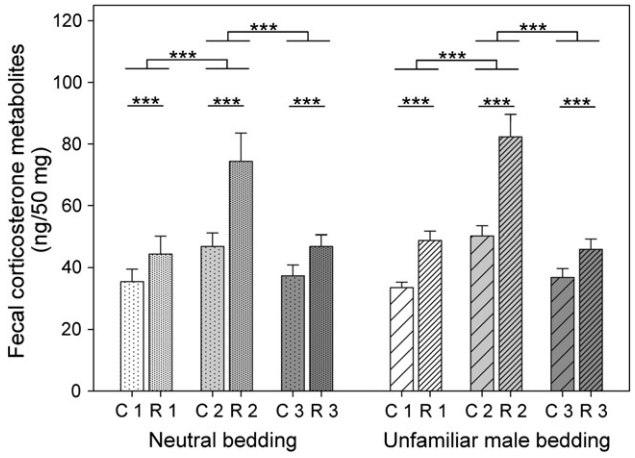

Fig. 2. Fecal corticosterone metabolites of neutral bedding (NB) and unfamiliar male bedding (UMB) treated mothers before (control = C) and after (reaction = R) the first prenatal treatment (C1/R1), the last prenatal treatment (C2/R2) and the third postnatal treatment (C3/R3). Data are shown as mean + SEM. Statistics: ANOVA, post hoc testing: Bonferroni, ***$p<0.001$. There were significant main effects of reaction (C vs. R; $p=0.001$) and measurement time point (measurement 1 vs. 2 vs. 3; $p=0.0001$), as well as an interaction effect of both ($p=0.001$). Data were obtained from 17 NB mothers and 20 UMB mothers.

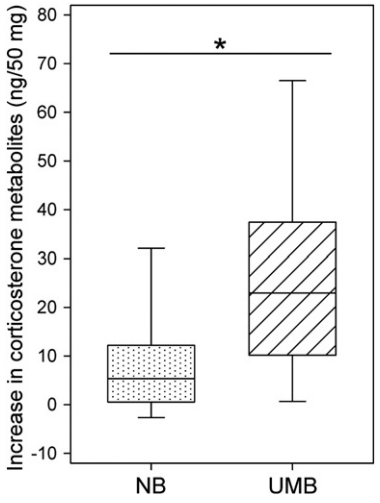

Fig. 3. Increase of fecal corticosterone metabolites (CM) of neutral bedding (NB) and unfamiliar male bedding (UMB) treated mothers following the first prenatal treatment. Data are given as box plots with medians, 25–75% quartiles and 5–95% ranges. Statistics: Mann–Whitney *U* test, *$p<0.05$. Data were obtained from 17 NB mothers and 20 UMB mothers and were calculated by subtracting CM control values (before treatment) from CM reaction values (after treatment).

mothers rose in the time from the first to the second measurement, whereas they were lower again at the third measurement during lactation. This variation over time was significant for mothers of both treatments ($F_{(2,70)}=46.666$, $p=0.0001$; post hoc testing: measurement 1/3 vs. 2: $p=0.0001$). Furthermore, there was a significant interaction between the measurement time point and the CM reaction ($F_{(2,\ 70)}=7.714$, $p=0.001$), meaning that the difference between control and reaction CM values was highest at the second measurement. Consistently, the CM increase (R-C) of NB and UMB females differed between the measurement time points (NB: $\chi^2=15.176$, $p=0.0001$; UMB: $\chi^2=13.300$, $p=0.001$; Fig. 3), with highest values at the second measurement (NB: R1-C1 vs. R2-C2 $p<0.01$, R2-C2 vs. R3-C3 $p<0.01$; UMB: R1-C1 vs. R2-C2 $p<0.1$, R2-C2 vs. R3-C3 $p<0.01$).

Control and reaction CM values did not differ between NB and UMB mothers at any of the time points ($F_{(1,\ 35)}=0.449$, $p=0.507$). However, the CM increase (R-C) after the first prenatal treatment was significantly higher in UMB mothers than in NB mothers (R1-C1: $U=93$, $p=0.018$), but not at the other time points (R2-C2: $U=152$, $p=0.598$; R3-C3: $U=159$, $p=0.752$).

Effects of treatment on maternal behavior

Maternal behavior was observed before any postnatal treatments (before PNT), after the first postnatal treatment (1. PNT) and after the second postnatal treatment (2. PNT). Parameters measured were the durations of nursing (NUR, Fig. 5), arched-back nursing (ABN, Fig. 6), blanket nursing (BN), passive nursing (PN), licking and grooming the pups (LG, Fig. 7), nest building (NEB) and the time the mother spent on the nest (MON, Fig. 4). Before PNT, there was no significant difference between NB and UMB mothers in any of the parameters (NUR: $U=156.5$, $p=0.689$; ABN: $U=157$, $p=0.707$; BN: $U=142$, $p=0.382$; PN: $U=168$, $p=0.958$; LG: $U=130.5$, $p=0.235$; NEB: $U=125.5$, $p=0.178$; MON: $U=164.5$, $p=0.874$). The comparison of maternal behavior before PNT, after the 1. PNT and after the 2. PNT revealed that the treatment with NB did not lead to significant changes in most of the parameters (NUR: $\chi^2=1.463$, $p=0.508$; ABN: $\chi^2=1.059$, $p=0.611$; PN: $\chi^2=1.969$, $p=0.389$; LG: $\chi^2=1.882$, $p=0.415$; NEB: $\chi^2=0.413$, $p=0.844$; MON: $\chi^2=1.463$, $p=0.508$), with the exception of blanket nursing (BN: $\chi^2=6.157$, $p=0.043$). However, pair wise comparisons did not reveal significant differences concerning blanket nursing in NB mothers between time points ($p>0.05$). In contrast, the treatment with UMB led to significant changes over time concerning nursing, arched-back nursing, licking and grooming the pups and time spent on the nest (NUR: $\chi^2=7.872$, $p=0.017$; ABN: $\chi^2=7.3$, $p=0.028$; LG: $\chi^2=12.494$, $p=0.001$; MON: $\chi^2=8.4$, $p=0.014$). In detail, durations of nursing, arched-back nursing, licking and grooming the pups and the time spent on the nest were significantly decreased in UMB mothers after the 2. PNT, compared to the first observation before PNT (NUR: $p=0.05$; ABN: $p=0.05$; LG: $p=0.01$; MON: $p=0.05$). Furthermore, in comparison to the first observation LG was already significantly reduced after the 1. PNT (LG: $p=0.05$). However, the treatment with UMB did not lead to significant changes in blanket nursing, passive nursing or nest building (BN: $\chi^2=4.899$, $p=0.092$; PN: $\chi^2=2.88$, $p=0.251$; NEB: $\chi^2=0.532$, $p=0.783$).

When the behavior after the 1. and 2. PNT was compared between NB and UMB mothers, it was found that there were no significant differences after the 1. PNT (NUR: $U=151.5$, $p=0.582$; ABN: $U=136.5$, $p=0.315$; BN: $U=148.5$, $p=0.463$; PN: $U=146.5$,

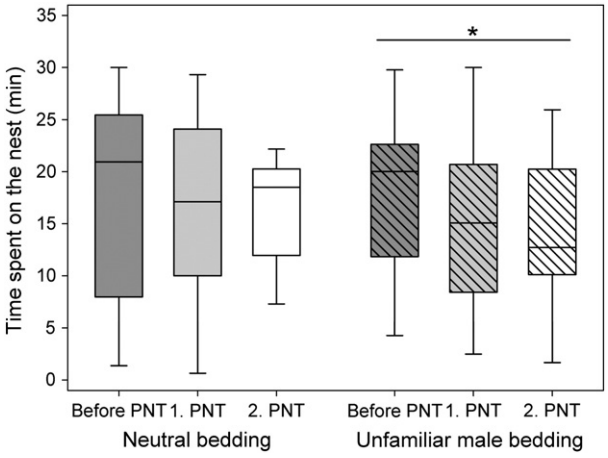

Fig. 4. Time spent on the nest of neutral bedding (NB) and unfamiliar male bedding (UMB) treated mothers before any postnatal treatments (before PNT), after the first postnatal treatment (1. PNT) and after the second postnatal treatment (2. PNT). Data are given as box plots with medians, 25–75% quartiles and 5–95% ranges. Statistics: Wilcoxon–Wilcox test, *$p<0.05$. Data were obtained from 17 NB mothers and 20 UMB mothers and refer to an observation time of 30 min per mother.

p = 0.480; LG: U = 107, p = 0.055; NEB: U = 149, p = 0.533; MON: U = 155.5, p = 0.667). Interestingly, after the 2. PNT UMB mothers spent less time arched-back nursing and licking and grooming their pups than NB mothers (ABN: U = 95.5, p = 0.022; LG: U = 84, p = 0.008), while the other parameters did not differ between mothers of the two treatment groups (NUR: U = 127.5, p = 0.200; BN: U = 118, p = 0.112; PN: U = 137, p = 0.315; NEB: U = 163.5, p = 0.851; MON: U = 141, p = 0.389).

Litter size and sex ratio

Litters of the two treatment conditions did not differ significantly concerning litter size (Mean: NB: 7.53, UMB: 7.45; T = −0.179, p = 0.859) or sex ratio (% males: NB: 52.86, UMB: 52.14; T = 0.116, p = 0.908).

Effects of genotype and treatment on offspring weight and behavior

Offspring of NB and UMB mothers with the genotypes 5-HTT +/+, +/− or −/− were weighed and subsequently tested for their anxiety-like and exploratory behavior in the Dark Light test (DL), the Open Field test (OF), the Elevated Plus Maze test (EPM), and the Free Exploration test (FE). Overall, these tests did not reveal clear and distinct effects of genotype and treatment on offspring weight and behavioral profile.

In detail, the weight of the animals on postnatal day 28–32 was not significantly influenced by genotype or treatment (Tables 2 and 3). Concerning the behavior, a significant effect of the genotype was only found in five out of eleven measured parameters (Table 2): in the DL differences between the genotypes were only shown in the parameter number of entries into the light compartment for UMB females ($\chi^2 = 9.605$, p = 0.008; supplemental Fig. A). More precisely, 5-HTT −/− females of the UMB group entered the dark compartment less often than 5-HTT +/− and +/+ UMB females (5-HTT +/−: U = 14, p = 0.0001; 5-HTT +/+: U = 29.5, p = 0.042). Concerning the path length in the OF, ANOVA detected a significant main effect of genotype for males ($F_{(2, 62)} = 9.639$, p = 0.0001; supplemental Fig. B), with 5-HTT −/− mice traveling shorter distances than 5-HTT +/− and +/+ mice (post hoc testing: p = 0.004 for both), but not for females ($F_{(2, 63)} = 2.844$, p = 0.066). Furthermore, in the EPM a genotype effect was detected for NB males in the parameter sum of entries into the open and closed arms ($\chi^2 = 10.481$, p = 0.005; supplemental Fig. C). In detail, 5-HTT −/− males of the NB group entered the open and closed arms of the EPM less often than 5-HTT +/− and +/+ NB males (5-HTT +/−: U = 19, p = 0.003; 5-HTT +/+: U = 14.5, p = 0.001). Concerning the FE, genotype effects were demonstrated for NB females in the parameters latency to enter the arena ($\chi^2 = 8.387$, p = 0.015; Supplemental Fig. D) and number of excursions into the arena ($\chi^2 = 7.431$, p = 0.024). 5-HTT −/− NB females entered the FE arena later than 5-HTT +/− and +/+ NB females (5-HTT +/−: U = 23, p = 0.002; 5-HTT +/+: U = 40, p = 0.028) and undertook fewer excursions into the arena than 5-HTT +/− NB females (U = 24.5, p = 0.002). Concerning all other parameters, no influence of genotype was found (p > 0.05).

Significant differences between NB and UMB mice were only found in three out of eleven behavioral parameters (Table 3). In detail, 5-HTT +/− males from the UMB group spent a significantly lower percentage of time in the FE arena than 5-HTT +/− males from the NB group (U = 40, p = 0.033). Furthermore, 5-HTT +/− females from the UMB group entered the open arms of the EPM significantly less often (U = 35, p = 0.029) and spent a significantly lower percentage of time on them than 5-HTT +/− females from the NB group (U = 33, p = 0.022). Concerning all other parameters UMB mice did not differ significantly from NB mice (p > 0.05).

Table 2
Comparison between genotypes (5-HTT +/+ vs. 5-HTT +/− vs. 5-HTT −/−) in offspring of neutral bedding (NB) and unfamiliar male bedding (UMB) treated mothers. Statistics: ANOVA for the path length in the OF, Kruskal–Wallis test for all other parameters. Bold values indicate significance; for directions of the differences see the text. DL: dark light test, OF: open field test, EPM: elevated plus maze test, FE: free exploration test.

		NB		UMB	
Test	Parameter	Males	Females	Males	Females
/	Body weight (postnatal day 28–32)	$\chi2 = 0.967$; p < 0.617	$\chi2 = 0.082$; p < 0.960	$\chi2 = 0.029$; p < 0.986	$\chi2 = 3.658$; p < 0.161
DL	Latency to enter the light compartment	$\chi2 = 0.026$; p < 0.987	$\chi2 = 1.306$; p < 0.521	$\chi2 = 0.496$; p < 0.780	$\chi2 = 1.832$; p < 0.400
	Number of entries into the light compartment	$\chi2 = 2.193$; p < 0.334	$\chi2 = 0.336$; p < 0.845	$\chi2 = 3.907$; p < 0.142	**$\chi2 = 9.605$; p < 0.008**
	Percentage of time spent in the light compartment	$\chi2 = 0.324$; p < 0.850	$\chi2 = 1.597$; p < 0.450	$\chi2 = 0.240$; p < 0.887	$\chi2 = 3.910$; p < 0.142
OF	Path length		**Males: F = 9.639; p < 0.0001** Females: F = 2.844, p < 0.066		
	Percent of time spent in the center	$\chi2 = 5.164$; p < 0.076	$\chi2 = 1.528$; p < 0.466	$\chi2 = 4.509$; p < 0.105	$\chi2 = 2.792$; p < 0.248
EPM	Percentage of time spent on the open arms	$\chi2 = 0.556$; p < 0.757	$\chi2 = 4.330$; p < 0.115	$\chi2 = 0.124$; p < 0.940	$\chi2 = 0.951$; p < 0.622
	Percentage of entries into the open arms	$\chi2 = 0.812$; p < 0.666	$\chi2 = 0.805$; p < 0.669	$\chi2 = 0.586$; p < 0.142	$\chi2 = 0.176$; p < 0.916
	Sum of entries into the open and closed arms	**$\chi2 = 10.481$; p < 0.005**	$\chi2 = 2.352$; p < 0.308	$\chi2 = 5.278$; p < 0.071	$\chi2 = 3.173$; p < 0.205
FE	Latency to enter the arena	$\chi2 = 5.619$; p < 0.060	**$\chi2 = 8.387$; p < 0.015**	$\chi2 = 0.722$; p < 0.697	$\chi2 = 0844$; p < 0.656
	Number of excursions into the arena	$\chi2 = 3.221$; p < 0.200	**$\chi2 = 7.431$; p < 0.024**	$\chi2 = 2.673$; p < 0.263	$\chi2 = 3.719$; p < 0.156
	Percentage of time spent in the arena	$\chi2 = 1.142$; p < 0.492	$\chi2 = 3.379$; p < 0.185	$\chi2 = 0.483$; p < 0.785	$\chi2 = 0.213$; p < 0.899

Discussion

In a recent study we showed that confronting pregnant and lactating mice with the odor cues of unfamiliar males, and thus simulating a "dangerous world", resulted in increased anxiety-like and reduced exploratory behavior in the offspring. Here we found that confronting females with unfamiliar male bedding (UMB, "dangerous environment"), as opposed to neutral bedding (NB, "safe environment"), during pregnancy and lactation led to distinctly reduced active maternal care and caused an increased stress response after the first treatment during gestation. We favor the hypothesis that in particular the decreased maternal care behavior could be the mechanism conveying effects of growing up in a "dangerous world" to offspring behavior. Surprisingly, in the present experiment no clear differences between early adolescent NB and UMB offspring were found.

Effects of a "dangerous world" on maternal stress hormones

Maternal stress hormones following the treatments were assessed non-invasively by measuring concentrations of corticosterone metabolites (CM) in the feces before (control), and after (reaction) the first prenatal treatment, the last prenatal treatment, and the third postnatal treatment. NB and UMB females did not differ concerning their control CM values and did both show an increase of basal CM in the time from the first to the second measurement during pregnancy,

Table 3
Comparison between offspring of neutral bedding (NB) and unfamiliar male bedding (UMB) treated mothers (NB vs. UMB) with different 5-HTT genotypes. Statistics: ANOVA for the path length in the OF, 1-tailed Mann–Whitney *U* test for all other parameters. Bold values indicate significance (NB>UMB). DL: dark light test, OF: open field test, EPM: elevated plus maze test, FE: free exploration test.

		5-HTT +/+		5-HTT +/−		5-HTT −/−	
Test	Parameter	Males	Females	Males	Females	Males	Females
/	Body weight (postnatal day 28–32)	U = 67; p<0.394	U = 36; p<0.106	U = 68.5; p<0.427	U = 68; p<0.416	U = 41; p<0.170	U = 59; p<0.234
DL	Latency to enter the light compartment	U = 63; p<0.309	U = 44.5; p<0.259	U = 68.5; p<0.427	U = 59.5; p<0.243	U = 48.5; p<0.477	U = 55.5; p<0.177
	Number of entries into the light compartment	U = 67; p<0.393	U = 31; p<0.054	U = 59.5; p<0.242	U = 47.5; p<0.081	U = 38; p<0.198	U = 65; p<0.350
	Percentage of time spent in the light compartment	U = 69; p<0.444	U = 38.5; p<0.142	U = 62; p<0.295	U = 61; p<0.271	U = 46.5; p<0.419	U = 71; p<0.483
OF	Path length	Males: F = 0.875; p<0.353 Females: F = 0.019, p<0.892					
	Percent of time spent in the center	U = 60; p<0.370	U = 38; p<0.139	U = 64; p<0.330	U = 60; p<0.252	U = 35; p<0.087	U = 65; p<0.351
EPM	Percentage of time spent on the open arms	U = 47; p<0.200	U = 49; p<0.377	U = 66; p<0.378	**U = 33; p<0.022**	U = 54.5; p<0.495	U = 60; p<0.257
	Percentage of entries into the open arms	U = 50; p<0.254	U = 43; p<0.225	U = 63.5; p<0.320	**U = 35; p<0.029**	U = 41.5; p<0.178	U = 55.5; p<0.177
	Sum of entries into the open and closed arms	U = 39.5; p<0.088	U = 50; p<0.397	U = 55; p<0.168	U = 56.5; p<0.287	U = 54.5; p<0.493	U = 61.5; p<0.280
FE	Latency to enter the arena	U = 57.5; p<0.208	U = 49; p<0.396	U = 55; p<0.170	U = 47.5; p<0.082	U = 52; p<0.431	U = 49; p<0.088
	Number of excursions into the arena	U = 68; p<0.416	U = 35; p<0.091	U = 55.5; p<0.177	U = 57.5; p<0.207	U = 46.5; p<0.284	U = 51.5; p<0.114
	Percentage of time spent in the arena	U = 50.5; p<0.112	U = 50; p<0.395	**U = 40; p<0.033**	U = 64.5; p<0.340	U = 52; p<0.432	U = 56; p<0.177

and a subsequent decrease to lower levels at the third measurement during lactation. This pattern is typical for mammals and also has been described for mice: plasma corticosterone levels were found to increase in the course of pregnancy with a peak at gestational day 16 or 17, before they decrease again at day 19 and stay low during lactation (Barlow et al., 1974; Dalle et al., 1978). Compared to control CM values, reaction CM values were significantly higher at all time points for both, NB and UMB mothers. Thus, the treatment with either type of bedding seems to have caused a stress response, although it cannot be excluded that heightened corticosterone levels in response to the control CM measurement contributed to this finding. Interestingly, the CM increase in response to the first prenatal treatment was significantly higher in UMB mothers than in NB mothers, indicating that being confronted with male-soiled bedding was more stressful for the mothers than being treated with neutral bedding. These results are in accordance with previous findings showing that UMB dams spent more time in the quarter of the cage where the bedding was inserted, than NB dams (Heiming et al., 2009). However, the difference in CM increase was restricted to the first prenatal treatment and the absolute reaction CM values did not differ between NB and UMB mothers at all three measurement time points, indicating that the hormonal stress response following the treatment was only mildly affected by the type of bedding used. Notably, although UMB dams seemed to habituate to the treatment concerning their CM increase, UMB mothers have previously been shown to explore the bedding material equally long during the first and fifth treatment, whereas NB females habituated to the treatment procedure (Heiming et al., 2009). Taken together, male odor stimuli, which signal the presence of potentially infanticidal males and the resulting risk of infant killing, seem to represent an ethologically relevant stimulus for pregnant mice.

Exposure to high levels of glucocorticoids can permanently affect offspring glucocorticoid receptor expression (Bale, 2005; Welberg and Seckl, 2001), e.g., in the amygdala, which in turn is associated with altered anxiety-like behavior in rodents (Welberg et al., 2000). However, in humans, as well as animals, the influence of prenatal stress on later behavior and stress responses critically depends on the timing of the stress exposure (Bale, 2005; Matthews, 2000; Weinstock, 2001). In rats, most of the hormonal and behavioral stress effects reported

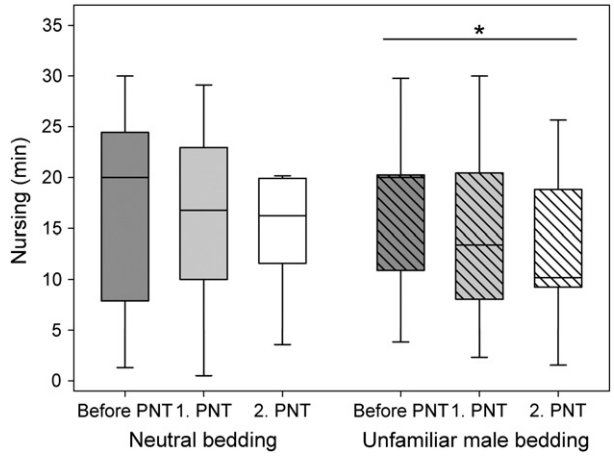

Fig. 5. Duration of nursing shown by neutral bedding (NB) and unfamiliar male bedding (UMB) treated mothers before any postnatal treatments (before PNT), after the first postnatal treatment (1. PNT) and after the second postnatal treatment (2. PNT). Data are given as box plots with medians, 25–75% quartiles and 5–95% ranges. Statistics: Wilcoxon–Wilcox test, *p<0.05. Data were obtained from 17 NB mothers and 20 UMB mothers and refer to an observation time of 30 min per mother.

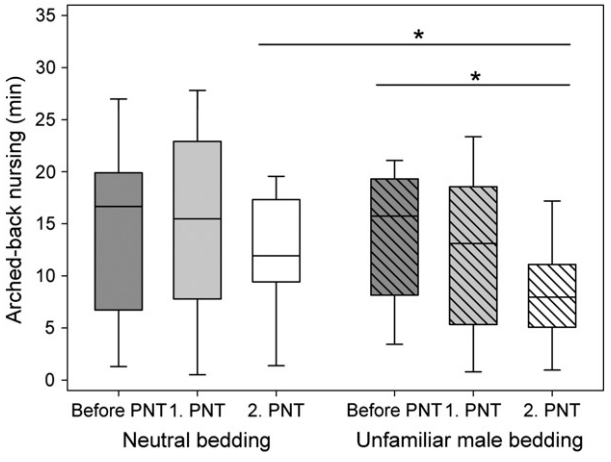

Fig. 6. Duration of arched-back nursing shown by neutral bedding (NB) and unfamiliar male bedding (UMB) treated mothers before any postnatal treatments (before PNT), after the first postnatal treatment (1. PNT) and after the second postnatal treatment (2. PNT). Data are given as box plots with medians, 25–75% quartiles and 5–95% ranges. Statistics: within treatments: Wilcoxon–Wilcox test; between treatments: Mann–Whitney *U* test. *p<0.05. Data were obtained from 17 NB mothers and 20 UMB mothers and refer to an observation time of 30 min per mother.

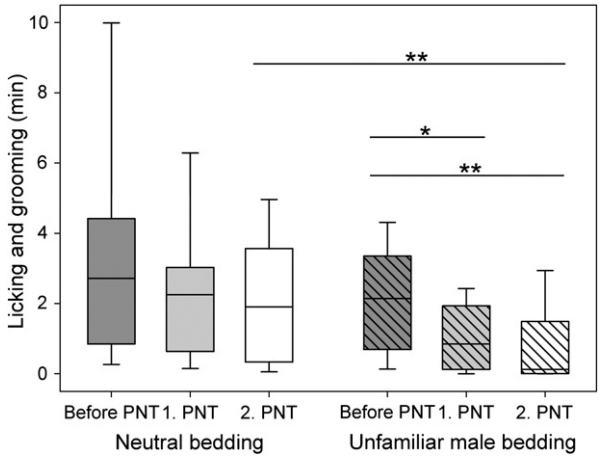

Fig. 7. Duration of licking and grooming shown by neutral bedding (NB) and unfamiliar male bedding (UMB) treated mothers before any postnatal treatments (before PNT), after the first postnatal treatment (1. PNT) and after the second postnatal treatment (2. PNT). Data are given as box plots with medians, 25–75% quartiles and 5–95% ranges. Statistics: within treatments: Wilcoxon–Wilcox test; between treatments: Mann–Whitney *U* test. $^{**}p<0.01$, $^{*}p<0.05$. Data were obtained from 17 NB mothers and 20 UMB mothers and refer to an observation time of 30 min per mother.

seem to be caused by fetal glucocorticoid exposure during the last week of gestation (Kofman, 2002; Matthews, 2000; Welberg and Seckl, 2001). In mice, on the other hand, the effects of prenatal stress on offspring fearfulness are less consistent and seem to depend on the timing, type and strength of the stressors (Chung et al., 2005; DeFries, 1964; Fonseca et al., 2002; Miyagawa et al., 2011; Mueller and Bale, 2008; Nishio et al., 2006; Pallarés et al., 2007). Since in the current study an elevated stress response in UMB mothers was only found following the first treatment during mid pregnancy (gestational days 7–10), but not after the last prenatal treatment in late pregnancy (gestational days 14–17), it seems unlikely that effects of UMB on offspring anxiety-like and exploratory behavior are mediated directly by the shortly elevated corticosterone levels during midpregnancy, although this possibility cannot be completely ruled out.

Alternatively, the higher increase in CM levels in UMB dams might have acted on the offspring indirectly by altering maternal care, as it has been shown for rat dams subjected to prenatal restraint stress (Champagne and Meaney, 2006) or corticosterone injections (Brummelte and Galea, 2010) during late pregnancy. However, since the elevated corticosterone increase in UMB dams was found only in mid pregnancy, it probably had minor, if any, impact on later maternal care. Furthermore, maternal behavior of NB and UMB females did not differ before the first postnatal treatment, indicating that the treatment with UMB during the prenatal phase and the consequently changed maternal stress hormones did not exert an influence on early postnatal maternal behavior.

Effects of a "dangerous world" on maternal care behavior

Strikingly, although the confrontation with UMB during lactation did not cause a different stress response than the treatment with NB, maternal care was significantly influenced by the type of bedding the females received. Maternal behavior was observed before any postnatal treatments (before PNT), after the first postnatal treatment (1. PNT) and after the second postnatal treatment (2. PNT). NB and UMB females showed no differences in maternal care before PNT. Interestingly, the treatment with UMB significantly reduced levels of maternal care behaviors, namely nursing, time spent on the nest, arched-back nursing (ABN) and licking and grooming the pups (LG), leading to significant differences between NB and UMB dams concerning ABN and LG, but not regarding the other parameters. It is not likely that the changes in maternal behavior reported here were caused by extended investigation of the bedding material by UMB dams, since the time the mother spent on the nest and the total duration of nursing did not differ between NB and UMB females at any of the observation time points. Our findings of reduced maternal care in mothers confronted with male-soiled bedding are in accordance with previous results showing that the presence of odor cues from potentially infanticidal males decreased one aspect of maternal behavior, namely the mother's latency to reach the pups after 30 min of separation (Mandillo and D'Amato, 1999). Furthermore, simulation of a dangerous environment by confronting lactating mothers with predator odor (mice dams: rat odor, rat dams: cat odor) also brought about variations in maternal care (Coutellier et al., 2008; Mashoodh et al., 2009; McLeod et al., 2007).

In our study, the most striking effects on maternal behavior were found for the duration of LG, which decreased significantly already after the first, and even more after the second postnatal treatment with UMB, whereas the treatment with NB had no such effect. Consequently, UMB dams spent significantly less time licking and grooming their pups following the 2. PNT than NB dams. Similarly, they performed significantly less ABN, although this decrease did not emerge until the 2. PNT. Similar as for LG and ABN, the duration of nursing and the time spent on the nest decreased in UMB mothers. However, these parameters were not significantly different from those of NB mothers at any of the observation time points. This finding is consistent with previous results, showing that mothers classified as displaying high or low maternal care do not differ in the time they nurse or spend in contact with the pups, but only concerning the frequency of ABN and LG (Caldji et al., 1998; Liu et al., 1997; Wei et al., 2010). LG provides tactile stimulation for the pups and thereby regulates offspring physiology and biobehavioral development (Kuhn et al., 1979; Kuhn and Schanberg, 1998; Moore, 1984; Myers et al., 1989; Wang et al., 1996). Apparently, the effects of maternal deprivation on the rat pup (e.g., decreased levels of growth hormone, elevated ACTH secretion) can even be eliminated just by stroking the pups with a paintbrush, mimicking the mother's licking pattern (Champagne, 2008; Schanberg and Field, 1987; Suchecki et al., 1993; van Oers et al., 1998).

Our results show that mothers confronted with UMB reduce levels of active maternal care (ABN and LG). However, since maternal behavior was only assessed at three time points, it is not clear whether the observed changes in maternal care persisted during the remaining time of lactation. Considering the increasing effect of UMB from the 1. PNT (postnatal days 4–6) to the 2. PNT (postnatal days 7–10), it is likely that active maternal care stayed low or even decreased further after the remaining four treatments. Notably, since observations after the onset of PNT were only conducted following the treatments, it cannot be excluded that maternal behavior in UMB dams was only altered in the periods after the treatment and returned to normal on the following day. However, even temporary changes in maternal care around postnatal days 7–10 can permanently influence offspring development, since during the first two weeks of life juvenile mice and rats seem to be especially sensitive to environmental influences (Cabib et al., 1993; Caldji et al., 1998; Champagne et al., 2003; D'Amato et al., 1998; Levine and Lewis, 1959; Liu et al., 1997; Meaney and Aitken, 1985; Romeo et al., 2003; Veenema et al., 2007). Altogether, our findings show that confrontation of lactating mice with UMB leads to distinct reductions in ABN and LG, which has been found to cause high fearfulness in the offspring (Calatayud et al., 2004; Carola et al., 2006; Wei et al., 2010). Therefore, maternal care could well act as a mediator between the dangerous environment simulated by UMB and the offspring.

Functional significance of altered maternal care

It was argued that the confrontation of pregnant and lactating mice with odor cues from several male mice of different strains

simulates a habitat populated by a high number of potentially infanticidal males, and thus a threatening environment for the mother and her young (see Introduction). In such a "dangerous world" it would probably be advantageous for mothers to program their offspring to exhibit higher anxiety-related and lower exploratory behavior. Therefore, the alterations in the behavioral profile of UMB offspring were suggested to represent adaptive maternal effects (Heiming et al., 2009; Heiming and Sachser, 2010), meaning that the mothers maximize their own Darwinian fitness by adjusting their offspring to the prevailing and/or future environmental conditions (Kaiser and Sachser, 2005; Kaiser and Sachser, 2009; Mousseau and Fox, 1998; Sachser et al., 2011). Consistently, several authors argue that the increased fearfulness (Calatayud et al., 2004; Carola et al., 2006) and higher endocrine responsivity to stress (Anisman et al., 1998; Liu et al., 1997; Meaney, 2001; Plotsky and Meaney, 1993) in rodents due to poor maternal care might be advantageous under conditions of increased environmental demand (e.g., high predator density or scarce resources), since these responses promote detection of potential threat, avoidance learning, and metabolic responses that are essential under the increased demands of adversity (Meaney, 2001). Thus, being the major link between the pup and its habitat (Levine, 1994), maternal care behavior might serve to "program" behavioral and endocrine responses to threatening stimuli in the offspring in a possibly adaptive way (Caldji et al., 1998; Fish et al., 2004; Meaney, 2001; Sachser et al., 2011).

Effects of a "dangerous world" on offspring behavior

In a previous study we found increased anxiety-like and reduced exploratory behavior in UMB offspring, especially in 5-HTT −/− individuals (Heiming et al., 2009). Furthermore, and in accordance with the literature (Holmes et al., 2003a, 2003b; Jansen et al., 2010; Lesch, 2005), 5-HTT −/− mice were shown to display higher levels of anxiety-like behavior and less exploratory locomotion than 5-HTT +/+ and +/− mice. Surprisingly, the present experiment revealed no clear and consistent effect of treatment or genotype on offspring behavioral profile. However, the few differences that were found all point in the same direction as previous findings: if different, UMB mice showed higher anxiety-like and lower exploratory behavior than NB mice, and 5-HTT −/− mice displayed increased anxiety-like and reduced exploratory behavior compared to 5-HTT +/− and 5-HTT +/+ mice. Regarding the lack of clear genotype and treatment effects in the current experiment, two explanations might be possible. Firstly, the offspring were tested at a younger age than in our previous study, namely between postnatal days 28 and 42. Thus, one possible explanation for the inconsistent results could be that the effects of UMB treatment and 5-HTT genotype are more pronounced during later phases of life. Notably, to our knowledge 5-HTT knockout mice have not been tested at a younger age than 50 days to date. Thus, the effects of reduced 5-HTT expression might not manifest in behavioral alterations before late adolescence. Similarly, it is possible that the consequences of treatment with UMB are deferred to a later phase of life and might have been detected in the offspring during late adolescence or adulthood. Delayed effects of stressors early in life also have been suggested by others; for example chronic, variable physical stress during the peripubertal–juvenile period produces delayed deficits in hippocampal morphology, cognition, and stress axis function in rats (e.g., Isgor et al., 2004).

Concerning the lack of a distinct UMB effect, there could be a second explanation: in our previous study the mortality among the mothers was quite high (Heiming et al., 2009), indicating that, besides the treatment, there might have been other stressors of unknown nature acting on the dams. It seems possible that this additional burden reduced maternal care behavior, resulting in such low levels of maternal care in UMB dams that offspring behavior was changed, whereas NB dams could buffer these effects. Thus, the maternal care in our previous study might have been even more decreased than in the present study.

Given the discrepancy between the results of the present and our previous study, the current version of our paradigm might require further refinements. Thus, in future experiments it could be advisable to increase the number of postnatal treatments in order to maximize the impact of UMB on maternal care, and consequently offspring behavioral profile. Furthermore, the bedding for the UMB treatment could be soiled by an increased number of different males.

Conclusions

Mothers treated with unfamiliar male bedding showed a significantly higher increase of fecal corticosterone metabolites following the initial treatment during mid pregnancy, than mothers treated with neutral bedding. Furthermore, living in a "dangerous world" distinctly and consistently reduced maternal care behavior, particularly the durations of ABN and LG, in UMB dams. Thus, the odor stimuli of potentially infanticidal males seem to represent an ethologically relevant stimulus for pregnant and lactating mice.

Since the stress response of UMB mothers habituated to the treatment and was not different from the stress reaction of NB mothers during late pregnancy, we argue that a short-term increase in the corticosterone response during mid gestation is not likely to exert a major influence on offspring anxiety-like and exploratory behavior. In contrast, maternal care behavior was consistently decreased in UMB treated females. Although our study demonstrated that the anxiety-like and exploratory behavior is not necessarily affected in offspring of UMB mothers at all ages, the sensitivity of maternal behavior towards environmental cues suggests maternal care as a major candidate mediating between a "dangerous world" and the offspring's behavioral profile.

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