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Effects of prenatal stress on hypothalamic–pituitary–adrenal (HPA) axis function over two generations of guinea pigs (*Cavia aperea* f. *porcellus*)

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ABSTRACT

Prenatal stress can alter hypothalamic–pituitary–adrenal axis function with potential consequences for later life. The aim of our study was to examine in guinea pigs (*Cavia aperea* f. *porcellus*) the effects of stress experienced during F0 pregnancy on glucocorticoid levels in plasma and feces, as well as challenge performance, in F1 offspring ($n = 44$) and fecal glucocorticoid levels in F2 offspring ($n = 67$). F1 animals were either born to F0 dams that had been stressed with strobe light during early to mid pregnancy, resulting in a short term increase but long-term down-regulation of maternal glucocorticoid levels, or to undisturbed F0 dams. The same stressor was used as a challenge for F1 offspring at age 26 days and around 100 days. Basal plasma cortisol concentrations during early F1 development, as well as overall glucocorticoid levels at challenge tests, were lower in F1 animals that were prenatally stressed than in control animals. Fecal cortisol metabolites were initially at lower levels in prenatally stressed F1 animals, relative to control animals, but shifted to higher levels around day 68, with an additional sex difference. Effects were also seen in the F2 generation, as male but not female offspring of prenatally stressed F1 animals had significantly higher levels of cortisol metabolites in feces after weaning. We conclude that stress exposure of F0 dams resulted in lower basal glucocorticoid levels in F1 offspring during the pre-pubertal phase and during stress exposure, but higher glucocorticoid levels in post-adolescent F1 animals. Also in males of F2 generation effects of stress exposure of F0 dams were detected.

1. Introduction

Environmental factors experienced during development, along with genetic background, can shape the phenotype of an organism and lead to permanent modifications which may persist during a lifetime [32,54,58]. This organizational phenomenon is termed developmental programming, reflecting the action of factors operating during crucial periods of development with potential long-term effects on structure and functioning of organs (e.g. [86]). Confirmed programming effects were found in many taxa (reviews in mammals: e.g. [29,8,63]; birds: [37]; fish: [1]; amphibians: [39]) pointing to a well conserved phenomenon. The concept of programming was linked to an evolutionary perspective by Gluckman and Hanson [30], who argued that if environmental conditions are harsh, mothers can pass environmental cues to the fetus and adjust its physiology to the predicted future. This would result in potential advantages of early survival and increased reproductive success, however at the cost of a potentially higher risk of disease in later life.

Not only poor nutrition as consequence of harsh conditions, but also infection, stress and synthetic glucocorticoids were found to have this programming ability [10,44,94], with long-term effects on offspring physiology demonstrated in various human and animal studies (for review see [9]).

The different causes of programming have been stated to have a major common underlying mechanism in placental animals, namely increased glucocorticoids passing the placenta from the mother to the fetus [82]. Therefore, the focus of the majority of studies has been on the short-term increase in glucocorticoids of mothers. However, if stress exposure is continued for a longer period, primary stress mediators such as glucocorticoids may also be decreased as a result of negative feedback and/or down-regulation of hypothalamic-pituitary-adrenal (HPA) axis [35,79,96]. This phenomenon is sometimes falsely interpreted as habituation, while actually the organism will show a desensitization of HPA axis and still perceive the situation as stressful [16]. It needs to be

Abbreviations: ACTH, adrenocorticotropic hormone; FCM, fecal cortisol metabolites; F0, parental (maternal) generation; F1, first filial generation; F2, second filial generation; HPA-axis, hypothalamic–pituitary–adrenal axis; SEM, standard error of the mean.

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determined whether next to increased levels of glucocorticoids also reduced levels of glucocorticoids can program the offspring's HPA axis and lead to alterations in later life.

Many prospective human as well as experimental animal studies have explored effects of glucocorticoids, either through administration of pharmaceutical doses or exposure to stress on offspring, with various and sometimes contradicting findings (for review see humans: [29], animals: [93]). Some reasons stated for these inconsistent reports are the numerous experimental designs used: species differences in HPA axis (e.g. cortisol or corticosterone as primary stress mediator) and development (altricial or precocial species), time window of stress exposure (early, mid or late gestation), sex (male, female, both), stressor used (report of anxiety, experience of disaster, heat stress, bright light, social instability, synthetic glucocorticoids, ACTH-test, etc.) and parameter tested in offspring (behavior, plasma glucocorticoids, receptor density, etc.).

As glucocorticoids are crucial for fetal development, especially for lung maturation, synthetic glucocorticoids are commonly administered to pregnant women during late gestation when they are at risk for preterm delivery [45]. Even a single course of administration of synthetic glucocorticoids during late pregnancy enhances lung development and thereby increases chances of survival in case of preterm birth [33]. However, it becomes clear from epidemiological as well as experimental studies that next to the obvious advantageous effects of glucocorticoids for survival, there are also effects on physiology that may persist long-term and increase the risk of developing metabolic diseases in later life [8,19,20,57]. That is why most experimental studies have focused on examining the effects of increased glucocorticoid values either through administration of pharmaceutical doses or exposure to stress during late gestation (gp: [4,24,42,43]). Yet, glucocorticoids operate on development during whole gestation and there is some evidence that stress during early time periods may likewise program later physiology [48,64,65,68]. The long-term alterations in phenotype may be even greater due to potentially substantial influences on later formed cell groups. There are even descriptions of programming effects seen in offspring after prenatal adverse conditions before or around conception for which uterine environment is discussed to be transferring maternal cues to the early developing fetus instead of the not yet formed placenta [51]. The study effects of stress during early gestation on long-term alterations in physiology may therefore be of great relevance from a medical point of view as pregnancies are often not discovered until a couple of weeks after conception and no precautions can be taken to avoid potential adverse stress effects. In this study we evaluate effects of stress starting shortly before conception and continuing during early and mid gestation on offspring. Guinea pigs are used as a model species, as they combine well described physiology, easy handling and breeding and similarities with humans that are: cortisol as the major glucocorticoid and a hemomonochorial placental structure. In the guinea pig, until now programming experiments were focused on effects evoked by prenatal stress during late gestation [4,24,42,43] and the whole duration of gestation [41,46,47]. Amongst others, strobe light has been used as stressor in this species to explore programming effects of stress. We applied the same stressor to pregnant guinea pigs in our study, however at a time period during which no data are available in the existing literature on experimental prenatal stress.

Causes, but also consequences of programming vary enormously, including effects seen in almost all organ systems (e.g. kidney: [63], skeletal muscle: [23], brain: [34], immunity: [67]). Especially endocrine function seems to be altered and influences the later phenotype. Prenatal stress, for instance, was found to be capable of altering HPA axis function in later life (e.g. [15,43]). The major HPA axis effector hormones – glucocorticoids – are circulated in the blood stream to reach every cell, increase in case of stress and have additional, e.g. metabolic, functions. Thus, it becomes clear how important a well regulated system is to avoid adverse effects and risk of disease.

The aim of our study was thus (i) to examine HPA-axis function in F1 offspring from guinea pig dams (*Cavia aperea* f. *porcellus*) that had been exposed to stress during early- to mid-pregnancy. As described elsewhere [81], stress exposure in these dams induced a short-term elevation of glucocorticoids but, in the long run, resulted in a down-regulation of HPA-axis activity. We therefore wanted (ii) to examine if also effects of long-duration stress (i.e. chronic stress) on females physiology could program offspring. In addition, in view of the literature on intergenerational transmission of programming effects (e.g. [22,26]), we (iii) further examined whether effects could also be detected in the F2 generation. We analyzed HPA axis activity and reactivity to challenge tests in F1 offspring and HPA axis activity in F2 offspring during the first 124 days of life. We predicted that if the mother is capable of transferring the cues of a stressful environment to the fetus, we will find reduced basal glucocorticoid levels and increased stress reactivity in F1 offspring. In F2 offspring, however, due to the relatively mild stressor used in F0 pregnancy (namely strobe light exposure) and the undisturbed F1 pregnancy, we did not expect to find any effect at all. Furthermore, sex hormones are known to modulate HPA-axis function. While testosterone has been shown to inhibit HPA axis at a hypothalamic level [90], estrogens are likely to affect steroidogenesis directly at the adrenal – either by increased sensitivity to ACTH [2,53], stimulation of enzymes involved in synthesis of glucocorticoids or steroid precursors [69,84,87,88]. We predicted reduced levels of glucocorticoids during time of increased sex hormone levels. Furthermore, we expected these results not to be the same for female and male offspring. Alternatively, because of puberty being a time period of increased social interactions that may lead to high stress levels, the predicted reduction of glucocorticoids may be evened out.

2. Material and methods

All husbandry and experimental procedures were performed with the permission of the institutional ethics committee and the Austrian Federal Ministry of Science and Research (GZ 68.205/0211-II/10b/2008).

2.1. Animals and housing conditions

For this study a total of 111 animals from a breeding colony of shorthaired, multicolored domestic guinea pigs (*C. aperea* f. *porcellus*) from our institute were used and could be identified by individual natural markings in the fur. Animals comprised two generations (F1: first generation, $n = 44$; F2: second generation, $n = 67$). F1 animals were born to F0 females that had either been exposed to repeated stress during the first two thirds of pregnancy (i.e. high frequency strobe light in a dark environment between 9 and 11 am and 4 and 5 pm during gestational days −7, 0, 7, 14, 21, 28, 35, 42; gestation length ~68 days) or were left undisturbed. Effects of this stress exposure (handling, isolation and strobe light exposure in a dark and unfamiliar room) are described in detail by Schöpper et al. [81] while here a short overview is given. The stress procedure was effective in inducing in a short-term increase in plasma cortisol concentrations of the dams, when comparing non-stress situations with samples taken right after stress exposure, both during early and mid-gestation. However, when looking at fecal cortisol metabolite levels over time stress exposure resulted in a significant down-regulation of glucocorticoid levels, which persisted even after termination of stress. In addition, it

attenuated body weight gain of F0 females, leading to a mean body weight at day 63 of gestation that was around 80 g lighter than that of undisturbed control females. Still, stress exposure and the effects seen in gestating mothers had no influence on their reproductive performance (described in detail in [81]).

The F1 generation was therefore either prenatally stressed (PS, $n = 20$, ♀8♂12) or left undisturbed during prenatal development (control, $n = 24$, ♀11♂13). To rear F2 offspring, F1 control females were mated with either F1 control or F1 PS males, while F1 PS females were mated with F1 control males. We abstained from pairing F1 PS females with F1 PS males due to limited sample size. All pairs consisted of unrelated animals. To monitor reproductive stage, the status of vaginal membrane of F1 females was checked daily at 9 am, as this is described as a suitable external marker of cycle stage in guinea pigs [85]. The first day a fully ruptured membrane occurred was defined as estrus (day 0 of the cycle), which correlates well with physiological estrus [97]. First estrus sets the start of reproductive maturation in female guinea pigs, occurring between days 23 and 50 in our study. In male guinea pigs puberty is not a single event and therefore difficult to determine. Surge of testosterone is seen starting already around day 30, while peaks are described between day 60 and 90, which is considered as the time of puberty in males [36,73]. In most cases we observed mating and defined it as day of conception (=day 0 of gestation). Otherwise the last day a fully ruptured vaginal membrane was observed was defined as day 0 of gestation. Expected day of birth was gestational day 68. F2 offspring were born into one of the following three groups: (i) both parents were control animals (con/con, $n = 20$, ♀12♂8), (ii) the father was prenatally stressed, while the mother was a control animal (faPS, $n = 16$, ♀9♂7) or iii) the mother was prenatally stressed, while the father was a control animal (moPS, $n = 31$, ♀18♂13).

Prior to and after pairing, F1 animals were housed in same-sex groups of two to four non-related animals of similar age. During pairing, F1 animals were transferred to a cage with fresh bedding and were directly observed for signs of aggression. If aggression occurred or there was no display of lordosis, animals were separated immediately. The pairs were reintroduced some hours later (maximum three times during the day of estrus) to finally meet during the receptive period. With this procedure we were able to minimize harassment for yet non-receptive females and to ensure exact mating dates for later calculation of the duration of gestation. Pregnant females were housed with familiar females until separation from the group approximately three days before expected parturition, when they were housed in a littering cage to be able to deliver undisturbed. F2 offspring were housed together with their mother during lactation until weaning on day 21. Subsequently F2 pups were housed in same-sex groups of two to four animals of similar age that were non-siblings.

All animals were housed under standard conditions with a LD 12:12 light regime (lights on at 7am), mean ambient temperature of 22.9 °C (SEM: 0.2 °C) and mean relative humidity of 43.4% (SEM: 0.5%). Enclosures (with a ground area of 100 cm × 50 cm for 3–4 animals, 85 cm × 50 cm for pairs and mother with litter) were bedded with wooden material and renewed on a weekly basis. Animals were fed 15 g d^{-1} of standard chow (Altromin 3013, Altromin GmbH, Lage, Germany) supplemented with fresh fruit and vegetables (40 g) and a handful of hay. During times of increased energy demand (last trimester of gestation and during lactation) we offered F1 females additional 20 g d^{-1} of pellets. Pups received 10 g d^{-1} standard chow and 10 g d^{-1} of fresh food while being housed with the mother until weaning at day 21. Water was available *ad libitum*.

2.2. Challenge tests in F1

To compare stress reactivity of control versus PS animals in F1 generation, a challenge test was performed at the age of 26 days: the animals were stressed via exposure to a standard strobe light with a pulse rate of about 10 Hz (Mini-Flash DK-011, China, distributed by Conrad Electronics, Austria), the same stressor PS mothers (F0) had experienced during their pregnancy. Exposure took place in an unfamiliar dark room, with the strobe light placed approximately one meter above the cage. The animals were housed individually during the challenge and were transferred back to their home cage after termination of stress exposure. Animals were stressed at different times of the day, because according to Sachser [76] there is a distinct diurnal rhythm in cortisol levels in guinea pigs with low levels in the morning and a peak around 4 pm. Therefore, we performed the stress procedure for two hours during the morning (from 9 to 11 am) to meet low levels and for 1 h during the afternoon (from 4 to 5 pm) of the same day to meet high levels of basal cortisol.

To test stress reactivity during young adulthood, the same challenge test was again performed at an age of about three and a half months. At this age, females were already exhibiting a reproductive cycle and we accounted for a potential influence of ovarian hormones on HPA-axis by testing F1 females twice: first, at day 7 of the 4th cycle (at an age of 100–115 days), which according to Garris [28] corresponds to the mid-luteal phase and coincides with peak progesterone levels [7], and again at the day of estrus (day 0) of the following cycle, which is dominated by estrogens [97]. Adolescent F1 males were exposed to strobe light only once. Half of the males were tested at an age similar to females during their mid luteal phase (mean age for both sexes: 102 days), the other half was exposed to stress at an age similar to females at the day of estrus (mean age for both sexes: 111 days). So in total three challenge tests were performed: at day (d)26, ∼d102 and ∼d111 (with F1 males being stressed on d26 and either ∼d102 or ∼d111, while females were stressed at all three times).

2.3. Blood sampling in F1 and analysis of plasma cortisol

We took blood samples from F1 individuals to measure plasma levels of cortisol during different stages of development. In males, samples were taken weekly at 9 am, starting at age 12 days up to day 124 (d12, d19, d26, d33, d40, d47, 54, . . ., d124). In females, samples were taken at the same regime prior to first estrus (d12–d40). After their first estrus we took blood samples for six consecutive cycles, always on the day of estrus (Cy1d0, Cy2d0, Cy3d0, Cy4d0, Cy5d0, Cy6d0), also at 9 am. With this procedure we covered the same age range in both sexes and avoided any confounding influence of the estrous cycle on cortisol levels of females. Additional blood samples were taken in both males and females immediately after the challenge tests at 11 am and 5 pm on d26, ∼d102 and ∼d111. Basal levels of cortisol were determined either from samples taken on the day prior to stress exposure (d25 and ∼d101), or for the last challenge test one day after stress exposure (∼d112), as the day of stress exposure first had to be identified as the day of estrus of the 5th cycle. To minimize any influence of diurnal fluctuations in hormonal levels, all blood sampling took place at the same time of day (11 am and 5 pm).

Collection of blood (100 μl) followed the protocol described by Sachser and Pröve [75]: marginal ear veins were punctured with a sterile lancet and samples were collected with a heparinized capillary tube without anesthesia. The whole procedure took less than three minutes per guinea pig, including removing the animal from the cage and returning it afterwards. Samples were centrifuged immediately and the plasma stored at −20 °C until further analysis.

For the analysis of blood samples, we extracted plasma (diluted in assay buffer to a total of 500 μl) with 5 ml of diethyl ether. Cortisol, the principal glucocorticoid in guinea pigs [56], was measured with an enzyme immunoassay (EIA) and all samples were

run in duplicate. Details of the EIA, including cross-reactions of the antibody, are given by Palme and Möstl [66]. The intra- and interassay coefficients of variation were 8.9% and 11.1%, respectively.

2.4. Feces collection in F1/F2 and analysis of cortisol metabolites

In F1 and F2 animals we also analyzed fecal cortisol metabolites (FCM), which mirror the overall status of the animal rather than showing a momentary picture, since the delay of hormone excretion in the feces of guinea pigs ranges from 14 to 20 h [6].

We collected fecal samples twice weekly starting at age 19 days (F1) and 22 days (F2). In F1 animals, we collected additional fecal samples immediately before the challenge tests (reflecting the non-stress situation, sampling on d26, ~d102, ~d111) as well as on the morning of the following day (reflecting the stress exposure of the previous day, sampling on d27, ~d103, ~d112). For collection of fecal samples, animals were briefly separated (usually less than 5 min) from their companions between 9 and 10 am and relocated to a cage with fresh bedding. After defecation, each individual was returned to its home cage and feces were collected from the bedding. Due to limited personnel and resources, we only analyzed fecal samples of all F1 individuals from the following days: d22, d33, d40, d54, d61, d68, d82, d96, d110 and d124 as well as d26, d27, ~d102/~d111 and ~103/~d112. Our main interest in F2 animals was the stress hormone status around the time of weaning (d21) and during puberty, which occurred in females of the F1 generation between d22 and d54 and in males around d75 to d82; therefore, we analyzed feces of all F2 animals from: d22, d26, d54 and d61; and additionally for F2 females from: d33, d40; and for F2 males from: d68, d75, d82 and d89.

After sample collection, feces were immediately frozen and stored at −20 °C until further analysis. Urine-contaminated feces were excluded from the analysis. Extraction of fecal samples followed the procedure described by Palme and Möstl [66], with slight modifications: dried samples (85 °C for two hours) were homogenized and a total of 0.1 g was suspended in 1.8 ml of 80% methanol, vortexed for 30 min and centrifuged at 2500 g for 15 min. Afterwards an aliquot of the supernatant was diluted (1:10) with assay buffer and stored at −20 °C until further analysis. We used a group-specific 11-oxoetiocholanolone-EIA measuring FCM with a 3α-OH-11-one structure [61], which was previously successfully validated for measuring adrenocortical activity in guinea pigs [6]. The intra- and interassay coefficients of variation were 9.5% and 11.3%, respectively.

2.5. Statistical analysis

For statistical analysis R 2.9.1 [72] with the additional package nlme [70] was used. We performed linear mixed effects models (LME) to adjust for repeated measurements. For the analysis of plasma and FCM during development, group (F1: PS vs. control, F2: faPS, maPS and con/con), time (used as a 2nd order polynomial when the quadratic term was significant) and sex (only for the analysis of sex differences in early plasma cortisol and FCM in F1) were entered as fixed factors, and different intercepts per animal as random factor in all cases. Because of the different sampling regimes in males versus females, the analysis of plasma cortisol concentrations after d40 in F1 animals, as well as of FCM in F2 animals was performed separately for each sex. Plasma cortisol and FCM levels before and after challenge tests in F1 animals were also analyzed using an LME model, with group (PS vs. control), day of stress (d26, ~d102 and ~d111), stress exposure (without vs. after stress), time of day (11am vs. 5 pm, only for the analysis of plasma samples) and sex as fixed factors and animal as random factor. Including the mother as second random factor increased model complexity but did not decrease residual deviance therefore, we used the model without this random factor. All response variables were Box–Cox-transformed for the analyses to ensure that residuals were normally distributed, which was ascertained by visual inspection of histograms and qq-plots of residuals. All models were limited to two-way interactions and reduced stepwise by exclusion of non-significant interactions. Results of these models are presented as *F*-values with degrees of freedom and corresponding *p*-value. Note that numerator degrees of freedom of 2 for the time effect indicate the use of a quadratic polynomial. Post hoc multiple comparisons were carried out using function glht from the R-package multcomp [38]. Observed differences were considered significant at p-values < 0.05.

3. Results

3.1. Concentrations of glucocorticoids during development

3.1.1. Concentrations of plasma cortisol in F1 generation

Comparing concentrations of plasma cortisol in F1 animals during the first 40 days of life, we found a significant difference between groups, with lower levels found in PS compared to control animals (Fig. 1A, LME: time: $F_{1/155} = 0.587$, $p = 0.445$, sex: $F_{1/41} = 0.004$, $p = 0.947$, group: $F_{1/41} = 7.187$, $p = 0.011$, time:sex: $F_{1/155} = 9.101$, $p = 0.003$). Additionally a significant sex-effect over time was detected, with cortisol concentrations of females staying at relatively stable levels around 150 ng/ml plasma, while males initially had slightly lower concentrations that decreased further to mean concentrations of under 50 ng/ml plasma at d40 (Fig. 1B).

After d40, in F1 females, plasma cortisol levels increased slightly over the course of the analyzed first six cycles. No significant difference between PS and control group was found (Fig. 2A, LME: time: $F_{1/92} = 4.181$, $p = 0.044$, group: $F_{1/17} = 0.970$, $p = 0.339$).

In F1 males after d40, cortisol concentrations were lower compared to F1 females (Fig. 2A/B), varied significantly over time from d47 to d124 but showed no significant difference between groups (Fig. 2B, LME: time: $F_{2/266} = 5.044$, $p = 0.007$, group: $F_{1/23} = 2.258$, $p = 0.147$). Generally, levels of cortisol stayed relatively low, around 50 ng/ml plasma, until d68 and rose to maximal mean levels of around 100 ng/ml plasma at d82 and fluctuated around 50 ng/ml thereafter, with slightly higher values found in control males.

3.1.2. Concentrations of FCM in F1 generation

Analyzing FCM of F1 animals, we found a significant effect of sex over time, with lower levels in males until around d40 to 54 and higher levels afterwards (Fig. 3A). In addition, the time course of FCM concentrations differed significantly between groups. Initially, PS animals showed lower levels compared to controls, then shifting to higher levels around d61 to 82 while FCM levels of control animals dropped (Fig. 3B, LME: group: $F_{1/41} = 1.336$, $p = 0.254$, sex: $F_{1/41} = 3.036$, $p = 0.089$, time: $F_{2/433} = 12.610$, $p < 0.001$, time:group: $F_{2/433} = 4.305$, $p = 0.014$, time:sex: $F_{2/433} = 9.519$, $p < 0.001$). In control animals, FCM values were around 1100 ng/g at d21, dropped to half at d47 and then increased to less than 800 ng/g, where they remained relatively stable until the end of sampling.

3.1.3. Concentrations of FCM in F2 generation

In order to cover the periods of weaning and puberty, the latter occurring at an earlier age in females, we applied different sampling regimes between the sexes in F2 animals and thus analyzed both sexes separately.

In F2 females, concentrations of FCM did not differ significantly between the three treatment groups over the course of sampling from d22 to d61 (LME: time: $F_{1/201} = 0.122$, $p = 0.727$,

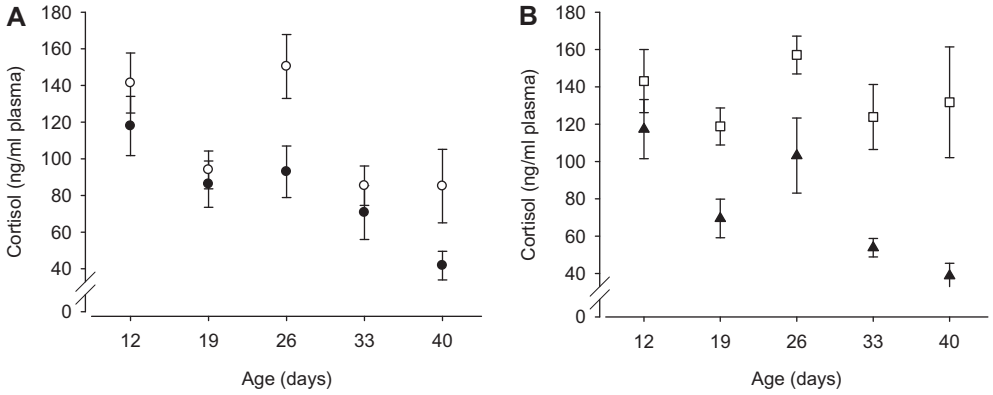

Fig. 1. Plasma cortisol concentrations (mean ± SEM) of F1 animals during the first 40 days of life (A) separated by group (control, open circles, *n*(animals) = 24, except d12 (23), d33 (22) and d40 (19) and PS, closed circles, *n*(animals) = 20, except for d26/d40 (16) and d33 (17)) and (B) separated by sex (females, open squares, *n* = 19, except for d26 (18), d33 (14) and d40 (10), males, closed triangles, *n* = 25, except d12 (24) and d26 (22)).

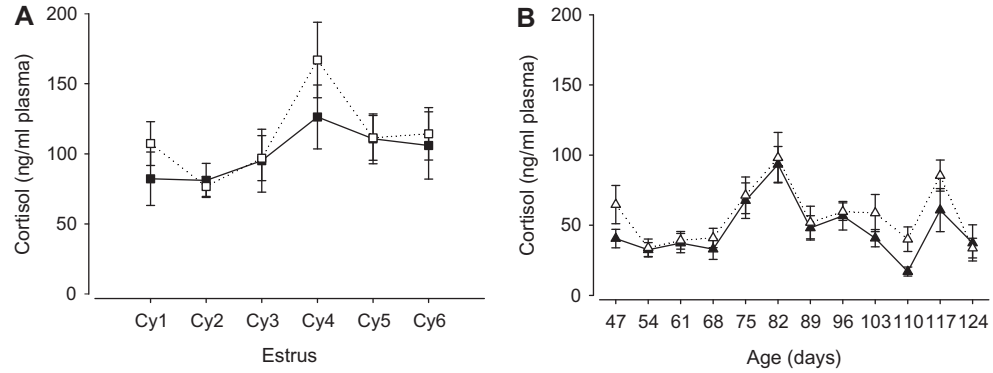

Fig. 2. Plasma cortisol concentrations (mean ± SEM) of (A) F1 females during the first day of their first six cycles (control, open squares, *n*(animals) = 11, except Cy5 (10) and PS, closed squares n (animals) = 8, except Cy1 (7)) and (B) F1 males between d40 and d124 (control, open triangles, *n*(animals) = 13, except d96 (11) and d110 (12) and PS, closed triangles, *n*(animals) = 12, except d110 (11) and d124 (9)).

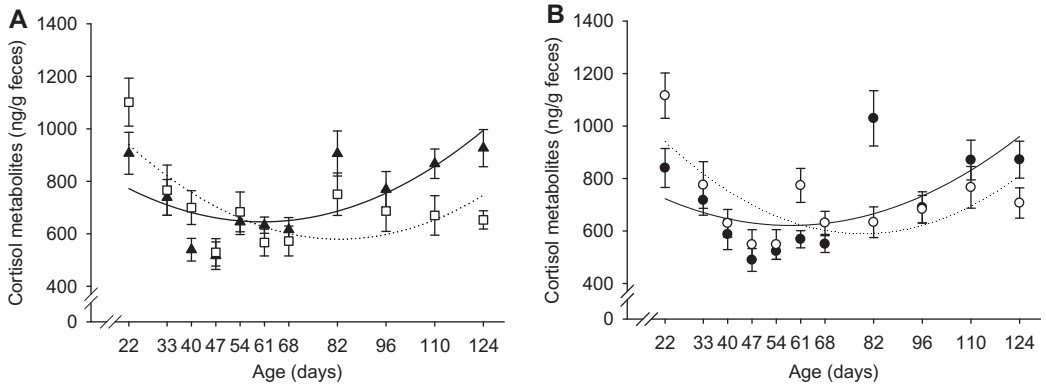

Fig. 3. Fecal cortisol metabolite concentrations (mean ± SEM) of F1 animals from d22 through d124 (A) separated by sex (females, open squares, *n* = 19 except for day d33 (18), and males, closed triangles, *n* = 25) and (B) separated by group (control, open circles, *n* = 24 and PS, filled circles, *n* = 20 except for d33 (19)). Values are given at d22 and d33, followed by weekly measurements until the age of 124 days.

group: $F_{2/36} = 0.068$, $p = 0.935$). Concentrations of FCM showed means around 450 ng/g without significant fluctuations over time.

In F2 males, levels of FCM differed significantly between groups and over the course of time, with a significant interaction found between group and point of time (Fig. 4, LME: time: $F_{7/148} = 4.647$, $p < 0.001$; group: $F_{2/25} = 11.798$, $p < 0.001$; time: group: $F_{14/148} = 2.133$, $p = 0.013$). Generally, FCM levels were between 200 and 600 ng/g at an age of 22 days and slowly increased over time. Post hoc tests revealed significant differences between con/con and moPS as well as between faPS and moPS at the day of first measurement (Post hoc d22: con/con – faPS: $p = 0.6978$, con/con – moPS: $p = 0.0371$, faPS – moPS: $p < 0.001$). Concentrations of FCM where higher in moPS animals compared to con/con, while faPS showed decreased levels of glucocorticoids after weaning.

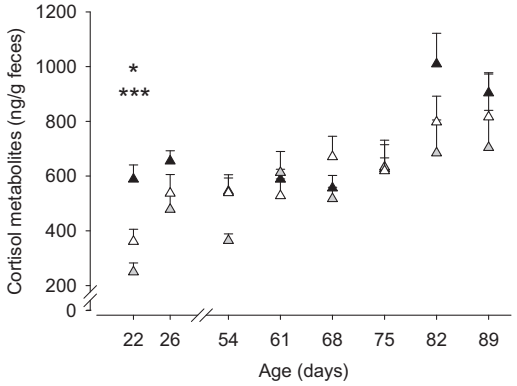

Fig. 4. Fecal cortisol metabolite concentrations (mean +, positive SEM to maintain clarity) of F2 males subdivided according to parental group affiliations (both parents derived from control animals (con/con), the mother was prenatally stressed (moPS) or the father was prenatally stressed (faPS)) from d22 until young adulthood (values are given at d22, d26, d54 and five following weeks (con/con, open triangles, n = 7/8/7/8/8/7/6/7, moPS, filled triangles, n = 10/13/12/13/12/12/12/12 and faPS, gray triangles, n = 6/5/3/7/7/6/4/5). $^{*}p = 0.037$ con/con – moPS, $^{***}p < 0.001$ faPS – moPS.

3.2. Stress effects on concentrations of glucocorticoids

3.2.1. Concentrations of plasma cortisol before and after challenge tests in F1 generation

Plasma cortisol concentrations in F1 animals both before and after challenge tests were significantly affected by group (PS vs. control) and sex, with lower levels found in PS than control animals, as well as in males than females. In addition, significant interactions of stress event (d26, ~d102, ~d111) with stress exposure (basal vs. stress) and time of day (morning vs. afternoon), as well as of stress exposure and time of day, were found (Fig. 5, LME: stress exposure: $F_{1/371} = 7.525$, $p = 0.006$, stress event: $F_{2/371} = 11.386$, $p < 0.001$, time of day: $F_{1/371} = 42.743$, $p < 0.001$, sex: $F_{1/41} = 37.116$, $p < 0.001$, group: $F_{1/41} = 4.448$, $p = 0.041$, stress exposure:stress event: $F_{2/371} = 3.772$, $p = 0.024$, stress exposure:time of day: $F_{1/371} = 7.874$, $p = 0.005$, stress event:time of day: $F_{2/371} = 3.495$, $p = 0.031$). Cortisol levels were lower, but the cortisol increase after stress exposure was higher, in the afternoon compared to morning samples, with lowest cortisol values found at challenge test d102. In addition, during the first stress event, basal cortisol concentrations were higher, and hence the cortisol increase was lower, than during the later challenge tests.

3.2.2. Concentrations of FCM before and after challenge tests in F1 generation

Concentrations of FCM in F1 animals before and after challenge tests did not differ significantly between sexes. There was, however, a significant interaction between stress event and group as well as between stress event and stress exposure (Fig. 6, LME: stress exposure: $F_{1/158} = 1.232$, $p = 0.269$, stress event: $F_{2/158} = 5.180$, $p = 0.007$, sex: $F_{1/41} = 1.279$, $p = 0.265$, group: $F_{1/41} = 4.104$, $p = 0.0493$, stress event:group: $F_{2/158} = 3.539$, $p = 0.031$, stress event:stress exposure: $F_{2/158} = 3.305$, $p = 0.039$). FCM levels increased after stress exposure only at challenge test d102 but not d26 and d111, with higher basal FCM levels at challenge test d111 than d26 and d102. In addition, FCM concentrations were lower in PS compared to control animals at challenge test d26 but not d102 and d111.

4. Discussion

The environment *in utero* affected the glucocorticoid levels of F1 offspring both during development and challenge tests. Generally, we found that early prenatal stress reduced basal stress hormone levels during the pre-pubertal phase but shifted to higher levels afterwards, whereas challenge tests indicated advanced HPA-axis reactivity, as in contrast to control animals PS animals were capable of increasing plasma cortisol levels after stress exposure at an age of 26 days. At a later stage all animals had comparable abilities to increase glucocorticoid concentration in response to the stressor (see Fig. 5). Furthermore, effects were transmitted to the subsequent generation.

It is generally presumed that effects of prenatal stress on later HPA-axis activity are mediated by glucocorticoids of maternal origin that pass the placental barrier and affect fetal hormonal status [18]. In addition, prenatal stress can modify placental 11ßHSD-2, the enzyme converting the active cortisol into inactive cortisone, which subsequently alters fetal hormone levels [95]. After adrenal maturation, the fetus itself is able to produce glucocorticoids in response to stress [14]. However, even before placental formation and organ development cues of the maternal environment are transferred to the developing organism via uterine characteristics [5,51]. Therefore, many different ways of glucocorticoids affecting the offspring are to be considered.

In contrast to common understanding of programming, that typically, stress exposure results in increased glucocorticoid concentrations of the mother, in our study the stress exposure of pregnant F0 dams led to a long-term decrease of glucocorticoid levels in response to stress (strobe light during early to mid gestation). So despite the brief cortisol increase immediately after stress expo-

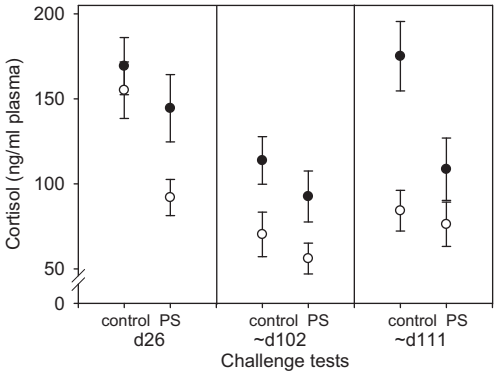

Fig. 5. Plasma cortisol concentrations (mean ± SEM) of F1 animals before and after the three challenge tests separated by stress event and group, irrespective of time of day (basal open circles, n(animals) = 24/18/17 and stress, filled circles, n(animals) = 20/14/14).

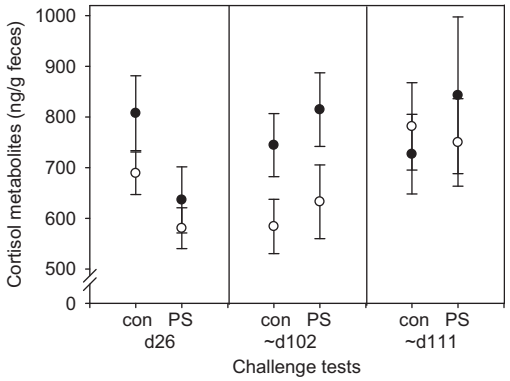

Fig. 6. Fecal cortisol metabolite concentrations (mean ± SEM) of F1 animals before and after challenge separated by stress event and group (basal, open circles, n = 20/14/14 and stress, filled circles, n = 24/18/17)).

sure, the maternal physiology of pregnant PS F0 dams was dominated by a down-regulation of stress hormone levels [81]. Our findings suggest that either the HPA-axis development of offspring can be programmed by decreased glucocorticoid levels, or the short-term increase in cortisol concentrations after stress in F0 dams was sufficient to program the F1 HPA-axis. Both explanations, however, are not mutually exclusive and may be extended by other effects or so far unobserved changes in stressed animals.

For example, the literature points to maternal behavior as another pathway for programming HPA-axis activity in offspring [13,25,26,60]. In our study, F0 dams exposed to stress during pregnancy were also tested for several behavioral parameters towards their offspring and aggression was the only parameter that differed significantly between groups [49]. Hence, slight differences in maternal behavior between stressed and control F0 dams may have contributed to the effects seen in F1. Also, body weight throughout pregnancy was lower in F0 dams exposed to stress than in control dams, even though no differences were found in the birth weight of the F1 offspring [81]. Nevertheless, potential effects of maternal body mass on offspring HPA-axis development cannot be excluded.

4.1. Concentrations of glucocorticoids during development

4.1.1. Concentrations of plasma cortisol and FCM in F1 generation

We found that plasma cortisol concentrations were usually lower in PS compared to control animals, although differences were significant only until d40. As the overall maternal endocrine status is dominated by down-regulated FCM levels [81], our results indicate that offspring physiology has been altered in the same direction.

A review of the available literature revealed variable findings of prenatal stress effects on later glucocorticoid levels in guinea pigs that depend on the type of stressor, the gestational period during which stress exposure took place, the time of sampling, sex and stage of reproductive cycle. For example, increased levels of basal cortisol were found in female offspring at an age of 60–90 days following prenatal adrenocorticotropin (ACTH) treatment during mid-gestation [40], and also in male offspring after weaning following exposure of pregnant dams to chronic variable stress during the second half of gestation [24]. Despite completely different time periods of exposure, similar findings, namely decreased cortisol levels were seen in the offspring of guinea pigs exposed to strobe light during late gestation [43]. Liu et al. [52] applied dexamethasone during pregnancy and also found reduced basal cortisol levels in the offspring. To our knowledge no comparable study of prenatal stress by exposure of strobe light during early to mid gestation is available. Therefore, we interpret our findings carefully, suggesting that young offspring's HPA axis was programmed to be prepared for stressful future and avoid excess levels of glucocorticoids.

Irrespective of treatment, we found that mean levels of plasma cortisol were lower in F1 males compared to F1 females during the first 40 days of life. After age 40 days, the comparison remained basically the same, however, owing to different sampling regimes, we abstained from statistical testing. In males but not females, cortisol levels increased significantly over time, possibly due to increased inter-individual aggression around puberty (personal observation). Dalle and Delost [17], in contrast, did not find differences between the sexes in basal cortisol levels in guinea pigs, yet animals were only up to three weeks of age.

Corresponding to the lower plasma cortisol levels in PS animals found during the early sampling period, FCM concentrations were lower in PS compared to control animals until d61 to 82, but shifted to higher levels thereafter. In addition, we found a comparable shift of FCM levels according to sex. Males initially had lower FCM concentrations than females, which increased to higher values around d54. The period of shifting FCM levels coincides with adolescence in young male guinea pigs [36,73], when increasing levels of gonadal hormones shape behavior and endocrine profiles [77,78,80,83]. As testosterone can act as a potential inhibitor of glucocorticoids [91], direct effects are an unlikely explanation for this shift. Rather, a growing number of studies indicate that adolescence is also a sensitive phase for programming (for review see: [59,74]) during which assessment of environment can redirect physiology if prenatal prediction does not match the current situation [55]. It is possible that the shift of stress hormone status in the PS animals might have been induced by such a reprogramming of HPA-axis in prenatally stressed animals maturing in a non-stressful environment. The discrepancy between plasma and FCM levels starting during the period of adolescence was unexpected and may be in part due to timing of sampling. Plasma samples were taken at 09:00 h and reflect the hormonal status at this time. Fecal samples were also taken in the morning however representing the hormonal status of a time period of the last afternoon. Therefore, it might be possible that adult FCM levels reflect general stress levels that are higher during the afternoon, which may not be reflected in plasma samples taken in the morning. Furthermore, not only general stress levels might be altered, but also circadian rhythm of glucocorticoid release. Clearly, more research is needed to explain this phenomenon.

From an evolutionary point of view it makes sense that the mother should adjust offspring to the predicted future environment using current cues, which may increase offspring survival and potentially fitness. After birth the offspring however has to evaluate the situation and attempt to match physiology to the actual setting. Our findings suggest that we mimicked harsh environmental conditions of the mother, which led to offspring with lower basal glucocorticoid levels, potentially to avoid overexposure in case of predicted stress. Furthermore, the earlier competence to respond to a stressor might be crucial for survival in a harsh setting in which mobilization of energy resources for flight might be life-saving. Puberty as a time of general hormonal resetting seems to be a crucial time period to assess current physiology and adjust it if necessary. In our case offspring were safe, secure and well nourished, apart from the two/three stress sessions, so that a change in basal glucocorticoid levels seems comprehensible. It would be very interesting to conduct further studies looking at the HPA axis development under harsh environmental conditions immediately, creating an environmental situation that matches the mother's prediction.

4.1.2. Concentrations of FCM in F2 generation

Studies primarily performed in rats have shown that programming effects can be transmitted to subsequent generations (for review see: [22]). To our knowledge only a single report on trans-generational effects is available for guinea pigs [50]. The authors report that administration of synthetic glucocorticoids during late F0 pregnancy resulted in alterations in HPA axis function in F2 offspring. Our study is the first showing trans-generational effects of stress exposure during early to mid F0 pregnancy on F1 and F2 glucocorticoid levels in this species. In F2 males, but not F2 females, concentrations of FCM were significantly higher in moPS offspring than in faPS offspring. In addition, males born to PS dams, showed higher concentrations of FCM compared to con/con animals, at age 22 days. Differences to con/con group were seen in moPS rather than faPS F2 males, indicating that the transmission of prenatal stress in F1 to F2 generation occurred primarily via the maternal line.

Several possible mechanisms might account for the effects seen in F2 animals, such as epigenetic alterations, by which DNA is modified without a change in DNA sequence resulting in changes in

gene expression that may be transferred to the subsequent generation [21]. Those changes include methylation state of DNA and three dimensional histone structure that have been shown to be altered by programming insults [27] and can be transmitted through multiple generations [11]. Transmission of epigenetic information by small RNA's with paternal sperm is another mechanism of transgenerational programming [71]. However, as main effects are seen in moPS animals in our study we believe differences to be transferred by mothers primarily. Therefore, another possible mechanism is the altered metabolic homeostasis of F1 females during their own fetal life that may have affected metabolic function even during adulthood, when these individuals become pregnant and thereby affecting F2 offspring [31]. FCM concentrations of pregnant F1 dams, however, did not differ between PS and control animals (data not shown). Further research is needed to elucidate the mechanisms of transmission and also to determine if effects are seen in the third generation, which is the first to be truly trans-generationally affected generation as F0 pregnancy included F1 offspring and F2-forming germ cells.

Sex-effects like those seen in our study are quite common in programming research and are described for both, males and females (e.g. [65,92]). Potential mechanisms include manipulations in expression of sex-specific genes as well as differences in placental transfer of glucocorticoids according to sex of the offspring [3,62].

4.2. Stress effects on concentrations of glucocorticoids

In all F1 animals, the challenge tests induced a robust increase of plasma cortisol concentration. Similar to our findings in F0 dams [81], this effect was manifest especially during the afternoon, when basal levels were significantly lower than those in the morning. This is in contrast to findings of Sachser [76] who showed a circadian rhythm of glucocorticoid levels in guinea pigs with nadirs during the morning. A possible explanation could be the general higher restlessness in response to feeding in the morning that led to increased basal morning cortisol levels in our study.

Basal plasma cortisol concentrations were higher during the first challenge test at d26 compared with the later challenge tests, especially in control animals. This finding is consistent with our results from plasma and fecal glucocorticoid measurements during F1 development. We assume that the putative stress burden of weaning, caused by separation from the dam and siblings coinciding with new surroundings and cage partners at d21, may have elevated cortisol levels, an effect that may still be present at d26. Interestingly, PS animals seemed to be less affected by this situation than controls, as PS animals showed comparable basal and stress levels of glucocorticoids in all three challenge tests. Maybe control animals were not yet able to react adequately to the stress event.

FCM concentrations only increased after challenge tests at ~d102, but not at d26 and ~d111. As FCM concentrations represent the overall status of an animal rather than a momentary picture, hormonal levels might be overlaid with the stress of weaning and regrouping at d26. At ~d111, all females (representing two thirds of the sample) were in estrus, so possibly gonadal hormones interacted with the HPA-axis and may have affected FCM concentrations. According to Viau and Meaney [89], sex steroids are the reason for differences in HPA-axis activity during the estrous cycle, because at the time of estrus concentrations of estrogens are elevated and subsequently HPA-axis function increases.

Taken together we found a group effect with significantly higher plasma cortisol concentrations found in control animals. Particularly, the cortisol increase after stress exposure was not apparent in control animals at d26, which might have resulted from elevated basal levels. We also found higher FCM levels in the feces of control animals than from PS animals during the first stress exposure (d26) but not during the second and third. These findings may point to a potentially earlier coping of PS animals with re-housing after weaning at d21, or even earlier maturation of HPA-axis function.

The existing literature on prenatal stress effects on HPA-axis reactivity is inconsistent due to varying stressors and timing of stress. Emack et al. [24] showed a blunted stress response to an open field test in young guinea pigs that had been exposed to chronic stress during fetal development. In contrast, Kapoor and Matthews [42] found no effects of prenatal stress on reaction to strobe light exposure in female guinea pigs around day 80. However, an ACTH challenge to the same animals revealed a significantly higher increase in cortisol concentrations of animals that had been stressed during late pregnancy. In accordance with our findings, Cadet et al. [12] found lower plasma ACTH and cortisol levels in response to strobe light exposure compared to control animals. As the exact hormonal situation of the mothers over the course of pregnancy is not sufficiently described, we cannot draw any conclusions from those effects seen in the offspring described in literature.

4.3. Conclusions

In summary, our findings indicate that prenatal stress affected the development of the HPA-axis in the F1 generation, resulting in lower basal glucocorticoid levels during the pre-pubertal period but higher levels in post-pubertal animals and an apparently earlier competence to respond to challenge tests. Effects were also transmitted to the F2 generation, probably via the maternal line, with a stronger effect found in males. These results suggest that maternal stress (induced by strobe light exposure that also included transfer of the animals to an unfamiliar dark room and single housing) during early- to midpregnancy was effective in inducing effects in the stress axis of two subsequent generations of offspring.

In contrast to the common literature, previous published results from our study indicate that maternal physiology of pregnant PS F0 dams exposed to stress was dominated by a down-regulation of stress hormone levels following a short cortisol increase immediately after stress exposure [81]. This finding was detected due to the long-term monitoring of the hormonal status of dams that other studies are lacking. Therefore, we propose three main findings of our study: (i) early- to midgestation is a time period during which maternal stress directly or indirectly (via alterations in maternal physiology and behavior) is capable of programming the offsprings HPA axis, (ii) next to short-term increase of cortisol concentrations after stress in F0 dams also decreased levels of glucocorticoids might code and transfer environmental cues for the prediction of a stressful future and program the offspring physiology and (iii) effects are even seen in a subsequent generation, however in a sex-specific way. Further studies are needed that take into account both short- and long-term effects of stress on HPA axis function in F0 dams to unravel the mechanisms underlying fetal programming of the HPA axis of F1 and F2 generations.

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