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Combined pre- and postnatal environmental enrichment programs the HPA axis differentially in male and female rats

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Summary Experimental environmental enrichment (EE) is usually applied in adulthood or immediately after weaning, with robust effects on physiology and behaviour. To investigate the effects of EE earlier in life, female rats were maintained under moderate enrichment during pregnancy and, together with their pups, during lactation until weaning. A separate group of dams housed under standard conditions during pregnancy and lactation served as controls. Dams housed under EE exhibited fewer nursing episodes and were off the nest more often, but the frequency of pup licking was not affected on postnatal days 3–5. EE effects on hypothalamus–pituitary–adrenal (HPA) axis responses to an acute stressor were determined in adult male and female offspring with and without previous exposure to the chronic stressor of constant light. In female offspring, chronic stress significantly increased basal corticosterone (CORT) levels, but not if rats had been exposed to early EE. Furthermore, while control females exposed to chronic stress showed a greatly reduced adrenocorticotropin (ACTH) response to an acute stressor, EE females did not display this desensitization. There was no significant effect of EE on basal ACTH and CORT levels in adult male offspring, nor did it alter their response to acute stress. Maternal licking frequency was moderately but significantly correlated with net corticosterone increases in response to acute stress, the direction of the correlation crucially depending on the offspring's sex and stress conditions. This study shows that EE during pregnancy and lactation has long-lasting effects on reactivity to acute and chronic stress in offspring and that these effects are dependent on the offspring's sex but not greatly on early postpartum maternal behaviour.

1. Introduction

A wealth of evidence has shown that environmental conditions early in life have profound influence on

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adult behaviour and vulnerability to stress (Heim et al., 2001; Ladd et al., 2000; Liu et al., 1997). The mechanisms by which this occurs are being explored in studies evaluating physiology and brain function in animals reared under aversive as well as enriched environmental conditions (Larsson et al., 2002; Moncek et al., 2004; Pham et al., 1999). In this regard, environmental enrichment (EE) usually involves housing several rats or mice together in a larger-than-usual cage that contains toys, tubes and/or a running wheel. The effects of EE can be roughly divided into three categories: consequences of EE *per se* on neurogenesis, neurochemistry and behaviour (Brown et al., 2003; van Praag et al., 1999; Kempermann et al., 2002; Lee et al., 2003); *prevention* by EE of lesion effects or symptoms associated with aging (Frick et al., 2003; Pham et al., 1999; Saito et al., 1994); and *reversal* by EE of changes induced by environmental impacts, lesions or genetic manipulation (van Rijzingen et al., 1997; Wagner et al., 2002).

Many EE studies have been performed in juvenile rats during the period immediately after weaning, a procedure sometimes referred to as 'EE rearing'. The brain during this period is particularly sensitive to environmental changes, as shown in studies of isolation rearing (Hall, 1998). EE during the post-weaning period has many effects (Fernandez-Teruel et al., 2002), notably on neuronal structure and survival (Berman et al., 1996; Young et al., 1999). EE during adolescence can also reverse the effects of an adverse early-life environment. Thus, the prolonged corticosterone (CORT) release in response to stress observed in prenatally stressed rats (Morley-Fletcher et al., 2003) or in rats exposed to repeated maternal separations during lactation (Francis et al., 2002) is normalized if these rats are reared under enriched conditions.

Notwithstanding the numerous effects of EE applied during adolescence or in adulthood, the perinatal period, during which rapid brain development takes place, is arguably even more susceptible to environmental manipulations. Indeed, both prenatal stress and repeated pup-mother separations during lactation have permanent 'programming' effects on offspring's sensitivity to stress later in life (for reviews see Ladd et al., 2000; Welberg & Seckl, 2001). Most animal studies investigating manipulations of the early life environment apply such treatments selectively prenatally or postnatally. In reality, it is likely that a particular milieu during pregnancy will remain the same during the postpartum period. Thus, in the study described here, female rats were environmentally enriched—to a moderate degree—during pregnancy and, together with their pups, during lactation until weaning. We determined the effects of EE on maternal behaviour and on hypothalamus–pituitary–adrenal (HPA) axis responses to an acute stressor in adult male and female offspring with and without previous exposure to chronic stress.

2. Methods

2.1. Animals and experimental protocol

Experiments were performed in accordance with NIH Guidelines for the care and use of laboratory animals and all protocols were approved by the Emory University IACUC. Unless stated otherwise, rats were maintained under regular animal husbandry conditions in temperature- and humidity controlled rooms with a 12:12 light-dark (LD) cycle (lights on 0700 h) and food and water available ad libitum.

A schematic overview of the experimental design is shown in Fig. 1. Timed-pregnant Long-Evans rats (Charles River Laboratories, Inc, Wilmington, MA) arrived in our facilities on day 12 of pregnancy. After a 2-day recovery period rats were housed singly either in a standard ($W\times L\times H$ 20×32×15 cm) cage with corn-cob bedding (C, $n=6$ dams) or in a moderately enriched environment (EE, $n=7$ dams) consisting of a large ($W\times L\times H$ 30×60×22 cm) cage containing a mixture of corn-cobs and pine shavings as bedding, and a 28 cm long white plastic tube (6.5 cm ø) large enough for a pregnant rat to walk through. Cages were changed one day before the estimated day of birth, and animals were then left undisturbed until the next cage change on postnatal day (PND) 14. Weaning took place on PND23. This involved removal of the dam and

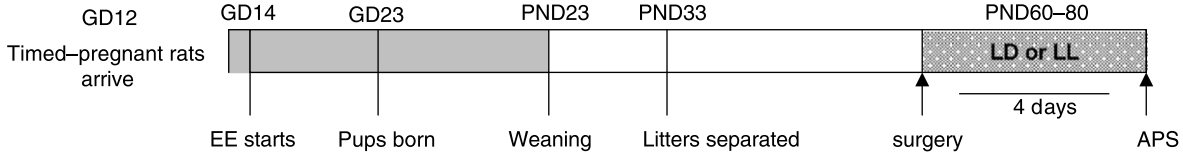

Figure 1 Experimental design of the study. GD, gestational day; PND, postnatal day; LD, housing under normal light cycle; LL, housing in constant light; APS, airpuff-startle stress test. Additional details are provided in Section 2.

placing the offspring in a clean cage. In the case of EE offspring, the whole litter was housed in a large cage, and in the case of control offspring, groups of 6–10 littermates were housed together in standard cages. On PND33 all litters were separated by sex and housed in standard cages. Twelve days later, rats were further divided into groups of 2–3 and left undisturbed except for regular animal husbandry, until testing in adulthood (2–3 months of age).

2.2. Maternal behaviour observations

Maternal behaviour was scored on PND 3-5 in four daily sessions consisting of eight observations per session (one observation every 5 min), yielding a total of 96 observations per dam. Types of behaviour scored included licking, arched-back nursing, blanket nursing, passive nursing, and being off the nest.

2.3. Offspring testing

Hormonal responses to stress were measured in adult male and female offspring from C and EE dams. In addition, adult offspring were housed under one of two lighting conditions: constant light (LL), serving as a non-invasive, cue-independent chronic stressor; or normal lighting (LD; see under 'Experimental housing conditions post-surgery'). Thus, a 2 (early environment) $\times$ 2 (lighting) design was used as shown in Table 1, and data from male and female offspring were analysed separately. No more than one male and one female offspring from each litter were used per adult lighting condition (Abbey & Howard, 1973).

Table 1 Experimental design and number of animals per treatment group to evaluate the effect of stressor on the HPA axis activity of the offspring.

Adult lighting condition[a]	Early life condition	
	C	EE
LD	6F, 6M	5F, 6M
LL	6F, 6M	7F, 6M

There were eight treatment groups in total (4 per sex: C/LD, C/LL, EE/LD, EE/LL). Experiments using F (female) and M (male) rats under LD (normal lighting) and LL (constant light) conditions were carried out in the same experimental room on separate days. C (control) = dams and litters maintained under normal rat husbandry conditions; EE = dams and litters maintained in an enriched environment. Post-weaning rearing conditions were similar for C and EE offspring.

[a] Treatment before presentation of the airpuff-startle (APS). Additional details are provided in Section 2.

2.4. Surgery

At 2–3 months of age, male and female offspring were weighed and anesthetized with an anesthetic mixture containing acepromazine–ketamine–xylazine and a catheter was placed into the right atrium via the right jugular vein using aseptic surgery (Thrivikraman et al., 2002). The distal end of the cannula was exteriorized at the nape of the neck, filled with a solution of gentamicin (5.0 μg/100 g BW; Schein pharmaceuticals Inc, Port Washington, NY) in sterile saline and closed. Animals were placed on heating pads to recover from surgery until they were ambulatory.

2.5. Housing conditions post-surgery

Upon recovery from surgery rats were housed in opaque plastic circular containers (28 cm ø $\times$ 35 cm height) in a quiet room for recuperation and habituation to the environment. Rats were maintained either on a regular 12/12 h light-dark cycle (LD; lights on 07:00 h) or under the chronic stressor of constant light (LL). Food and water were available ad libitum, and rats were briefly handled each day.

2.6. Stress test

Experiments were performed between 08:00 and 10:00 h. On the morning of the stress test, 4 days after surgery, jugular cannulae were connected to 50 cm long tubing extensions (PE50, VWR, Atlanta) containing heparinized (20 IU/ml) saline, connected to 1 cm^3 syringes, and extended outside the test cages. This minimized experimenters' contact with the rats and facilitated undisturbed blood sampling.

Immediately after connection of the tubing a 300 μl blood sample was drawn for determination of basal plasma adrenocorticotropic hormone (ACTH) and CORT levels. Rats were subsequently subjected to an airpuff startle (APS), a non-noxious aversive stimulus that reliably activates the HPA axis (Engelmann et al., 1996). APS was applied in repeated blocks from a pressurized air can (50-65 psi) to rats in their experimental cages. Each block consisted of three 1-s long air blasts applied over a 5 s period beginning at time 0. The airpuffs were directed towards the side of the head from a distance of 10–20 cm. Three blocks of airpuffs were applied with 1-min intervals separating the blocks. Sequential 300 μl blood samples were drawn at 5, 10, 15, 30, 45 and 60 min following APS. A replacement volume of heparinized saline was

infused after each blood sample to compensate for lost volume.

2.7. Blood samples and hormone assays

All blood samples were collected into microcentrifuge tubes containing 10 μl EDTA (100 mg/ml EDTA tetra sodium salt, Sigma, St. Louis, MO) and stored on ice until the end of the experiment. Samples were centrifuged at 4 °C and plasma aliquots were stored at −20 °C until assayed. Plasma ACTH (Allegro® HS-ACTH, Nichols Institute, San Juan Capistrano, CA) and CORT (ImmuChem™ Double Antibody, ICN Biochemicals, Costa Mesa, CA) were determined as previously described (Thrivikraman et al., 1997). The working ranges were 5–1500 pg/ml for the ACTH assay and 5–1000 ng/ml for the CORT assay, with intra- and inter-assay coefficients of variation below 10%.

2.8. Data analysis

2.8.1. Maternal behaviour

For each dam, a maternal-behaviour score was calculated for each of the 3 days of observation. An ANOVA for repeated measures was performed for licking behaviour, nursing behaviour (a composite of arched-back, passive and blanket nursing) and episodes spent off the nest. Where responses were significant, post-hoc multicomparisons were performed using Fisher's LSD test.

2.8.2. Adult offspring

Body weights from C and EE male and female offspring were compared using unpaired *t*-tests. Basal ACTH and CORT levels were evaluated using a two-way (early life conditions × lighting) ANOVA in male and female offspring separately. ACTH and CORT responses to APS were evaluated by ANOVA for repeated measures with early life housing conditions and lighting as main factors. In addition, because of the differences in basal plasma ACTH and CORT levels between groups, net increases of ACTH and CORT in response to APS were calculated by subtracting the basal value ($t=0$) from each subsequent value ($t=5$ through $t=60$), followed by summation of all of the resulting values. Net increases were compared between groups using a two-way ANOVA with early life housing conditions and lighting as main factors. When the overall response was significant by ANOVA, post-hoc comparisons were performed using Fisher's LSD test. For all statistical tests, significance was set at $p \leq 0.05$.

3. Results

3.1. Birth parameters and body weight

Housing conditions did not affect gestation length ($t=0.33$, $p=0.75$), number of pups per litter ($t=0.19$, $p=0.85$), or sex ratio ($t=0.94$, $p=0.37$) of the litters. Furthermore, EE did not affect body weight in adult offspring: Mean body weight in C females was 283 ± 9.9 g, and in EE females 278 ± 6.8 g ($t=0.42$, $p=0.67$). Control males weighed 416 ± 10.1 g, whereas males exposed to early EE weighed 428 ± 6.4 g ($t=1.08$, $p=0.29$).

3.2. Maternal behaviour

Because the frequency of blanket-, passive-, and arched-back nursing did not significantly differ between C and EE dams (data not shown), these three types of maternal behaviour were pooled into one 'nursing' category. Thus, ANOVAs for repeated measures were performed on three aspects of maternal behaviour: licking, nursing and episodes spent off the nest. There were no effects of group or time on the number of licking episodes (data not shown). There were effects of group ($F=5.50$, $p=0.039$) and time ($F=6.42$, $p=0.006$) on the number of nursing episodes but no interaction between the two. Post-hoc analysis revealed that dams housed in EE conditions spent significantly fewer episodes nursing their offspring, and that fewer episodes were spent nursing on PND 5 than on PND 3 ($p=0.001$) or PND 4 ($p=0.029$). For off-the-nest episodes there was a group effect ($F=11.08$, $p=0.007$) with EE dams spending more episodes away from the nest, but there was no significant effect of time or an interaction between group and time (Fig. 2).

3.3. HPA axis activity

3.3.1. Basal plasma ACTH and CORT

The effects of different treatment conditions on basal levels of plasma ACTH and CORT in the adult male and female offspring are shown in Fig. 3. Although EE tended to increase basal ACTH levels in females, a two-way ANOVA with lighting- and early life conditions as main factors showed that neither factor influenced basal ACTH levels significantly (Fig. 3A). There was also no main effect of early EE on basal CORT levels in females, but a significant effect of chronic stress (constant light) ($F=11.91$, $p=0.003$) and an interaction between lighting and early life conditions ($F=4.23$, $p=0.05$) were observed. Subsequent post-hoc analysis showed

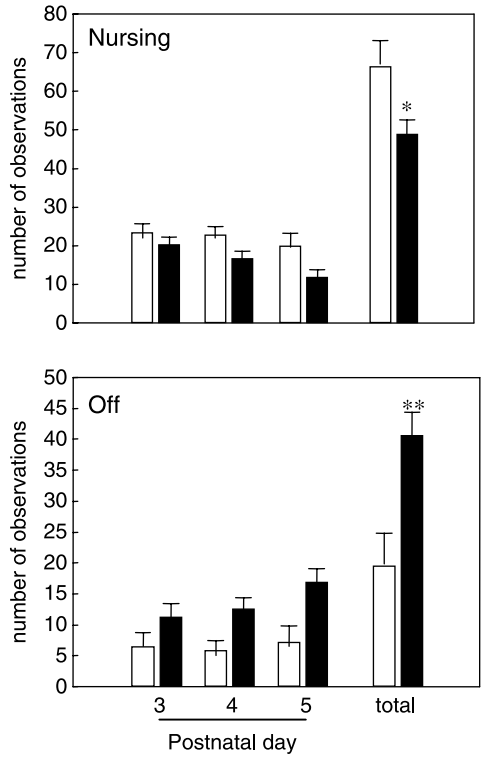

Figure 2 Maternal nursing behaviour (top panel) and episodes spent off the nest (bottom panel) in dams housed in control (white bars) and enriched (black bars) environments. *$p<0.05$, **$p<0.01$.

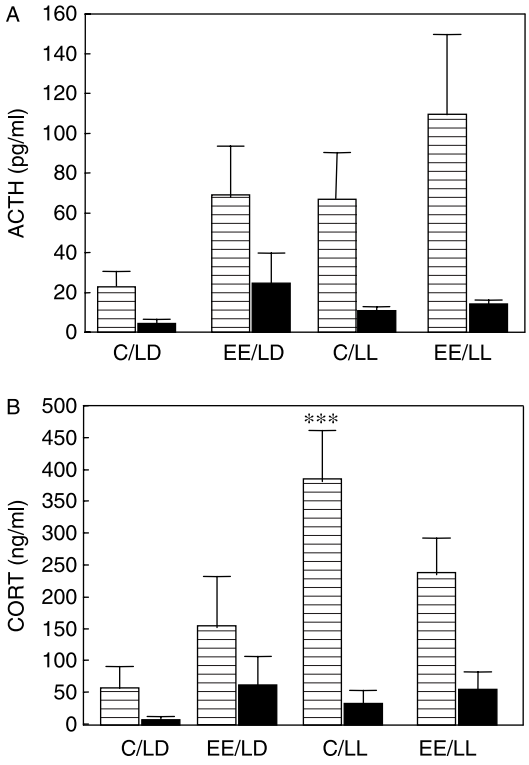

Figure 3 Basal plasma ACTH (A) and CORT (B) levels in female (striped bars) and male (solid bars) offspring. C, control; EE, early enrichment; LD, normal light cycle; LL, constant light. ***$p<0.001$ *vs* C/LD

that chronic stress increased basal CORT levels in control females (C/LD *vs* C/LL; $p=0.0008$), but not in females exposed to EE (EE/LD *vs* EE/LL; $p=0.34$) (Fig. 3B).

A two-way ANOVA with early-life environment and lighting as main factors showed that neither condition significantly affected basal ACTH or CORT levels in male offspring, nor was there a significant interaction between the two factors (Fig. 3).

3.3.2. Stress response in female offspring

An ANOVA for repeated measures showed no main effects of early-life conditions ($F=1.21$, $p=0.285$) or chronic stress ($F=1.32$, $p=0.264$) on the ACTH response to APS in females. However, there was a significant interaction between the effects of chronic stress and early-life conditions ($F=5.23$, $p=0.033$) as well as a significant three-way interaction ($F=2.77$, $p=0.015$). Subsequent post-hoc analysis revealed that compared to housing under regular lighting, the chronic stress of constant-light housing caused a significant decrease in the ACTH response to APS in C females (C/LD *vs* C/LL; $p=0.024$), but not in EE females (EE/LD *vs* EE/LL; $p=0.434$), as shown in Fig. 4A and B. Indeed, early EE rats housed under constant light have a higher ACTH response to APS than C females housed in constant light (EE/LL *vs* C/LL; $p=0.021$) (Fig. 4B). Fig. 6A shows that this effect of chronic stress was also noticeable in the net increase in ACTH in response to APS ($F=4.56$, $p=0.044$), as was an interaction between the two factors ($F=8.5$, $p=0.009$; Fig. 6A): Constant-light housing reduced the net ACTH release in control females ($p=0.002$; C/LD *vs* C/LL), but not in EE females (EE/LD *vs* EE/LL). Indeed, control females housed under chronic stress had a significantly lower net release of ACTH than EE females under chronic stress ($p=0.016$; C/LL *vs* EE/LL) (Fig. 6A).

As shown in Fig. 4C and D, the overall CORT response to APS was not affected by early-life conditions ($F=0.01$, $p=0.918$) or by chronic stress ($F=0.21$, $p=0.655$) (Fig. 4C and D). However, the net release of CORT (Fig. 6B) in response to APS was affected by lighting ($F=9.73$, $p=0.005$), and there was an interaction between early-life conditions and lighting ($F=5.85$, $p=0.025$). Subsequent post-hoc analysis showed that chronic stress (constant light) reduced the CORT release in control females (C/LD *vs* C/LL; $p=0.0008$), but not in EE females (EE/LD *vs* EE/LL), and that control females housed under chronic stress had a significantly lower net

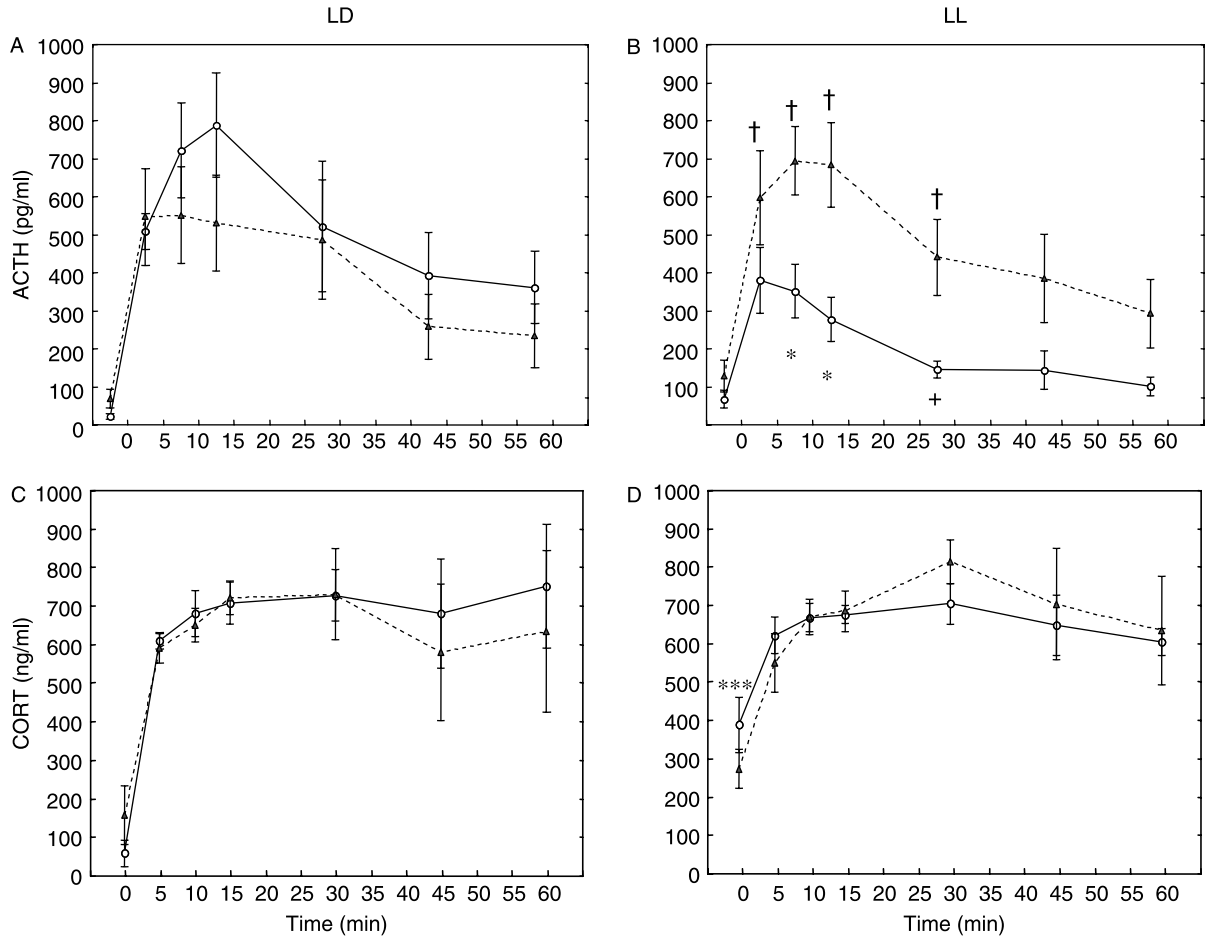

Figure 4 Plasma ACTH (top, A and B) and CORT (bottom, C and D) responses to airpuff-startle stress in female offspring that were either previously unstressed (A and C) or chronically stressed (B and D). Solid line, C; dotted line, EE; Panel A and C, normal light cycle (LD); Panel B and D, constant light (LL). ***$p<0.001$ *vs* C/LD; *$p<0.05$ *vs* C/LD; +$p=0.05$ *vs* C/LD; †$p<0.05$ *vs* C/LL.

release of CORT than EE females under chronic stress (C/LL *vs* EE/LL; $p=0.05$) (Fig. 6B).

3.3.3. Stress response in male offspring

A multifactorial Anova for repeated measures on data from all males showed that neither early-life enrichment nor the chronic stress of constant light significantly affected the ACTH (Fig. 5A and B) and CORT (Fig. 5C and D) responses to APS, although chronic stress tended to increase the overall CORT response ($F=3.90$, $p=0.062$). The net increases in ACTH and CORT were not significantly affected by early-life conditions or by chronic stress (Fig. 6).

3.3.4. Correlations

Correlations between maternal behaviour as observed on PND 3–5 and net increases in the offspring's ACTH and CORT levels in response to APS are shown in Table 2. Correlations were calculated per sex and per lighting condition. Significant correlations were only found for licking behaviour: It was negatively correlated with the net CORT release in females, but only if they were housed under chronic stress. The same correlation was found in male offspring housed under a normal LD cycle, whereas the net HPA response to APS in males under chronic stress was positively correlated with maternal licking on PND 3–5.

4. Discussion

This study showed that (1) moderate EE of rats during pregnancy and lactation had permanent effects on stress responsiveness of the offspring, especially in females; (2) these effects were not mainly mediated by changes in early maternal behaviour; and (3) chronic mild stress desensitized the ACTH response of control females to a subsequent stressor. It is perhaps not surprising that manipulation of the pre-weaning environment

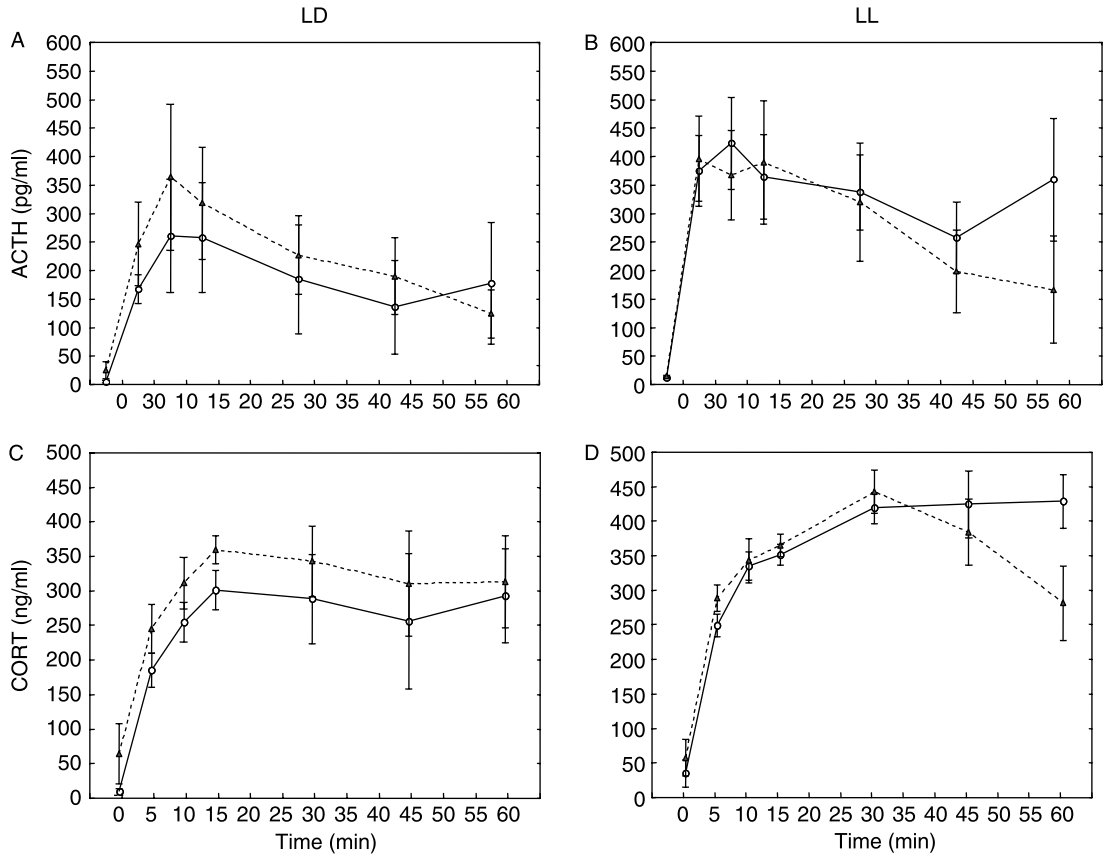

Figure 5 Plasma ACTH (top, A and B) and CORT (bottom, C and D) responses to airpuff-startle stress in male offspring that were either previously unstressed (A and C) or chronically stressed (B and D). Solid line, C; dotted line, EE; Panel A and C, normal light cycle (LD); Panel B and D, constant light (LL).

had such profound effects on offspring stress sensitivity. Early life stressors such as prenatal stress and mother-pup separations have been shown to permanently alter both the behavioural and hormonal stress response in the offspring (Ladd et al., 2000; Welberg & Seckl, 2001). Clearly, introducing enrichment, rather than stress, in the pre-weaning environment also has long-lasting effects.

In the present study, early EE altered the effect of chronic-stress exposure on the HPA axis response to a subsequent acute stressor: Control female offspring showed a severely reduced ACTH response to acute stress, which confirms a previous finding in female mice (Bhatnagar & Vining, 2004), whereas EE females did not. Significant interactions between housing conditions, prior stress and response to acute stress have been described in an earlier report (Larsson et al., 2002) in which both the direction and the strength of the response to an acute stressor—after previous exposure to stress—depended on the rats' housing conditions, although EE in that study took place in adulthood.

Housing under constant light has been used as a chronic stressor by other groups in both male and female rats (Larsen et al., 1994; Morimoto et al., 1975; Popovic et al., 1996) and has been shown to increase basal CORT levels in females (Dalal et al., 1994; Morimoto et al., 1975). Indeed, in our study constant-light housing also greatly increased basal CORT levels in control females. This may have produced a strong tonic feedback upon the HPA axis, resulting in desensitization of the axis to subsequent stressors and consequently an inappropriately reduced ACTH response to APS. Alternatively, it has been suggested that constant light induces a hyperoestrogenemic state in female rats (Hoffmann, 1978; Schwartz, 1982; Weber & Adler, 1979), although this was not tested in the present study. Fluctuations in oestrogen levels are known to affect HPA responsiveness (Carey et al., 1995), with pro-oestrus responses being higher than those in the oestrus phase of the cycle. Thus, the reduced sensitivity to APS of females housed in constant light may be due to increased oestrogen levels. Importantly however, early-life EE

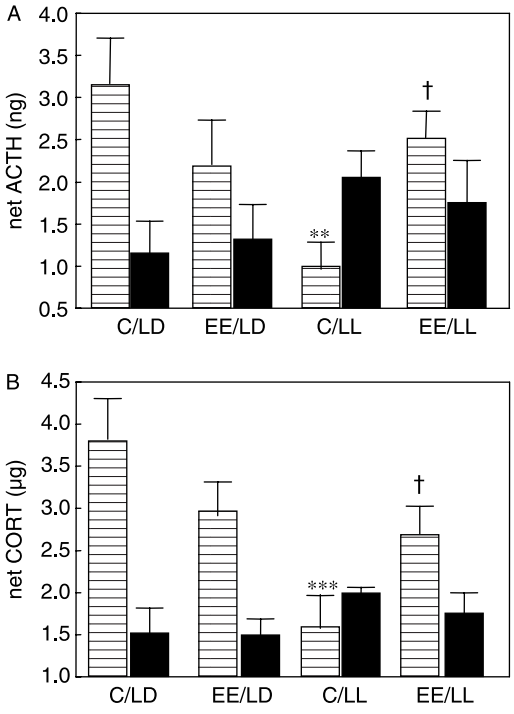

Figure 6 Net release of ACTH (A) and CORT (B) levels in response to airpuff-startle in female (striped bars) and male (solid bars) offspring. C, control; EE, early enrichment; LD, normal light cycle; LL, constant light. ***$p<0.001$ *vs* C/LD; **$p<0.01$ vs C/LD; †$p\leq0.05$ *vs* C/LL.

abolished this hyposensitivity, as EE females housed under a normal light cycle and those housed in constant light showed similar ACTH responses to APS.

The CORT response to APS was similar, though very high, in all females. It is possible that females from all treatment groups had reached their maximum CORT output and that therefore differences in ACTH levels were not reflected in the CORT response to APS. Alternatively, the chronic stress of constant-light housing may have increased adrenal sensitivity to ACTH, so that a reduced ACTH output in C/LL females nevertheless resulted in a CORT output equal to that of the other female rats.

The finding that effects of early EE were more pronounced in female than male offspring is consistent with earlier studies showing greater sensitivity of female rats to effects of prenatal stress (e.g., McCormick et al., 1995) and of EE performed in adulthood (Belz et al., 2003; Klein et al., 1994). It is possible that an effect of early EE on the HPA response to acute stress would become visible in males only after a stronger challenge to the system. The fact that constant-light housing did not significantly affect male rats' basal HPA activity or their response to acute stress indicates that 4 days of LL may not have acted as a stressor in the male offspring in this study. Indeed, other studies investigating the effects of LL typically house rats in constant light for 10 days or longer (e.g., Fischman et al., 1988; Honma et al., 1978). However, it is important to note that the power of this study is limited due to the relatively small sample sizes, and conclusions that LL or EE did not affect males' basal or net HPA activity should be interpreted with caution.

Interestingly, a blunted ACTH response to stress such as that seen in C/LL female rats in the present study, has also been observed in humans with disorders associated with exposure to stress such as chronic fatigue syndrome (Demitrack et al., 1991; Gaab et al., 2002; Gaab et al., 2005). The present study suggests that a stimulating environment in early life could act as a protective factor against development of such stress-related disorders. Furthermore, our data indicate that female rats exposed to chronic stress may serve as a model for at least the neuroendocrine aspects of chronic fatigue syndrome, although additional assessments such as CRF stimulation- and dexamethasone suppression tests are needed to validate this possibility.

Early-life manipulations in animal studies generally are applied selectively prenatally or

Table 2 Correlations between maternal behaviour and HPA responses to an acute stressor in male and female offspring.

	Licking		Nursing		Off the nest	
	Net ACTH	Net CORT	Net ACTH	Net CORT	Net ACTH	Net CORT
LD females	−0.16	−0.11	0.39	0.45	−0.36	−0.44
LD males	−0.19	−0.58[a]	0.19	0.44	−0.15	−0.29
LL females	−0.16	−0.59[a]	−0.29	−0.11	0.34	0.28
LL males	0.58[a]	0.59[a]	−0.10	−0.11	−0.04	−0.03

'Nursing' behaviour consists of arched-back, passive and blanket nursing; LD, rats were housed post-surgery under a normal light-dark cycle; LL, rats were housed post-surgery under constant light.

[a] significant correlation ($p<0.05$).

postnatally, while in reality there is no reason to assume that pre- and postpartum environments are radically different. The current study was therefore designed to assess the effects of a prolonged period of early EE. It employed a moderate form of EE that was simple to execute. Both dams and pups were observed to spend much time inside or on top of the tube used to enrich the environment. By the nature of its design, it was not possible in this study to distinguish between prenatal- and postnatal effects of EE, or between indirect maternal effects of EE and direct effects on the offspring itself. EE of pregnant females alone has been shown to improve maze learning in offspring (Kiyono et al., 1985) and in one study even EE of the dam before mating changed her offspring's behaviour (Dell & Rose, 1987). Recently it has become clear that manipulation of the early environment often exerts its effects through alterations in specific aspects of maternal behaviour, notably licking and arched-back nursing (Liu et al., 1997). Only one previous study has investigated effects of EE during pregnancy and lactation on maternal behaviour in mice and found that it significantly increased licking, which likely accelerated the timing of eye opening in the mouse pups (Cancedda et al., 2004). In contrast, EE in the present study tended to *decrease* maternal licking on PND 3–5. Furthermore, the EE dams spent more time off the nest than control dams, and engaged less in nursing behaviour, but these measures were not correlated with HPA responses in adult offspring.

Liu et al. (Liu et al., 1997) showed that high levels of maternal licking and nursing can program, among other measures, an efficient HPA response in offspring later in life. In the present study maternal licking was indeed correlated with net CORT responses to APS, but the direction of correlation depended on the sex and lighting conditions of the offspring. Since males and females in the different lighting conditions came from the same litters, this means that a dam's licking frequency may correlate positively with one of her pups' stress response, but negatively with another's. Thus, male offspring of mothers displaying a higher frequency of licking would have a high HPA response to acute stress if they were already chronically stressed, but would show a relatively low CORT response if housed under a normal LD cycle. A speculative interpretation of this finding could be that maternal licking programs appropriate stress responses in offspring: Under normal circumstances, an acute stressor like APS may only require a modest HPA response, but under conditions of chronic stress with an additional acute stressor a more robust HPA response may be appropriate. However, EE did not significantly alter maternal licking frequencies on PND 3–5, and it is therefore unlikely that maternal behaviour on these days mediated the effects of EE in this study. Behaviour on later days was not recorded, and an effect of maternal care after PND five on offspring HPA axis activity cannot be excluded, although it has been suggested that the first 5 days after birth may constitute the critical period for effects of early life events on stress responsivity (Levine & Lewis, 1959).

Alterations in maternal and/or pup CORT levels induced by EE may have mediated the effects on HPA responsivity in the offspring. EE changes plasma CORT levels, usually increasing them (Kempermann et al., 2002; Moncek et al., 2004), though this is not reported in all studies (Belz et al., 2003; Morley-Fletcher et al., 2003). Considering the powerful programming effects of perinatal exposure to glucocorticoids (Welberg & Seckl, 2001), EE-induced changes in maternal plasma CORT concentrations may have influenced offspring development in utero and/or during lactation. As we did not measure CORT levels in maternal plasma, this possibility remains to be investigated. However, Devenport et al. (1992) showed that EE-induced increases in cortical thickness in rats were not abolished by prior adrenalectomy, suggesting that corticosteroids do not mediate EE-induced augmentation of neuronal growth, at least when EE is performed during adolescence. Of note, it is possible that early EE shapes adult HPA reactivity without affecting on neuronal growth. Indeed, Kohl et al. (2002) showed that pre-weaning enrichment had no effect on adult hippocampal neurogenesis.

Many EE studies have been performed in juvenile rats during the period immediately after weaning, but few of these have investigated the effects of EE per se on HPA axis activity and the findings are inconclusive: post-weaning EE has been reported to have no effect on basal or stress-induced CORT in males (Morley-Fletcher et al., 2003; Schrijver et al., 2002), to reduce basal ACTH and CORT and stress-induced ACTH levels in males and females (Belz et al., 2003), or to have no effect in male rats that were neonatally handled (Francis et al., 2002). Discrepancies between the outcomes of these studies may be due to differences in the duration of EE, in the timing of testing, and possibly also the in social component of the enrichment (e.g. Belz et al., 2003, although see Schrijver et al., 2002). However, EE appears to *reverse* the effects on HPA axis activity of earlier manipulations such as prenatal stress (Morley-Fletcher et al., 2003) and maternal separation (Francis et al., 2002). Similarly, in the present study it normalised the desensitized ACTH response of chronically-stressed

female rats to a subsequent stressor. It is possible that beneficial effects of EE, either pre-weaning or post-weaning, manifest themselves more clearly on a background of some adversity and may induce resilience to future stressors. In this light, one might speculate that neonatal handling and pre-weaning enrichment both create an improved early environment, which then programs an efficient hormonal response to stress later in life. Indeed, similar to EE in the present study, neonatal handling normalised the HPA axis response to a novel stressor in chronically-stressed rats, although here the novel stressor induced an exaggerated ACTH and CORT release in non-handled animals (Bhatnagar & Meaney, 1995). Importantly, neonatal handling clearly has effects on its own: It reduces basal HPA axis activity and its response to acute and chronic stress (Meaney et al., 1991; Meaney et al., 1993; Panagiotaropoulos et al., 2004). Furthermore, handling has effects in both males and females (Panagiotaropoulos et al., 2004), whereas early EE strongly affected only females' stress responses. Another important difference between the neonatal handling and early EE paradigms is that the former has been shown to increase maternal behaviour (Liu et al., 1997), whereas in the present study EE dams spent more time off the nest and less time nursing. Clearly, neonatal handling and early EE are both capable of programming adult HPA axis activity, possibly creating a more stress-resilient animal, but the mechanisms involved may be different.

Although previous studies of EE employed much more elaborate forms of EE (Morley-Fletcher et al., 2003; Pham et al., 1999; Venable et al., 1989), simple forms of cage improvement have also been tested. Rodents appear to have a preference for cages with chewable objects (Belz et al., 2003; Chmiel & Noonan, 1996) and nesting material (van de Weerd et al., 1997a; van de Weerd et al., 1997b). The current study showed that a single tube, more space and different bedding provided enough enrichment to have persistent effects on the offspring. The profound effects of such moderate and simple EE may leave one wondering how deprived 'control' laboratory mice and rats living in standard housing conditions actually are. In addition, this study underlines the importance of housing conditions during pregnancy and lactation. Perhaps many animals used in stress research have unwittingly been made more or less vulnerable to exactly those influences that are being investigated. Interestingly, other studies showed that early life conditions that are clearly adverse such as prenatal stress, also altered HPA axis adaptations to chronic stress (Bhatnagar et al., 2005; Chung et al., 2005), in a sex-specific way (Bhatnagar et al., 2005).

Summarizing, this study showed that housing conditions during pregnancy and lactation have long-lasting effects on reactivity to acute and chronic stress in offspring and that these effects are dependent on the offspring's sex but not greatly on postpartum maternal behaviour on PND 3–5. Furthermore, our finding that EE prevented the chronic-stress induced desensitization to a subsequent novel stressor in female rats suggests that a favourable early-life environment may protect against development of stress-related disorders associated with a hyporesponsive HPA axis such as chronic fatigue syndrome.

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