The newborn rat's stress system readily habituates to repeated and prolonged maternal separation, while continuing to respond to stressors in context dependent fashion

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Abstract

Adrenal corticosterone secretion of newborn mice rapidly desensitizes to repeated maternal absence. The present study investigated the effects of novelty exposure, maternal care and genotype on this phenomenon. Maternal separation (MS) took place on postnatal days (pnd) 3–5. In Wistar rats, the degree of novelty in the MS-environment was varied by exposing pups to: (i) “home separation”: pups remained in the home cage; (ii) “novel separation”: pups were placed individually in a novel cage. Maternal care was recorded on pnd 1 to 4. To investigate the effect of genotype, we also examined Long Evans in the “home separation” condition. Basal and stress-induced ACTH and corticosterone levels were measured. Adrenal tyrosine hydroxylase (TH) and melanocortin receptor-2 (MCR-2) proteins served as markers for adrenal function. We show, in both rat strains, that the rise in plasma corticosterone induced by a single 8 h-MS on pnd 5 was abolished, when this separation procedure had also been performed on pnd 3 and 4. Habituation to maternal absence occurred irrespective of housing conditions. However, pups in the “home separation” condition received less maternal care upon reunion than those placed in the “novel separation”. These “home separation” pups appeared more responsive to a subsequent acute novelty-stressor, and their adrenal TH and MCR-2 were higher. Long Evans rats appeared more stress responsive than the Wisters, in the home separation condition.

In conclusion, separation environment, maternal care and genotype do not affect adrenal desensitization to repeated 8 h-MS itself, but may modulate the adrenal stress-responsiveness of separated pups.

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Introduction

Aberrant HPA axis activity and corticosterone (CORT) secretion induced by adverse early life experiences is considered a major risk factor for the development of psychiatric disorders in humans (Heim et al., 2008; Lupien et al., 2009). Therefore, rodents deprived as pups from maternal care have been widely used as a laboratory model for early adversity to study the underlying mechanism of CORT-enhanced vulnerabilities (Plotsky et al., 2005; Pryce et al., 2001a). Several adversity paradigms are currently used. One common approach is a single episode of 24 h of maternal absence. Another approach is repeated daily maternal separations (MS), which include repeated periods of 3–8 h absence of the dam during the first two postnatal weeks. It has been proposed that pups experiencing repeated MS display as adult enhanced stress responsiveness, increased anxiety, helplessness and anhedonia, deficits of sensorimotor gating and increased propensity for the intake of addictive drugs (Biagini et al., 1998; Brake et al., 2004; Moffett et al., 2007; Plotsky et al., 2005; Zhang et al., 2005). Interestingly, these rodent behaviors, programmed by early adversity, resemble clinical endophenotypes of depression and schizophrenia, hence providing face and construct validity to these models (Pryce and Seifritz, 2011). However, the outcome of early adversity depends on strain and gender of the animals as well as the frequency, duration, age and time point (within the light cycle) of MS (Claessens et al., 2011; Lehmann and Feldon, 2000). Moreover, the environmental context (housing in groups or in isolation, inside the home cage or in a novel environment) and the ambient temperature have an effect (Lehmann and Feldon, 2000; Ruedi-Bettchen et al., 2004). Cumulatively, these observations have raised the question to what extent the HPA axis is actually activated during repeated MS. Schmidt and colleagues (Schmidt et al., 2004; Schmidt et al., 2006) found that,
in mice, after 8 h of a single MS the basal level of circulating CORT has slowly reached peak levels, while the stress hyporesponsive period (SHRP) has become disrupted resulting in an enhanced responsiveness of the adrenocortical secretion of CORT to mild stressors and exogenous ACTH administration (Levine et al., 1991; Rosenfeld et al., 1992b). Enthoven and colleagues hypothesized that 3 consecutive daily 8 h-MS from postnatal (pnd) 3–5 would amplify neuroendocrine responses to both separation and novelty exposure. Surprisingly, they reported instead that the increase in HPA axis basal activity that is first observed after the initial 8 h-MS was rapidly abolished after repeated 8 h-MS. This rapid desensitization of the neonate’s HPA axis to repeated daily separations from the dam was not due to metabolic factors such as ghrelin, and also did not occur because of enhanced glucocorticoid negative feedback (Enthoven et al., 2008). In spite of this rapid desensitization of the HPA axis to the effect of daily separation, the SHRP remained disturbed because a subsequent novelty stressor triggered an enhanced plasma CORT and c-fos mRNA response in the PVN (Enthoven et al., 2008). This finding raised the question whether the environmental context experienced by the pup during MS can influence the outcome (Enthoven et al., 2008).

In the present study we have extended these findings to the rat by examining the immediate effects of 3 daily repeated 8 h-MS from pnd 3–5. The objective of these experiments was to investigate further the apparent “desensitization” of HPA axis activity to repeated MS in different separation contexts in two rat strains. The degree of novelty was varied in the separation environment using: (i) “home separation”: the environment was the home cage and pups remained grouped together; (ii) “novel separation”: the environment did not contain any element of the home cage and pups were additionally isolated from their littersmates. The effect of the different MS protocols was investigated on basal and novelty stress-induced ACTH and CORT levels on pnd 5. Since in the study of Enthoven et al. (2008), the apparent adrenal sensitivity to ACTH was altered dramatically during the repeated separations we also measured two biomarkers for adrenal function: tyrosine hydroxylase (TH) levels as index for adrenal medullary function and the level of melanocortin 2 receptor (MC2R) as an index of adrenal sensitivity to ACTH. Moreover, maternal care was measured for the first 4 postnatal days to explore its possible implication in the outcome of the different separation procedures.

Similar to what we observed in mice, we also found that the MS-induced CORT response is readily abolished in rats if the separations are repeated daily, but that the animals’ ability to respond to a novelty stressor depends on the separation context. Genotype and maternal care upon reunion did not affect the desensitization phenomenon, but rather appeared to be associated with stress responsiveness of the pup to an acute novelty stressor.

Materials and methods

Animals

Wistar rats (originally obtained from Harlan, Horst, The Netherlands) and Long Evans rats (originally obtained from Elevage Janvier, Le Genest-St-Ieule, France) were used in this study and housed in our animal facility under a 11:13 h light/dark cycle (lights on at 08:30 h, illumination inside the cage: 20–30 lux, temperature: 20 ± 1°C, relative humidity: 60 ± 10%) and low volume background noise (40 dB). Food (RM3, Special Diet Services, Witham, Essex, UK) and water (containing 0.02% HCL) was ad libitum. Upon arrival males and females were housed in groups of 2 or 3 in macrolon-polycarbonate type IV cages with wire lid; 60×38×20 cm; containing sawdust bedding and tissue, and used for breeding at least after a habituation period of one week.

Animal experiments were approved by the Local Committee for Animal Health, Ethics and Research of Leiden University and carried out in accordance with European Communities Council Directive 86/609/EEC.

Breeding

Two or three females of F1 generation, which were group housed for at least a week, were mated with a male in Type IV macrolon-polycarbonate cages with wire lid. After 10 days, male were removed from the cage and pregnant females were transferred individually to clean cages (macrolon-polycarbonate type III cages with wire lid; 42.5×26.6×18.5 cm) containing sawdust and two sheets of paper towels for nest material. Pregnant females were checked for litters daily at 19:30 h starting from 20 days after the start of breeding. If litters were present, the day of birth was defined as pnd 0 for that litter. On the day after parturition, pnd 1, each litter was culled to 8–10 healthy pups (ratio males:females = 1:1) and remained undisturbed until used in the study.

Maternal behavior

The maternal behavior of each dam was observed and scored for five-60 min periods per day during the first 4 days post partum using a procedure originally described before (Champagne et al., 2003; Myers et al., 1989). Observations were performed at three periods during the light phase (10:00, 13:30, and 17:00 h) and two periods during the dark phase (07:30 and 19:30 h; under 2 × 60 W red TLD-light). The behavior of each mother was scored every 3 min (20 observations per period, 100 observations per day).

We scored the following maternal behaviors: retrieval: the dam retrieves her pups from either a location outside the nest to the nest or from inside the nest to a new location, maternal contact: the dam is in contact with the pups but not nursing or licking, licking and grooming (LG): the dam is licking and grooming either the whole body or specifically the anogenital area of the pup, passive nursing posture: the dam is in a passive posture; she is lying either on her back or side while the pups are nursed, away from nest: there is no maternal contact, nest building: the dam gathers material to a nest side, redistributes nest material, creates nest or changes shape of nest, burying her pups. Finally, arched-back nursing was also measured with a distinction of (i) (passive) low arch: dam in a passive posture where she lays over (some or all of) her pups flat (blanket nursing) or with a low arch. (ii) (active) low arch: the dam is positioned less passively over (some or all of) her pups; her possible other activities are licking pups, moving nest material, self grooming, repositioning pups in the nest, eating etc. (iii) Middle arch: in this position the dam shows a greater arch and her other activities are only pup-oriented (licking pups), (iv) High Arch: in this position the limbs of the dam are extended so the pups have full access to her nipples. We considered as (overall) passive nursing the sum of the passive nursing posture and the (passive) low arch back nursing scores. The other three nursing postures (active low arch, middle arch, high arch) were considered (overall) active nursing (AN).

Other dam non-maternal care behaviors were also observed like eating, drinking water, chasing tail, self grooming, digging, and sleeping. Note that some behavioral categories were not mutually exclusive. For, example, licking and grooming often occurs while the dam is nursing the pups. Other litter conditions were noticed: split litter (pups divided over two positions) and buried pups. We analyzed the percentage of observations in which: 1) the dams displayed each behavior or 2) litters were in a certain condition.

In the result section, we report frequencies of AN (as % of observations) and LG (as % of observations). Note that AN and LG frequencies both include instances where both of the behaviors occurred simultaneously and those instances are quite frequent.
Maternal separation (MS; Fig. 1)

MS occurred at either pnd 3, 4 and 5 or only pnd 5, lasting 8 h each. Litters were randomly distributed over experimental conditions.

Dams’ transfer from the litter (“Dam out”)

At 9:00 h, dams selected for MS were removed from their cage (“home” cage), placed in a cage of the same type and transferred to an adjacent room (“dams’ room”). In the “dams’ room, the environmental conditions were the same except the lighting intensity was higher (illumination inside the cage: 50–60 lux).

Separation procedure

After the dam was relocated to a new cage, litters were kept without any food or water available for 8 h (9:00 to 17:00 h). The home cage was placed on heating pads (33–38 °C; TM 22, Beurer, Ulm, Germany) to maintain the body temperature of the pups. To acquire the desired temperature, heating pads were turned on 30 min prior use.

We used two following separation contexts:

- “Home separation” (HOME SEP; Fig. 1A). The pups remained in their familiar environment (housing room, home cage) together with their littermates.
- “Novel separation” (NOVEL SEP; Fig. 1B). The pups were moved to an adjacent unfamiliar room, with similar conditions as the housing room. Pups were put individually in new clean cages (macronol-polycarbonate type II, which were divided in compartments of 18 × 20 × 14 cm, containing fresh sawdust bedding) and placed on heating pads. The separated pups housed in this unfamiliar novel context, experienced the absence of their dam, the home cage environment and proximal contact with their littermates (isolation).

Reunion (“Dam back”)

At 17:00 h, the pups were returned to their home cage followed by their dams. Dams of separated pups in home and novel contexts were reunited with their litter at the same time.

Control litters

Non-separated (NON SEP) litters remained undisturbed with their dams in the housing room until the time of testing.

Testing: novelty exposure (pnd 5)

We determined the HPA axis responsiveness to a mild stressor at 17:00 h on pnd 5. Pups were removed from their separation environment (home or novel) and either sacrificed immediately by decapitation or placed individually in new clean cages (same type as in the novel separation), containing fresh sawdust. Novelty exposure was carried out in a separate room, the “novelty exposure” room, under similar environmental conditions as the housing room. The cages were placed on heating pads (33–38 °C) to maintain the body temperature of the pups. After 30 min from the onset of the stressor, the pups were sacrificed.

Note that this manipulation is different for pups of the different groups: for the HOME SEP pups (1st HOME SEP, 3rd HOME SEP), it is the first time they experience a novel and unfamiliar cage, for the NOVEL SEP pups it is a relatively familiar manipulation since they are coming from a similar environment. Therefore, the perceived degree of novelty may be different.

Experimental design (Fig. 2)

Litters for the endocrine experiments: in order to minimize inter-litter differences, every treatment group consisted of 4–7 litters and, within each litter; we distributed the pups equally in terms of time point of sacrifice and sex. Number of animals per time point in every treatment group was 8–18.

- Experiment I (Fig. 2A): to determine the effects of repeated separations in different separation context on body growth, ACTH & CORT secretion and maternal care, litters were divided into three treatment groups: single home separation (1st HOME SEP), repeated home separation (3rd HOME SEP), and repeated novel separation (3rd NOVEL SEP). We sacrificed rat pups in three different testing conditions: basal levels (basal), 8 h of separation (separated), 8 h of separation + 30 min of novelty (novelty). A total of 12 litters were used (4 litters for each group with 8 pups in every time point).
- Experiment II (Fig. 2B): to determine the effects of repeated separations in home context on adrenal activity, litters were divided into two treatment groups: single home separation (1st HOME SEP) and repeated home separation (3rd HOME SEP). We sacrificed rat pups in two different testing conditions: basal levels (basal) and 8 h of separation (separated). A total of 8 litters were used (4 litters for each group with 8 pups in every time point).
- Experiment III (Fig. 2C): to determine the effects of repeated separation in home context on ACTH&CORT secretion of another rat strain (Long Evans), litters were divided into two treatment groups: single home separation (1st HOME SEP) and repeated...
home separation (3rd HOME SEP). We sacrificed rat pups in three different testing conditions: basal levels (basal), 8 h of separation (separated), 8 h of separation + 30-min of novelty (novelty). A total of 13 litters were used (6 litters for the 1st HOME SEP group with 16 pups in every time point, 7 litters for the 3rd HOME SEP group with 18 pups in every time point).

Litters for maternal care observations: maternal care measurements were performed for the litters of Experiment I together with some extra litters that were not used in the endocrine experiments: 6 non separated litters, 5 repeatedly separated litters on pnd 3 and 4 in a home (HOME SEP) or novel context (NOVEL SEP). On pnd 3 & 4, for the 3rd HOME SEP/HOME SEP and 3rd NOVEL SEP/NOVEL SEP groups, dams were not in contact with the pups at two time points (10:00 and 13:30) because they were separated, and, therefore, we could not collect maternal care data.

See Table 1 for a synopsis of litters used in the different experimental groups.

**Collection of blood plasma and adrenals**

At the designated time point, the pups were sacrificed by decapitation. Trunk blood from all pups was collected individually in 1.5 ml EDTA-coated microcentrifuge tubes. All blood samples were kept on ice and later centrifuged for 15 min at 13,000 rpm at 4 °C. Plasma was transferred to clean 1.5 ml microcentrifuge tubes. All plasma samples were stored frozen at -20 °C until the determination of ACTH and CORT. After decapitation, adrenals were dissected and snap frozen in isopentane on dry ice and stored at -80 °C until used for Western blotting.

**Measurements**

Body weight (gr) was measured just before every experimental manipulation with an electronic precision scale (MXX-2001, Denver Instrument, Göttingen Germany; readability 0.1 g, linearity 0.2 g). Since the groups were different in birth weight (pnd 1 weight), we calculated the ratio (in %) of the body weight measurements to birth weight.

**Table 1**

<table>
<thead>
<tr>
<th>Exp. group</th>
<th>Exp. I</th>
<th>Exp. II</th>
<th>Exp. III</th>
<th>Maternal care observations</th>
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<tr>
<td>1st HOME SEP</td>
<td>NON SEP</td>
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<tr>
<td>3rd HOME SEP</td>
<td>HOME SEP</td>
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<td>3rd NOVEL SEP</td>
<td>NOVEL SEP</td>
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Note. First separation (1st HOME SEP) or Non separated pups (NON SEP) had no previous history of treatments, third separation animals were exposed to 8 h of maternal separation on pnd 3 and 4 in a home (3rd HOME SEP) or novel context (3rd NOVEL SEP). Note that novelty exposure is different for pups of the different groups: for the HOME SEP (1st HOME SEP, 3rd HOME SEP) pups, it is the first time they experience a novel and unfamiliar cage, for the 3rd NOVEL SEP pups it is a relatively familiar manipulation since they are coming from a similar environment.
ACTH was measured by radioimmunoassay (MP Biomedicals, LLC, NY, USA; sensitivity 10 pg/ml, intra-assay variation 4.1%, interassay variation 4.4%). Samples were determined in a 50% dilution, starting with 25 μl blood plasma. All samples were analyzed in one assay to exclude inter-assay variability.

CORT was measured by radioimmunoassay (MP Biomedicals, LLC, NY, USA; sensitivity 1.25 ng/ml, intra-assay variation, 4.4%, interassay variation 6.5%). Concentrations were determined in duplicate from an extended standard curve (0, 6.25, 12.5, 25, 50, 100, 250, 500 and 1000 ng corticosterone/ml), since we noted that the lower boundary provided by the kit was not sensitive enough to measure neonatal basal plasma concentrations. All samples were analyzed in one assay to exclude inter-assay variability.

Tyrosine hydroxylase (TH) & melanocortin receptor type 2 (MCR-2) protein levels

Adrenals were homogenized in 400 μl lysis buffer (Triethanolamine, NaCl, DOC, SDS, triton-X-100) and protease inhibitor was added to inhibit proteins’ degradation. This lystate was spun down and supernatant was kept and stored in −20 °C. Concentration of proteins present in the supernatant was determined using a Thermo Scientific Pierce BCA Protein Assay. Therefore, a calibration curve (Bovine Serum Albumin in 5 dilutions) was done.

Western blotting was performed, according to a previously described method (Brinks et al., 2007), in duplicate on the supernatant of the homogenized adrenals to measure TH and MCR-2 protein levels. Each sample supernatant was loaded in a concentration of 1 mg/ml (by varying the amount of H2O added to the sample). The samples also included a standard volume of sample buffer and were denaturized at 95 °C (5 min) and subjected to SDS-PAGE.

After electrophoresis, the proteins were transferred to a membrane (blotting) overnight (4 °C, 125 mA). The day after, the blots were blocked in 10 mM Tris–HCl (pH 8.0), 150 mM NaCl and 0.05% Tween 20 containing 5% non-fat dried milk powder and, then, incubated with the primary antibody and the secondary antibody consecutively. For TH, the primary antibody used was rabbit anti Tyrosine Hydroxylase (TH) (AB152) ordered from Millipore in a 1:1000 concentration. The secondary antibody used was goat anti mouse IgG-HRP in a 1:10,000 concentration. For MCR-2, the primary antibody used was mouse anti Melanocortin Receptor-2 (MCR-2) ordered from Chemicon in a 1:1000 concentration. The secondary antibody used was goat anti mouse IgG–HRP in a 1:5000 concentration. For TH, we used liver tissue as negative control and adult rat adrenal tissue as positive control. For MCR-2, we used human skin samples as positive control and water as negative control. Samples were also tested on their α-tubulin levels, as well, to correct for the total amount of protein. The primary antibody used was anti-mouse α-tubulin in a 1:5000 concentration and the secondary antibody used was goat anti mouse IgG–HRP in a 1:10,000 concentration.

After washing of the antibodies, blots were incubated with peroxidase-conjugated antibodies (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoreactive bands were visualized by enhanced chemiluminescence and the blots were exposed to films. The autoradiographs (films) were scanned and optical density (OD) of the TH, MCR-2 and α-tubulin bands were determined using Image J software. The TH and MCR-2 values of the samples were corrected for total protein (α-tubulin); therefore, the ratios between the TH and α-tubulin levels and MCR-2 and α-tubulin levels were calculated. In order to compare samples ran in different gels we used also one sample (“standard sample”) that was loaded in all gels. Group size n = 8 adrenals (from 8 separate animals) per time point of each treatment group were used for the measurements.

Statistical analysis

The results were analyzed by analysis of variance (ANOVA) with the level of significance set at p ≤ 0.05. Where appropriate, simple and interaction main effects were investigated further with subsequent post-hoc comparisons (by Tukey test). The statistical analysis was adjusted for non-equivalent groups when needed. The initial analysis of pups’ measurements included sex as a factor; once it was determined that sex was not a significant factor, data from males and females were pooled. Data are presented as mean ± SEM. The level of significance was set at p ≤ 0.05.

Results

Experiment I

Wistar pups were exposed to repeated MS under different separation contexts with the goal to examine the “desensitization” of the endocrine responses.

Body weight (data not shown)

Repeated measures ANOVA, for the ratio of body weight to birth weight, revealed main effects of time (F1,45 = 4027.35; p ≤ 0.001) and the interaction of time and treatment (F1,45 = 34.36; p ≤ 0.001). The treatment effect was significant in all time points (p ≤ 0.001). On pnd 5, HOME SEP and NOVEL SEP were not different, but both were lighter than the NON SEP (p ≤ 0.001).

HOME SEP vs. NOVEL SEP: The two repeatedly separated groups were followed for body growth in more time points (pnd 3 and pnd 4) than the controls. Therefore, we performed a separate statistical analysis for their comparison. Repeated measures ANOVA revealed main effects of time (F6,180 = 1315.76; p ≤ 0.001), but not of treatment or the interaction of time and treatment. The two groups (HOME SEP and NOVEL SEP) were not different in weight at any time point.

ACTH (data not shown)

ACTH basal levels for naïve Wistars on pnd 5 were 90.08 ± 11.38 (pg/ml). Two-way ANOVA did not reveal any main effects of treatment, time or their interaction. ACTH is not different after a single event of 8 h of MS on pnd 5. Rat pups also did not respond to 30 min novelty if it was implemented immediately after the first or third MS period. There was no effect of treatment on ACTH. However, if repeatedly separated groups were compared separately, ACTH levels of NOVEL SEP pups, after the third MS, were lower than the ones of the HOME SEP pups (p = 0.046).

CORT (Fig. 3)

Two-way ANOVA revealed effects of treatment (F2,63 = 51.54; p ≤ 0.001), time (F2,63 = 17.50; p ≤ 0.001) and their interaction (F4,63 = 10.97; p ≤ 0.001). The interaction of time and treatment (F2,63 = 51.54; p ≤ 0.001) or to the combination of MS with novelty desensitization was set at p ≤ 0.001, as well as the interaction of time and treatment. The two groups (HOME SEP and NOVEL SEP) were not different in weight at any time point.

1st HOME SEP

Naïve rat pups responded on pnd 5 with a three-fold increase of CORT to 8 h-MS (p ≤ 0.001) or to the combination of MS with novelty exposure (p ≤ 0.001). The novelty exposure did not, however, create an additional increase over the MS levels.

1st HOME SEP vs. 3rd HOME SEP/3rd NOVEL SEP

If on pnd 3 and 4 pups were separated from their mother, on pnd 5, CORT levels, in response to the third period of MS, was not altered regardless if the MS happened in a home or a novel context (“separated” vs. “basal” levels in 3rd HOME SEP or in 3rd NOVEL SEP). CORT levels after MS or after the combination of MS and novelty (“separated” and “novelty” levels) were lower for the repeatedly
levels upon reunion after 8 h-MS for the second time (for the dams of NON SEP (p≤0.001) and HOME SEP: p=0.003, for the dams of NOVEL SEP: p≤0.001), as compared to before separation levels. Interestingly, the effect of MS on maternal care did not habituate. Before the second MS the dams of NOVEL SEP pups displayed the least LG (p=0.011 vs. NON SEP). When they were reunited with their pups, after the second MS, dams of separated pups in both contexts displayed higher maternal care compared to the controls (for the dams of HOME SEP: p=0.038, for the dams of NOVEL SEP: p=0.005).

Active nursing (Fig. 5)
Repeated measures ANOVA reveal main effects of time (F(19,475) = 16.20; p≤0.001) and the interaction of time and treatment (F(38,475) = 7.37; p≤0.001) on AN (Fig. 5A), and of treatment (F(2,23) = 3.62; p=0.041) on the overall mean of AN across the first postnatal days (Fig. 5B). The time effect was significant for all groups (for the dams of NON SEP pups p=0.008, of HOME SEP pups p≤0.001 and of NOVEL SEP pups p≤0.001). Dams of HOME SEP pups, compared to the controls (NON SEP), displayed different levels of AN across the individual time points (p=0.042) and ended-up with less overall mean across pnd 1–4 (p=0.039). We conducted further post hoc analysis for the post separation hour observation period on pnd 3 and pnd 4. On both days, there is no treatment effect before or after the first 8 h-MS indicating there is no post-reunion increase of AN in both days.

Experiment II
Because the endocrine blood levels imply changes in adrenal sensitivity to subsequent stressors upon repeated MS in the home context we have examined two biomarkers that may give an indication how adrenal function is affected.

TH (Fig. 6A)
Two-way ANOVA revealed effects of time (F(1,31) = 5.43; p=0.027) and the interaction of treatment and time (F(2,31) = 4.52; p=0.009). If, on pnd 3 and 4, pups were separated from their mother in a home context, on pnd 5, a 65% reduction in TH level is displayed compared to naïve pups (basal levels 1st HOME SEP vs. 3rd HOME SEP; p=0.014). The reduction is followed by a four-fold increase in response to the third period of MS (p=0.006) ("separated" vs. "basal" levels in 3rd HOME SEP).

MC2 receptor (Fig. 6B)
Two-way ANOVA revealed effect of the interaction of treatment and time (F(2,31) = 6.09; p=0.020). If, on pnd 3 and 4, pups were separated from their mother in a home context, on pnd 5 receptor levels display a 50% reduction compared to naïve pups (basal levels 1st HOME SEP vs. 3rd HOME SEP), but it was not significant (p=0.055). The reduction is followed by an increase (three-fold) in response to the third period of MS (p=0.047) ("separated" vs. "basal" levels in 3rd HOME SEP).

Experiment III
In order to assess possible strain differences the effect of repeated MS in home context, Long Evans rats was used.

ACTH (data not shown)
ACTH basal levels for naïve Long Evans on pnd 5 were 35.56 ± 1.96 (pg/ml). Two-way ANOVA revealed effects of treatment (F(1,110) = 80.51; p≤0.001), time (F(2,110) = 8.25; p=0.002) and their interaction (F(2,110) = 9.25; p≤0.001).

1st HOME SEP. Naïve rat pups on pnd 5 responded with an ACTH increase to the combination of MS with novelty exposure (p≤0.001;
50% increase). The novelty exposure creates an additional increase over the MS levels \((p = 0.030)\).

3rd HOME SEP. If on pnd 3 and 4 rat pups were separated from their mother, on pnd 5 ACTH levels, in response to the third period of MS, were decreased (“separated” vs. “basal” levels in 3rd HOME SEP; \(p = 0.038\)) and they even became lower than basal levels of naïve pups of pnd 5 \((p = 0.002; 35\% \text{ decrease})\).

1st HOME SEP vs. 3rd HOME SEP. ACTH levels, either basal, after MS or after combination of MS and novelty levels of repeatedly separated pups are reduced compared to the respective values of the singly separated pups \((p = 0.031 \text{ for “basal” values}, p \leq 0.001 \text{ for “separated” values}, p \leq 0.001 \text{ for “novelty” values})\).

CORT (Fig. 7)
Two-way ANOVA revealed effects of treatment \((F_{1,110}=103.63; \ p \leq 0.001)\), time \((F_{2,110}=86.12; \ p \leq 0.001)\) and their interaction \((F_{2,110}=37.76; \ p \leq 0.001)\).

1st HOME SEP. Naïve rat pups on pnd 5 responded with a CORT increase to 8 h-MS \((p \leq 0.001; \text{ four-fold increase})\) or to the combination of MS with novelty exposure \((p \leq 0.001; \text{ five-fold increase})\). The novelty exposure resulted in an additional increase over the MS levels \((p = 0.004)\).

3rd HOME SEP. If, on pnd 3 and 4, rat pups were separated from their mother, CORT levels were not altered on pnd 5, in response to the third period of MS (“separated” vs. “basal” levels in 3rd HOME SEP). The HOME SEP pups displayed an increase in CORT over basal levels on pnd 5 if they were additionally exposed to novelty after MS \((p \leq 0.001; \text{ two-fold increase})\), over separated levels \((p = 0.015)\) and over CORT levels of naïve pups \((p \leq 0.001)\).

1st HOME SEP vs. 3rd HOME SEP. CORT levels after MS or after combination of MS and novelty (“separated” and “novelty” levels) were reduced in the repeatedly separated pups compared to the respective values of the singly separated pups \((p \leq 0.001 \text{ for both comparisons})\).
Discussion

The present study was designed to extend previous data on the immediate outcome of repeated daily maternal separations on the HPA axis from the mouse to the rat. Our previous studies had revealed the remarkable phenomenon that these repeated daily maternal separations in mice resulted in a desensitization of the CORT-response to the separation procedure itself, while the pups continued to respond to an acute novelty stressor (Enthoven et al., 2008). In this study we demonstrate that also the rat readily adapts to repeated daily separations. Specifically, we show that after 8 h of maternal separation CORT levels increased markedly in the 5 day old Wistar and Long Evans rat pup. However, if the pups had been exposed also the two preceding days to 8 h-MS this rise in CORT was abolished. The adrenal desensitization induced by the homotypic daily repeated 8 h-MS was not affected by genotype, separation environment or maternal care upon reunion. However, repeatedly home separated pups show a subtle enhancement of the adrenal CORT response to a heterotypic acute novelty exposure. Additionally, the adrenal TH and MCR-2 protein content was higher than observed in pups exposed to solely a single 8 h-MS. This increased stress response to novelty after...
repeated episodes of home rather than novel 8 h-MS was observed in both genotypes, with the Long Evans pups displaying a relatively higher stress-response compared to the Wistar pups.

**Differential ACTH and CORT time course during maternal absence**

In line with expectations, CORT levels following a single 8 h-MS on pnd 5 were significantly increased relative to basal levels. Both in rats and mice, a single prolonged period of maternal separation of ≥8 h during the SHRP is needed for a large increase of CORT (Enthoven et al., 2008; Rosenfeld et al., 1992b; Schmidt et al., 2004; Stanton et al., 1988; Walker et al., 1991). As far as ACTH levels are concerned, in both rat strains, they were not increased after 8 h of maternal absence. However, in mice it is clearly shown by Schmidt et al. that during the course of a 24 h-MS, ACTH levels are increased between 8 and 12 h from the onset of separation (Enthoven et al., 2008; Schmidt et al., 2004). Hence, the lack of ACTH response in rats observed in the present study is probably a rat-mouse difference (Schmidt et al., 2002), since our ACTH findings are actually in agreement with other studies using rats. Walker et al. (1991) showed already at 30 min of maternal absence a rise in ACTH level, which then returned to baseline after 8 h of separation possibly by either depletion of the pituitary ACTH stores or glucocorticoid feedback inhibition of ACTH release. That the feedback inhibition already operates in newborn rats at pnd 5 was shown by injecting CORT or dexamethasone to naïve, deprived or adrenalectomized pups (van Oers et al., 1998a; van Oers et al., 1998b; Walker et al., 1990; Walker et al., 1986). Accordingly, a single 8 h-MS period permits a robust CORT increase, while at that time the ACTH response is suppressed. Our findings (in Long Evans) argue against the depletion of ACTH from the pituitary stores since we observed increased ACTH levels in response to 30 min of novelty following a single 8 h-MS.

**Desensitization of CORT response to maternal absence: a robust phenomenon**

The interesting novel aspect of our previous studies with mice was that upon repeated separations the pup readily adapts to maternal absence and as a result the separation-induced increase in CORT does not occur any longer. We report here that also in the newborn rat the basal CORT response to daily repeated 8 h-MS is abolished, both in Wistar and Long Evans 5 day old rat pups while their adrenals are able to respond to a single 8 h-MS. This habituation or adaptation of the pup to the experience of repeated MS was not due to a shift in the time course of the MS-induced CORT response (Enthoven et al., 2008). Also, repeated MS in a home context does not result in a cumulative CORT response irrespective of the duration (from 15 min to 8 h) of maternal absence (D’Amato et al., 1992; Rosenfeld et al., 1992b). It should be noted, however, that these authors did not report desensitization of the adrenal response to repeated maternal absence as was shown in Enthoven’s study for the mouse, and here in two rat strains. However, the current study differs from the mouse study in one aspect. While Enthoven et al. found a persistent decrease in basal CORT release after two daily separations; we did not see this decrease consistently in the two rat strains. Possibly this difference could be explained by small circadian fluctuation present already in that age (Enthoven et al., 2008). Enthoven measured basal levels at 9:00 h (16 h after previous separation) and we did at 17:00 h (24 h after the end of the previous 8 h-MS). However, the circadian fluctuation of CORT and the potential influence of early life experience have not been reported in neonate rodents so far earlier than the third week of life (Ader, 1969).

In the present study it was shown that if the pups are housed in a novel environment isolated from their littermates and deprived from all familiar cues during the 8 h-MS, the desensitization of the adrenal still happens. In the rat, comparable studies have been performed, however with variable outcomes. Studies using shorter periods of maternal absence in a novel environment (1 hour x 8 days) showed sensitization of the adrenal response to this procedure (Knuth and Etgen, 2005; McCormick et al., 1998). Other studies (15 min x 8 days, 1 h x 3 days) did not (McCormick et al., 1998; Vazquez and Akil, 1992). Thus, taken together, the duration and frequency of the daily separations might influence the outcome. Overall, the fact that adrenal corticosterone secretion under two widely different conditions displayed desensitization to maternal absence demonstrated the robustness of this phenomenon.

**Enhanced adrenal sensitivity to stress after repeated maternal absence**

While it is now well-established that the adrenal readily desensitizes to homotypic repeated maternal separations, it is also well-established that MS causes increased adrenal sensitivity to heterotypic stressors and exogenous ACTH. This enhanced adrenal sensitivity to a heterotypic stressor needs at least 8 h-MS to develop and is profound after 24 h of separation (Levine et al., 1991; Okimoto et al., 2002). Previously, it was reported that despite the rapid adaptation of the HPA axis to daily repeated maternal absence, the CD1 mouse pup stayed on alert and retained the ability to respond to stressors with an increase in ACTH and CORT levels (Enthoven et al., 2008), which indicates a large steroid production capacity and argues against ACTH depletion. Wistar pups, when exposed to novelty for 30 min immediately after the separation, show no ACTH response irrespective of whether the separation was the first (the Long Evans did) or the third. Also it did not matter whether the separation context was familiar (home cage) or unfamiliar (novel cage). However, a subtle CORT response to the novelty stressor still occurred despite desensitization to the homotypic repeated maternal separation both in Wistar and Long Evans pups.

Interestingly, in Wistars, repeated “home separation” vs. “novel separation” had a different outcome on the response to the subsequent 30 min novelty stressor. Rat pups separated for 8 h on 3 consecutive days in a novel environment apparently adapted to this condition because the additional 30 min of novelty stress (which in this group could be considered a homotypic stressor) could not trigger a response anymore. Apparently, the previous experience of maternal absence in a novel environment not only did prepare the pups for maternal absence but also for the experience of “novelty” itself. Both Wistar and Long Evans pups, in the “home separation” condition, still show a CORT response. Moreover, Enthoven et al. (2008) reported that a daily repeated exposure to a combination of 8 h home separation + additional 30 min novelty was not able to attenuate the response to a subsequent 30 min novelty exposure itself. This suggests that only if the environment, in which the novelty stress is experienced, is intrinsic to the housing conditions during separation, then the novelty stressor can be considered “homotypic” and the response to this stressor is abolished.

These observations underscore previous research showing that the neonatal adrenal function is altered after maternal absence (Okimoto et al., 2002). To further examine the altered adrenal function we measured ACTH receptors (MC2-R) in the neonate adrenals in an attempt to explain why adrenal sensitivity to the novelty stressor was increased in the face of unaltered circulating ACTH levels. We report that MCR-2 protein content was reduced 24 h after the second home separation, but enhanced after a third separation interval. This would predict enhanced responsiveness to exogenous ACTH as had been shown before, immediately after prolonged 24 h maternal separation (Okimoto et al., 2002; Rosenfeld et al., 1991). We also measured TH protein level as an indirect measure of medullary-catecholamine response to maternal absence (Okimoto et al., 2002). We show that the third home separation induced an increase of TH protein levels over the basal levels (which were reduced 24 h after the second period of MS). Apparently, the increase in adrenal activity develops after repeated 8 h episodes of maternal absence (in our study) or after prolonged 24 h of maternal absence (Okimoto study) and not after a single 8 h episode. These experiments provide some insights into the
mechanistic underpinning of enhanced adrenal sensitivity towards heterotypic stressors, which are in line with the elegant experiments of singly separated pups subjected to chemical sympathectomy (Walker, 1995).

**Maternal care upon reunion**

First, we would like to underline a possible methodological constraint in all the above studies (including the present). AN pnd 1–4 (alone or together with LG) in our study was 56% (of the observations), which is in the same range of previous reports on pnd 1–4 [49% (Pryce et al., 2001b), 60% (Macri et al., 2004) and] higher than previously reported values taking into account longer periods (pnd 1–8: 40%; [Pryce et al., 2001b), 47% (Champagne et al., 2003); (Long Evans), 43% (Macri et al., 2004)]. For LG we reported a mean of 6% (of the observations), which is approximately 2 fold lower than previously reported [14% (Pryce et al., 2001b), 12% (Champagne et al., 2001)]. Strain differences, culling of pups, litter size and litter's sex ratio could be important factors influencing baseline levels of LG in the various studies (Calessens et al., 2011). However, even the same research group, using the same strain of rats with small changes in the experimental procedure, reported large differences in the pnd 1–4 LG scores [approximate mean values: 11% (Caldji et al., 1998), 12% (Champagne et al., 2001) 14% (Liu et al., 2000), 15% (Champagne et al., 2003)].

The question raised in this study was to investigate to what extent maternal care the pups received, after reunion with the dam, could explain the effect of repeated separations on the HPA axis. It is believed that maternal absence can affect adrenocortical cell differentiation and function (Rosenfeld et al., 1992a) and this effect seems related to feeding rather than tactile stimulation provided by the dam. Feeding acts as an inhibitory factor to the neonate's baseline and stress-induced adrenal activity (Schmidt et al., 2006; Suchecki et al., 2004; van Oers et al., 1998b). Therefore, one other mechanism for the CORT desensitization to maternal absence could be related to the pattern of tactile stimulation (in the form of LG) or food intake (nursing) experienced by the pups after reunion with their dam.

In the current study we observed the following: (i) home and novel separated groups received overall similar care which, in the case of AN, was reduced compared to that of the controls; (ii) post separation bouts of LG were higher for novel separated than for home separated pups, (iii) separation did not induce an increase in AN upon reunion. These findings suggest that for some maternal behaviors (LG) the dam compensates upon reunion. However, for others (AN) she does not compensate, resulting in a lack of care. This altered pattern of care after MS might have led to a greater suppression of the HPA axis and result in blunted stress activation after the third 8 h-MS. However, differences in maternal care for pups experiencing MS in familiar or unfamiliar contexts, were not reflected in the CORT response to repeated absence. However, those differences in maternal care may explain the differences between separation contexts in the response to a heterotypic stressor.

Plotcky and colleagues have argued that maternal care is the major factor driving the effects of MS. Their arguments were based on experiments showing that dam’s exposure to foster litters while her pups where in MS, did not lead, in adulthood, to enhanced HPA axis’ responsiveness for the separated animals (Huot et al., 2004). In contrast, other investigators, using a similar design, observed that dams are actually able to distinguish the separated pups from non separated pups, by their altered vocalization behavior, leading to the post separation bouts of maternal care in favor of the separated pups (Zimmerberg et al., 2003a; Zimmerberg et al., 2003b). Cumulatively, the maternal mediation hypothesis maybe is not the sole mechanism explaining the effects of early life stress in the HPA axis activity, but it is proposed that environmental adversity and the maternal repertoire both underlie the lasting alteration on the offspring’s HPA response (Macri and Wurbel, 2006). Finally, an interesting possibility is that the response of the dams upon re-union might have worked as a cue used by the pups to predict maternal return after a separation experience.

**Mechanism of repeated maternal separations: neonatal learning**

The mechanism underlying the effects of repeated separations has been explored previously. It is known that food deprivation leads to increased adrenocortical output and sensitivity to stressors (Dallman et al., 1999). It was therefore reasonable to assume that metabolic factors are involved. However, since in the previous mouse study the rise in ghrelin and the decrease in glucose were identical after each separation we could rule out involvement of metabolism (Enthoven et al., 2008).

We also could eliminate enhanced glucocorticoid feedback as potential mechanism for the lack of CORT response to repeated separations, since a glucocorticoid antagonist that profoundly enhanced the CORT response to the 1st separation failed to do so after the third. Small effects of mineralocorticoid receptor antagonists were found though suggesting the involvement of higher brain regions in the effect of repeated separations (Enthoven et al., 2008). The involvement of higher brain regions raises the possibility that maternal separation could have a key role since recent reports showed one-trial odor learning in this age (Moricaud and Sullivan, 2006). It is important to underline that the odor system is fully developed at this time. When the dam is present in the nest, adversity towards the pups will be negligible and attachment to the dam care-giver is expected to develop irrespective the quality of maternal behavior (Raineki et al., 2010). Until approximately pnd 10, pups exhibit odor preference to novel odors even when they are paired with negative stimuli (Sullivan et al., 2000a). This odor preference is associated with enhanced co-activation of the locus coeruleus–olfactory bulb pathway (Sullivan et al., 2000b). In the post-sensitive period, odor-avoidance behavior appears and is associated with neural processes in amygdala and piriform cortex (Sullivan et al., 2000a). Interestingly, during the “sensitive” period when the dam is away, the odor avoidance neuronal system is activated prematurely and aversive memories can be formed as long as the CORT levels are elevated in blood and amygdala (Moricaud and Sullivan, 2006; Moricaud et al., 2006).

In our experiment, the pups were at an age (pnd 3–5 during the SHRP) that permitted formation of memories only during long-term absence of the dam. After being separated from their mothers for the first time, the pups may have learned to predict the return of the mother and thus the reinstatement of maternal care. In other words, the pups previously separated do not respond to the homotypic separation itself (habitation), but do respond to the heterotypic stressor (30 min of novelty). This notion calls for study of brain areas involved in processing of novel information for memory storage. The PVN c-fos expression data in Enthoven’s experiments support this line of reasoning (Enthoven et al., 2008), and recent findings from our laboratory have demonstrated particularly in the amygdala a rise in c-fos expression if MS rats were exposed to an heterotypic stressor (Daskalakis et al., 2009). As mentioned before also maternal cues might have helped in the potential contextual associations. However, we have to be cautious since there is no reported evidence yet of contextual fear learning the first week of life apart from the studies of odor fear learning. The possibility that the odor cues of the familiar or novel context are more important still remains to be tested.

**Conclusion**

Taken together, the current study shows that the effect of repeated separations on the HPA axis activity previously observed in mice can be generalized to rats (Enthoven et al., 2008). To explain this we favor the reasoning that the newborn rats readily learn to predict the return of the dam after the first experience of 8 h absence irrespective of whether the pups are housed in the home or the novel environment.
This adaptation or habitation of the pup to maternal absence manifests itself in the desensitization of the adrenocortical CORT output normally observed after the first separation. It occurs irrespective of rat strain and separation context, while metabolic factors and maternal care upon reunion do not seem to be implicated. Following maternal absence the pups become more sensitive to heterotypic stressors on the adrenal level. We propose that the protocols employed in MS studies should be standardized because the current data predict a different outcome on stress responsiveness to an acute novel stressor depending on whether the pups were separated in home vs novel environments. It would be of interest in future studies to test that the hypothesis that these variations in early life experience have different outcomes for brain function and behavior in adulthood.

Conflict of interest

The authors have no conflict of interest to report.

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