Nurturing nature : testing the three-hit hypothesis of schizophrenia

Daskalakis, N.

Chapter 3

Beyond maternal absence: evidence for the role of peers and non-shared stressful experience in mediating the development of a fearful phenotype

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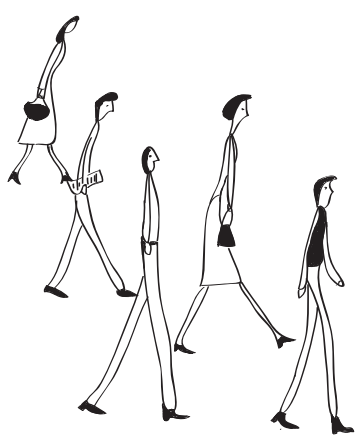

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Abstract

During the first postnatal-days (pnd) rodents show hypo-responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis to mild stressors and fail to display aversion to noxious stimuli. Maternal-separation (MS) disrupts stress hypo-responsiveness causing elevated basal and stress-induced corticosterone (CORT) levels that, in turn, can initiate amygdala-dependent fear aversion in the neonate. However, if MS is repeated, the newborn's HPA-axis ceases to respond to subsequent MS, but continues to respond to acute mild stressors. Here we test the hypothesis that it is the stressful experience of peer separation during repeated MS rather than maternal absence per se which programs an adult fearful phenotype.

Wistar rat pups were exposed to either: (i) "home-separation" (i.e. HOME SEP); during daily 8h absence of the dam, starting pnd 3 the pups remained together in the home cage; (ii) "novel-separation" (i.e. NOVEL SEP); the same separation procedure, but now the pups were individually housed in a novel environment. Non separated individuals were used as controls.

We measured stress-induced amygdala c-Fos mRNA expression, and ACTH and CORT plasma levels in both neonates (pnd5) and adult (pnd 240) offspring. We also assessed juvenile (pnd 30) social play and peri-pubertal (pnd 55) spatial memory.

Compared to HOME SEP rats, the NOVEL SEP rats showed enhanced stress-induced amygdala c-Fos mRNA expression and ACTH-release on pnd 5, while CORT-secretion was attenuated. NOVEL SEP juvenile rats also displayed deficits in social play but intact spatial memory in the peri-pubertal phase. As adults, NOVEL SEP offspring displayed more freezing when exposed to a contextual fear paradigm relative to the HOME SEgroup. Taken together, these findings suggest that the neuro-endocrine phenotype imposed by novel-separation persisted from early-life into adulthood with enhanced central stress-system activity and hypo-responsive adrenal CORT-secretion. In conclusion, an adult fearful phenotype linked to amygdala priming develops if individual pups are repeatedly isolated from peers in a novel environment, while away from the dam. These findings highlight the role of non-shared stressful experiences of the pups in the programming fearfulness rather than maternal absence per se.

1. Introduction

Rodents have postnatally a stress hyporesponsive period (SHRP) and only during maternal absence corticosterone (CORT) secretion slowly rises in the neonate, while stress responsiveness and avoidance behaviors are enhanced until the dam returns [1-4]. Recently, it was found that the activity of the hypothalamic-pituitary-adrenal (HPA) axis of pups readily adapts to repeated maternal separations (MS) [5, 6]. Already from the second episode of maternal absence, CORT levels no longer rise as if the pup has learned to predict the return of the dam [6]. However, in spite of this rapid adaptation to repeated MS that occurs irrespective of the degree of novelty in the separation context,

post reunion maternal care and genotype, the pup's stress system stays on alert; mild acute stressors continue to trigger an enhanced HPA-axis response and the stress hyporesponsive period (SHRP) remains seriously disrupted [5, 6].

We have also shown previously that while the separation context is not affecting the pup's adaptation to maternal absence, it has profound consequences for other aspects of the stress response. For instance we showed that if the neonates are housed individually in a novel cage during MS, the outcome is strikingly different than when the pups remain housed as a group in the home cage [5]. In the current study we aim to extend these findings by further investigating the impact of the MS context on behavioral and endocrine stress responsiveness as well as on the development of the amygdala fear aversion pathway. The study of the latter is important in the context of early-life stress since previous findings showing that the amygdala is hypo-active during the first 10 days of rodent life, allowing attachment of the neonate to the mother [7]. However, if the amygdala fear pathway is activated by early-life stress through increased circulating corticosterone (CORT), aversion learning develops prematurely [8]. Such a stressful early-life experience persistently influences adult contextual fear responses, possibly by programming the amygdala fear pathway. [9-12].

In the first experiment, one group of pups (cohort 1) was exposed to repeated daily 8h-MS starting on postnatal-day (pnd) 3 and 4. During MS, pups remained either in the home context (home-separation), or they were removed from the home cage and housed in isolation from peers (novel-separation). We assessed the lasting impact of these different separation contexts after a 24h post-reunion interval on stress-induced amygdala c-Fos expression and HPA-axis activity in neonates (pnd 5). In the second experiment, another group of pups (cohort 2) were exposed to repeated MS on pnd 3, 4 and 5 and were tested for their ability to engage in social play on pnd 30 (juveniles) as well as ability to form short-term spatial memory at pnd 55 (peri-pubertal period). In the third experiment, the adult offspring (pnd 240) from cohort 2 were also subjected to a contextual fear conditioning paradigm where amygdala c-Fos mRNA expression levels as well as behavioral and endocrine HPA-axis responses were measured.

We report that the stressful experience of isolation from peers and novelty exposure during MS rather than experiencing MS per se is associated with the priming of the amygdala fear pathway with enduring consequences for responsiveness of the stress-regulating system and related behaviors.

2. Materials & Methods

2.1 Animals.

Wistar rats (originally obtained from Harlan, Horst, The Netherlands & Taconic Europe, Ejby, Denmark) were used in this study and housed in our animal facility under an 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 3-4 in Type IV macrolon-polycarbonate cages (L60 x W38 x H20 cm) with wire lid. Each cage contained sawdust as bedding and tissue. These cages were also used for breeding following a one-week habituation period.
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.

2.2 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 cage. After 10 days, the male was removed from the cage and pregnant females were transferred individually to clean cages (Type III macrolon-polycarbonate cages with wire lid; L42.5 x W26.6 x H18.5 cm) containing sawdust and two sheets of paper towels for nest material. Pregnant females were checked for litters daily at 19:30h 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 (males:females = 1:1) and remained undisturbed until used in the study.

2.3 Early-life experience.
Litters were randomly assigned to the experimental conditions.
2.3.1 Non separated (NON SEP)
Non-separated litters remained undisturbed with their dams in the housing room until the time of testing.
2.3.2 Repeated Maternal Separation (MS) in home context (HOME SEP) or novel context (NOVEL SEP)
The procedure has been described before [5]. Depending on the experiment, MS was performed either on pnd 3 & 4 (cohort 1), or pnd 3,4 & 5 (cohort 2). Each MS period lasted 8h. The procedure was divided in 3 steps:
2.3.2.1 Dams' transfer from the litter ("Dam out")
At 9:00h, dams selected for MS experiment were removed from the "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 that the lighting intensity was slightly higher (illumination inside the cage: 50-60 lux).
2.3.2.2 Maternal separation contexts
After the dam was relocated to a new cage, litters were kept from having access to food and water for 8h (9:00 to 17:00h). 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 30min prior use.
We used two following separation contexts:
- Home-separation (HOME SEP). Following removal of the dam, pups were left untouched and remained in their familiar environment including housing room as well as home cage containing all the littermates. Therefore HOME SEP pups experienced the absence of their dam but not of their home cage environment, which includes proximal contact with their littermates.
- Novel-separation (NOVEL SEP). Following removal of the dam, pups were also removed from the home cage and transferred to an adjacent unfamiliar room, which had similar conditions as the

housing room. In addition to being transferred to a new room, pups were also transferred to a new clean cage (Type II macrolon-polycarbonate, which were divided in compartments of L18 x W20 x H14 cm, containing fresh sawdust bedding) and placed on heating pads. Note that as opposed to the HOME SEP context, pups were placed individually into novel cage and had no physical access to their respective littermates. Therefore NOVEL SEP pups experienced the absence of both their dam and their home cage environment, which includes proximal contact with their littermates.

2.3.2.3 Reunion ("Dam back")

NOVEL SEP pups were returned to their home cage at 17:00h. Immediately thereafter, dams of both HOME SEP and NOVEL SEP pups were reunited with their respective litters.

2.4 Cage cleaning – Weaning

From pnd 1-10 cages were not cleaned. From pnd 11, the cages were weekly changed. On pnd 21, the pups were weaned from their dams; males and females were separated. The males of the same litters were housed together in groups of 3-4 in Type IV cages.

2.5 Experimental design (Fig. 1).

We used two cohorts of rats: in Cohort 1, we assessed the immediate effects of MS context, in cohort 2 we assessed the mid and long-term effects around adolescence and in adult age. Blood samples and brain tissue of neonates and adult rats were examined.

2.5.1. Cohort 1 – Experiment I: Immediate impact of MS context (pnd 5)

2.5.1.1 Objective – Immediate outcome measures

We sought to determine the immediate neuro-endocrine responses to stress depending on the history of different MS context. Litters were divided in litters with history of home-separation the previous two days, pnd 3 & 4, (HOME SEP), and litters with history of novel-separation the previous two days, pnd 3 & 4, (NOVEL SEP). NON SEP litters were used to set the baseline of the outcome measures (Fig. 2; represented as dotted line). In total we used 4 litters per group.

We measured ACTH and CORT in the blood and c-Fos m-RNA in the amygdala in two conditions: at basal resting (basal) and after 30min of novelty-stress (novelty).

2.5.1.2 Blood sampling in neonates

We determined the HPA-axis responsiveness to a 30min novelty-stress consisting of exposing pups individually to a novel cage (same type as in the NOVEL SEP experiments) containing fresh sawdust. The timeline is as follows: at 17:00h, pups were removed from their nest and either sacrificed immediately by decapitation or immediately following 30min novelty-stress. Note that 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.

Trunk blood from all pups was collected individually in 1.5 ml EDTA-coated microcentrifuge tubes on ice (see below for subsequent blood samples handling). After decapitation, whole brains were snap-frozen in isopentane on dry ice and stored at -80°C until used for in situ hybridization.

2.5.1.3 Radioimmunoassays

Blood samples were kept on ice and were later centrifuged for 15min at 13000 rpm. at 4°C. Plasma was transferred to clean 1.5 ml microcentrifuge tubes. Plasma samples were stored frozen at 20°C until the determination of ACTH and CORT.

The hormone assays were performed as previously described [6]. ACTH (pg/ml) was measured

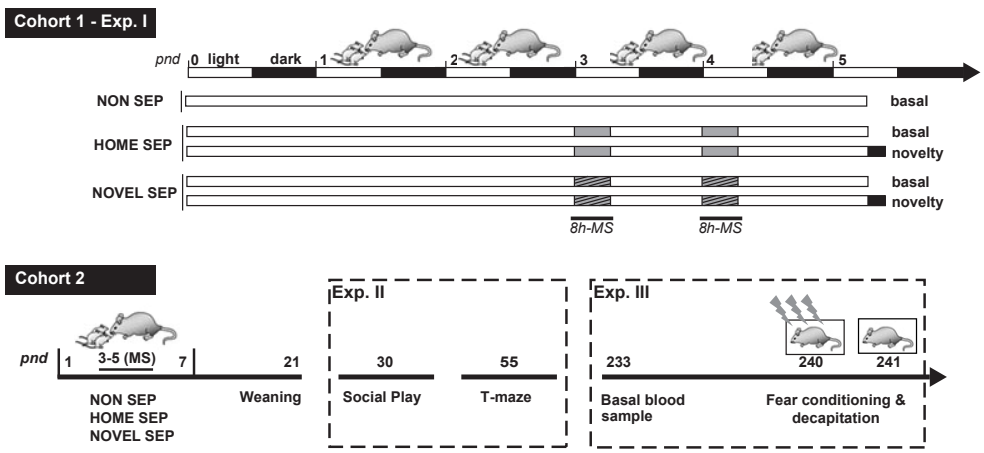

Figure 1. Graphical representation of the experiments. Cohort 1-Experiment 1 (immediate impact of MS context): Time bar from postnatal-day (pnd) 0 to 5; within each day in white is the light period of the light cycle and in black the dark period. Each white bar under the time bar represents different conditions of treatment in relation to the time of sacrifice. Within every white bar there are boxes indicating the experimental manipulations; gray box represents 8h of maternal separation (8h-MS) in home context, gray box with black stripes represents 8h-MS in novel context and black box represents 30 min of novelty exposure. Treatment groups: non separated (NON SEP) had no previous history of treatments, repeatedly separated groups were exposed to 8h-MS on pnd 3 and pnd 4 in a home context (HOME SEP) or novel context (NOVEL SEP). Maternal care was observed in the pre & post reunion periods (depicted with the design of the dam with the pups). Cohort 2: Time line of the longitudinal study. Litters were divided to three treatment groups: non separated (NON SEP), history of home-separation on pnd 3,4 & 5 (HOME SEP) and history of novel-separation on pnd 3,4 & 5 (NOVEL SEP). Maternal care was observed during the first postnatal week. Weaning happened at pnd 21. In Experiment II (mid-term impact of MS) we measured social play at pnd 30 and T-maze spontaneous alternation at pnd 55. In Experiment III (long-term impact of MS) we did a basal blood sampling at pnd 233 and acquisition and retrieval of fear conditioning, blood sampling & decapitation at pnd 240-241.

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 25µl blood plasma. CORT (ng/ml) 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 basal plasma concentrations. All samples were analysed in one assay to exclude inter-assay variability.

2.5.1.4 Amygdala c-Fos mRNA expression

Poly-L-Lysine coating: Uncoated frosted microscope glass slides were put for 6min in autoclaved DepC water, after which they were put for 6min in absolute EtOH. Thereafter they were put for 15min in a 110°C oven. The last step was for the glasses to be put in 0.01% poly-L-lysine dissolved in autoclaved DepC water. The slides were dried under the hood overnight in RNAse free boxes.

Cryosectioning: The neotate brains were cut with skull on Leica CM 1900 at -13°C in the coronal

plane at various levels (prefrontal cortex, PVN, anterior hippocampus). The slices were cut 16μm thick. Sections were thaw-mounted on poly-L-lysine coated slides., air-dried and kept at -80ºC. Three brain sections were put on each slide. For coordinates we used a stereotaxic map [13]. When cutting the brains, we consulted photos of an in-house photo-database (Cresyl-violet strained neonate brains; Long Evans pnd 7; Leica DM 6000).

Cresyl Violet staining: For every section to be used for in situ hybridisation, the subsequent section was kept for cresyl violet staining. This was in order to confirm that section was in the desired coordinates. For this staining we used the following protocol. Slides with sections were thawed. Sections were fixed by putting the slides for 30min in 4% paraformaldehyde solution (adult brain sections) or 4% paraformaldehyde/0,5% gluteraldehyde solution (neonate brain sections). After this, slides were rinsed in distilled water, immersed for 10min in Cresyl Violet solution (0.5%). Afterwards, slides were rinsed for 20sec in 50% acid EtOH and then were dehydrated in increasing concentrations of EtOH (70%, 80%, 100%, 100%, 2min/concentration). Last, slides were immersed in Xylene for 2min, then 4 drops of DPX were placed on the glass and a coverslip was used to seal the slide. The slides were air dried under the hood overnight.

In Situ hybridization: In order to visualize c-Fos mRNA expression, in situ hybridizations with ^{33}phosporus-labeled riboprobes were performed according to a previously described protocol [14]. Briefly, the neonate sections in a 4% paraformaldehyde/0.5% gluteraldehyde solution for 60min at room temperature and rinsed twice in PBS.

The tissue was permeabilized via immersion in 0.36% HCL with proteinase K [1 μg/ml in 0.1 m Tris (pH 8.0)] at 37 ºC for 10min. After a brief rinse in diethyl pyrocarbonate-treated water, brain sections were acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10min at room temperature and finally rinsed twice in 2x SSC. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol and air dried.

Sections were saturated with 100μl of hybridisation buffer (25 mM Tris-HCl (pH 7.4), 50% formamide, 350 mM NaCL, 1.2 mM EDTA (pH 8.0), 1x Denhardt's, 0.1 mg/ml yeast tRNA, 0.1 mg/ml herring sperm DNA, 10% dextran sulfate, 100 mM dithiothreitol, 0.1% SDS and 0.1% sodium thiosulfate containing approximately 1-3 x 10^6 cpm ^{33}Phosphor labelled ribonucleotide probe. Brain sections were coverslipped and incubated overnight at 55ºC. As a control, a few slides were hybridized with sense probe [14].

The next day, coverslips were removed and the slides were washed in 2xSSC at room temperature for 10min, treated with Rnase A [2 mg/100 ml in 0.5 m NaCl (pH 7.5)] at 37ºC for 10min, and washed three times in 2xSSC/50% formamide at 60ºC for 15min. After a short wash with 2xSSC, sections were dehydrated in an ethanol series and air dried.

Finally, stringency of post-hybridization washes was at 0.1 x standard sodium citrate at 65 °C. The slides were put in an x-ray exposure holder and apposed to Biomax MR film (Kodak,Rochester, NY) for 14-21 d. 14C (RPA 504 microscales; Amersham, Buckinghamshire, UK) were used to calibrate the signal. Films were scanned and quantified by using Image J software.

2.5.1.5 Maternal care

The maternal care behavior of each dam was observed and scored for five-60min periods per day during the first 7 days post partum using a procedure described before [5, 15, 16]. Observations were performed at three periods during the light phase (10:00, 13:30, and 17:00h) and two periods during the dark phase (07:30 and 19:30h; under 2x60 W red TLD-light). Note that the observation at 17.00 h the day of MS was the time of dam's re-union.

The behavior of each mother was scored every 3min (20 observations per period, 100 observations per day). We scored the following maternal behaviors: retrieval, maternal contact (the dam is nest building), burying, in contact with the pups but not nursing or licking, licking and grooming (LG), away from nest, passive nursing posture, arched-back nursing [blanket nursing/(passive) low arch, (active) low arch, middle arch, high arch). We considered as (overall) passive nursing the sum of the passive nursing posture and the (passive) low arched-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, 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 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. AN & LG frequencies both include instances where both of the behaviors occurred simultaneously and those instances are quite frequent.

2.5.2. Cohort 2: Mid-term and long-term impact of MS contexts (pnd 30, 55 & 240)

2.5.2.1 Objectives – Outcome measure

We sought to determine the mid-term (Experiment 2) and long–term impact (Experiment 3) of the different maternal separation contexts on neuroendocrine and behavioural measures at different life stages. Litters were divided to four treatment groups: non separated (NON SEP), history of 8h home-separation on pnd 3, 4 & 5 (HOME SEP), and history of 8h novel-separation on pnd 3,4 & 5 (NOVEL SEP). We used only the male offspring. In total we used 5-6 litters per group. We measured juvenile play-fighting, peri-pubertal short-memory and behavioral (freezing) and endocrine (ACTH, CORT) HPA-axis responses to conditioned fear in adulthood.

2.5.2.2 Experiment II: Mid-term impact of MS context

We choose two non-aversive behavioural measures in the period before adulthood. One is a naturalistic measurement of a normal behavior (social play) and the other is a spatial memory task in low-stress conditions (T-maze spontaneous alternation) in the housing room.

2.5.2.2.1 Social play in a novel setting at pnd 30

Juvenile social play behavior is regarded as one of the earliest forms of non-mother directed social behavior in rodents [17].

Apparatus. The recording was made with cameras placed above the test arenas connected to an automated video-recording system. During the video recording, the program Ethovision 3.1 (Noldus, Wageningen, Netherlands) was used to measure rats' proximity to each other and duration of interaction. We determined whether patterns of social interaction between individuals were differently influenced by the MS contexts.

Procedure. The social interaction experiment was performed on pnd 30 in a novel environment setting. Four hours of single housing was followed by 20min observation of social interaction in dyads of rats. Two days before the testing, rats were habituated to a dim-lighted (5-10 lux) room. On the actual experimental day, rats were single housed in a cage identical to the social-interaction testing cage (L55xW30xH44 cm) for 4h. The single housing of the juvenile rat increases the amount of social play [18]. During the first two hours, rats had access to a plastic tube (open in

one end) from which they could dig out light-weighted stones (from a total of 300gr). This was to assess the general behavioral function and muscular strength of the rats in an ecological relevant way [19]. Thereafter, the tube was removed and the quantity of stones (in gr) retrieved by the rat was calculated. The bedding was refreshed with new bedding (sawdust) and the rat remained in this setting for another 2h.

To observe social interactions, two rats of the same age that were unfamiliar to each but with same early-life history were put together for 20min in a novel testing cage. Behavior was videotaped and analyzed at a later time point. The 20min experiments took place during either a morning session at 9:00h or an afternoon session at 13:30h. Rats within and between experimental groups were counterbalanced between morning and afternoon session. In order to distinguish between rats during the analysis of the tapes, one rat was painted with odorless mark the previous day while the other was equally handled but not labelled. Note that there was only a marginal difference in weight (up to maximum of 10gr) between rats tested in pair. We believe that such difference is not likely to influence the results reported below.

Measurements. The scoring of the behaviors was done using the Observer XT program (Noldus, Wageningen, The Netherlands). The first 5min of each video recording were analyzed. Behaviors were scored according to a protocol previously described by Vanderschuren and colleagues [20, 21]: (i) Playful activities (initiation/response): chasing/evading, nape attack/ attacked, pinning/ supine, boxing, wrestling, (ii) Non-playful, but social activities: social grooming, crawl over/under, (iii) Non-playful, but non social activities : self grooming, exploration of the cage. For the following behaviors, both the duration as well as frequency at which an individual was engaged in a given behavior could be obtained: evade, chase, boxing/wrestling, self grooming, social grooming and exploration of the cage. For pinning, supine, nape attack and crawl over/under, the frequencies were scored. An additional analysis of rats' proximity was performed with the use of Ethovision 3.1 program (Noldus, Wageningen, The Netherlands). In order to get an overall picture from results of the individual behaviors, we added the frequencies of behaviors indicating initiation of social play sequelae.

2.5.2.2.1 Spontaneous alternation in a T-maze (short-term memory) at pnd 55

Spontaneous alternation in a T-maze is a short-term memory test. This test is proven to be sensitive to dysfunction of the hippocampus, but other brain structures are also involved [22].

Apparatus. A Plexiglas T-maze with transparent walls and a black floor was used. The T-maze was divided in three arms: start (L75xW12xH20 cm), left (L32xW12xH20 cm) and right (L32xW12xH20 cm). Two sliding doors permitted to close the entrance of the left and right arm respectively. A metal grid cover was additionally used so that the rats were not able to escape from the maze. The T-maze was placed in the housing room in such a way that the amount of luminescence was the same in the right and the left arm of the T-maze (15 LUX).

Spontaneous alternation protocol. The T-maze was used to investigate if the different rats would spontaneously alternate. An experimental session consisted of a sample trial and a choice trail. In the sample trial, the animal was placed in the start-arm of the T-maze and allowed to explore the whole maze. Once the animal entered one of the targeted arms, the sliding door was closed preventing the animal leaving this arm. The arm entered (left or right) was registered, as well as the latency of the entry. Head dips which were made in the arms before an entry were also registered. A maximum of 90sec was given for an entry. After 90sec (if an entry was not made) or

after 20sec after the time of entry, the animal was taken out of the maze and put into a Type III cage. The number of defecations and urinations were registered and the maze was cleaned with a 10% alcohol solution and dried with a tissue. Directly after the choice trial followed. The rat was returned to the start-arm, with the two arm-sliding doors open again, and allowed to explore again. After 90sec (if an entry was not made) or after 10sec after the time of entry, the animal was taken out of the maze and put back to its home cage. The arm entered (if any) in the second trial was registered. Two sessions were conducted per day (at 12:00h & 16:00h) for 3 days in a row. Measurements. The percentage of sessions (% spontaneous alternation) that a rat alternated in the choice trial was used as an output parameter.

2.5.2.3 Experiment III: Long-term impact of MS context

2.5.2.3.1 Contextual fear conditioning at pnd 240

We choose adult psychoneuroendocrine response to contextual fear. Fear conditioning in rats has been widely used throughout the literature to study fear formation, recall and extinction together with the relevant brain circuits [23, 24].

Apparatus. The fear conditioning box (L40xW40xH50 cm) was located in a room with similar environmental conditions as the housing room. The walls of the box were made of black Plexiglas. The floor of the box consisted of stainless steel rods, connected to a shock generator. The box was cleaned with a 10% ethanol solution before rats were placed inside. A video camera placed 20 cm above the box allowed each subject' behavior to be monitored as well as recorded digitally by a computer.

Procedure. Acquisition: Rats were individually transported in a cage from the housing room to an adjacent room containing the fear conditioning set up. The rat was placed in the shock box. After 2min, one electric foot shock (0.6 mA, 2sec) was given and 2min later, the rat returned to its home cage Re-exposure: 24h later, the same procedure was repeated however without delivery of the foot shock.

Measurements. Behavior of the rat was recorded by the camera during acquisition and re-exposure. An observer unaware of treatment conditions scored the videotapes using special software (Observer 9.0 XT). Behavior was classified as: (1) freezing (lack of all body movement except that necessary for breathing), (2) scanning (lack of body movement but swaying of the head and breathing), (3) rearing (animal is taking a new position while standing on his hind legs) or (4) default (other).

The factor "time" had three levels: during acquisition: (1) 2min after shock, during reexposure: (2) first 2min and (3) last 2min. No rat showed more than 10% freezing behavior before the shock, which we had set as exclusion criterion.

2.5.2.3.2 Blood sampling in adult rats

Immediately following the re-exposure to the fearful context (i.e. 4min exposure to the fear conditioning box), rats were individually transferred in a novel cage and brought to a different room (next to the housing room). The tail incision method used for collection of small volumes of blood has been described before [25]. Blood samples were taken 4, 15, 30, 120min after the onset of the stressor. Blood samples to determine hormone levels in basal resting conditions have been collected one week before the fear conditioning experiment. Blood was collected in 1.5 ml EDTA-coated microcentrifuge tubes and kept on ice (see 5.1.4 for subsequent procedures on

blood samples). Rats were decapitated either at basal conditions (no experience of conditioning experiment) or 30min after the onset of the re-exposure the fear conditioning context. Brains were removed from skulls, rapidly frozen on dry ice and stored at -80°C until used for in situ hybridization of c-Fos mRNA (as described above; only difference the post-fixation of the brain section took place in a freshly prepared 4% paraformaldehyde solution).
ACTH was measured at basal, 4min and 15min time points. CORT was measured at all time points30min. For the CORT response curve we could calculate the AUC using Prism Graph Pad software (version 5).

2.6 Statistical analysis

Data are presented as mean ± SEM and were analyzed by one-way, one-way repeated measures or two-way analysis of variance (ANOVA) with the significance level set at $p< 0.05$. Where appropriate, simple and interaction main effects were investigated further with subsequent post-hoc comparisons (by Tukey test or student t-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 neonate male and female rats were pooled.

3. Results

3.1 Cohort 1 - Experiment I: Immediate outcome of MS context.

In order to investigate if repeated 8h-MS in different contexts can induce a state of differential neuroendocrine responsivity to a 30min novelty-stressor, we exposed separated pups (twice on pnd 3&4 in home or novel context) to 30min of novelty-stress 24h later on pnd 5.

3.1.1 ACTH (Fig. 2A).

ACTH basal levels for naïve Wistars on pnd 5 were 90.08 ± 7.35 (pg/ml). Data of separated pups are presented as % of this value. Two-way ANOVA revealed effects of novelty-stress ($F_{1,31}$= 4.57; p=0.041). On pnd 5, ACTH increased 34% in response to 30min of novelty, if the separation on the previous days had happened in a novel context ("novelty" vs. "basal" levels in NOVEL SEP; p=0.023).

3.1.2 CORT (Fig. 2B).

CORT basal levels for naïve Wistars on pnd 5 were 4.96 ± 0.25 (ng/ml) Data of separated pups are presented as % of this value. Two-way ANOVA revealed effects of novelty-stress ($F_{1,31}$= 9.25; p=0.005). On pnd 5, CORT increased 86% in response to 30min of novelty, if the separation on the previous days had happened in a home context ("novelty" vs. "basal" levels in HOME SEP; p=0.023).

CORT & ACTH at pnd 3: To assess whether the effect seen on pnd 5 was already present earlier (pnd 3), we did an additional experiment, where we sacrificed pups after one MS episode in basal conditions (directly after MS) or after 30min of novelty-stress

(data not shown). MS causes a slow increase of CORT levels in pups in either context (for HOME SEP: p=0.021 & for NOVEL SEP: p=0.009). After a single episode of MS on pnd 3 there was no difference in the CORT response to stress between HOME SEP and NOVEL SEP. However, separated pups' stress-induced ACTH release was influenced by the MS context. Only in the NOVEL SEP there was a significant rise in response to novelty (p=0.019).

3.1.4 Amygdala c-Fos mRNA (Fig. 2C). Basal levels for naïve Wistars on pnd 5 were 7.10 ± 0.91 (grey scale arbitary units). Two-way ANOVA revealed a main effect of novelty-stress ($F_{1,23}$= 7.48; p=0.013). On pnd 5, amygdala c-Fos expression levels increased 72% in response to 30min of novelty, if the separation on the previous days had happened in a novel context ("novelty" vs. "basal" levels in NOVEL SEP; p=0.003).

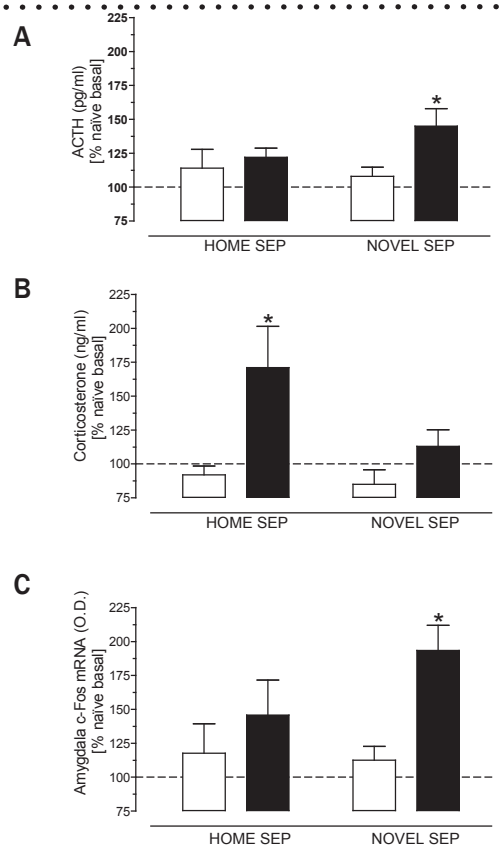

Figure 2. Endocrine and Amygdala responses to novelty-stress on postnatal day (pnd) 5. ACTH blood plasma levels (A) CORT blood plasma levels (B) and amygdala c-fos mRNA (C). Blood samples were taken at basal resting conditions (basal, white bar) and 24 hrs after maternal separation with 30min of exposure to novelty (novelty; black bar). Early-life conditions: separated pups had experienced 2 times 8h of maternal separation on pnd 3 & 4 in home context (HOME SEP) or novel context (NOVEL SEP). Data are presented as percentage (MEAN±SEM) of basal levels of naïve non-separated rats at pnd 5 (represented as a dotted line). The exact hormonal concentrations are given in the result section.. Significance level was set at p≤0.05. * vs. basal. For A & B, n=8 per time point for both experimental groups and for C, n=6 per time point for both experimental groups

3.2 Experiment II: Mid-term impact of MS context

3.2.1 Social play in a novel setting at pnd 30.

Burrowing: In the first 2h of the habituation phase to the novel setting, all groups burrowed to the same extent, indicating that all rats had similar muscular strength.

Explore cage (data not shown): During the 20min social interaction, rats of all treatment groups explored the cage to the same extent

Social play frequency (Fig. 3A): NOVEL SEP rats initiate less frequently social play compared to the other two groups (vs. NON SEP: p=0.037, vs. HOME SEP: p=0.034).

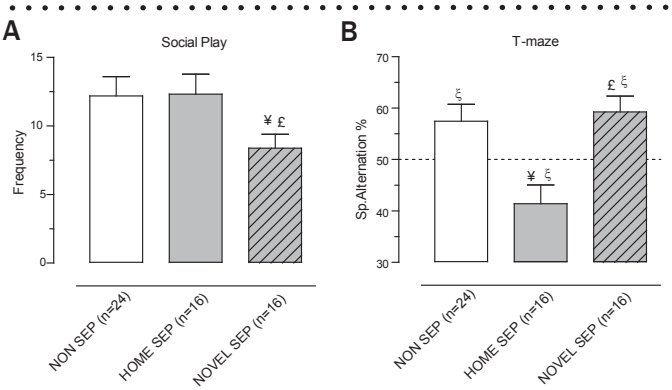

Figure 3. Social play frequency of juvenile rats (A) and peri-pubertal spontaneous alternation in a T-maze (B). Early-life conditions: non separated (NON SEP) had no history of separation before weaning and maternally separated rats had experienced 3 times 8h-MS on pnd 3, 4 & 5 in home context (HOME SEP) and novel context (NOVEL SEP). Data represent MEAN±SEM. Significance level was set at p≤ 0.05. ¥ vs. NON SEP, £ vs. HOME SEP, ξ vs. 50% (chance level). The number of rats used is indicated in the panels.

3.2.2 Spontaneous alternation in a T-maze (short-term memory) at pnd 55.

% Spontaneous alternation (Fig. 3B): One-way ANOVA revealed a main effect of treatment ($F_{2,55}$= 7.59; p=0.001). HOME SEP alternate less than rats of the other two groups (vs. NON SEP: p=0.004, vs. NOVEL SEP: p=0.003). Both NON SEP and NOVEL SEP groups perform higher than 50% chance level (p=0.027 & p=0.005 respectively), whereas HOME SEP performed lower than chance (p=0.025).

3.3 Experiment III: Long-term impact of MS contexts

3.3.1 Contextual fear conditioning at pnd 240

3.3.1.1 Freezing (Fig. 4A): Repeated measures one-way ANOVA revealed a main effect of time ($F_{2,98}$=8.70; p≤0.001). This effect was significant for HOME SEP (p=0.029) and NOVEL SEP (p=0.021). For the post-shock 2min period, one-way ANOVA analysis did not reveal any effect of treatment. For the first 2min of re-exposure (24h later), one-

way ANOVA revealed a main effect of treatment ($F_{3,67}$=3.47; p=0.021). NOVEL SEP froze more than the HOME SEP p=0.02). For the last 2min of re-exposure (data not shown), one-way ANOVA analysis did not reveal any effect of treatment.

3.3.1.2 Scanning (Fig. 4B): Repeated measures one-way ANOVA revealed a main effect of time ($F_{2,98}$=15.25; p≤0.001) and treatment ($F_{2,49}$=2.95; p=0.029). HOME SEP were overall scanning more than the NOVEL SEP (p=0.023). The time effect was significant only for the NON SEP (p=0.007) and HOME SEP (p≤0.001). For the post-shock 2min period, one-way ANOVA revealed a main effect of treatment ($F_{3,67}$=5.41; p=0.002). The HOME SEP scanned more than the NON SEP(p=0.021) and the NOVEL SEP(p≤0.001). For the first 2min and the last 2min of re-exposure, 24h later, (data not shown), one-way ANOVA analysis did not reveal any effect of treatment.

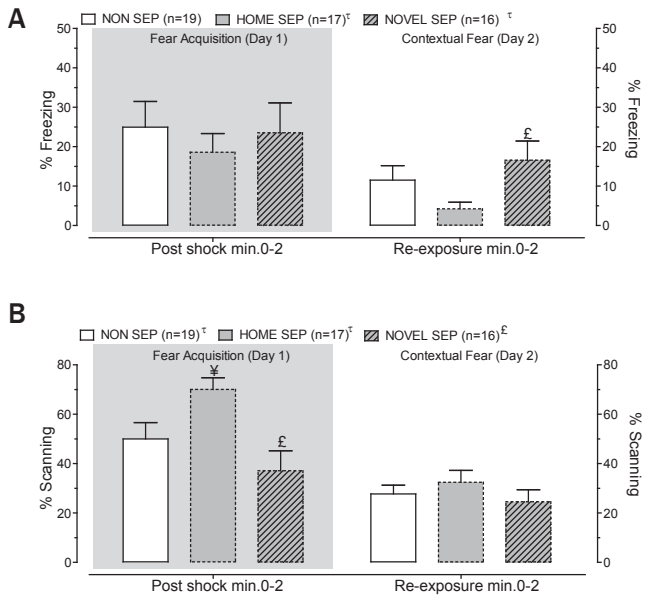

Figure 4. Contextual fear response as percentage of duration of freezing (A) and scanning (B). Early-life conditions: non separated (NON SEP) had no previous history of separation before weaning and maternally separated had experienced 3 times 8h-MS on pnd 3, 4 & 5 in home context (HOME SEP;) and novel context (NOVEL SEP). Data are represented as MEAN±SEM. Significance level was set at p≤ 0.05. ¥ vs. NON SEP, £ vs. HOME SEP. The exact number of rats used is indicated in the panels.

3.3.2 CORT (Fig. 5A & B)

Repeated measures one-way ANOVA revealed effects of time ($F_{4,96}$= 146.93; p≤0.001/ p≤0.001 for all treatment groups), treatment ($F_{2,27}$= 4.24; p=0.009) and their interaction(F8,98= 2.25; p=0.030). HOME SEP displayed overall higher CORT levels than the NON SEP and NOVEL SEP (p=0.029 & p=0.019 respectively). For more comparisons of the separate time points see Figure 5A.

For CORT-AUC (Fig. 5B), one-way ANOVA revealed a main effect of treatment ($F_{1,27}$=

3.74; p=0.039). HOME SEP pups secreted more CORT than NON SEP (p=0.048) and NOVEL SEP (p=0.025).

3.3.3 ACTH (Fig. 5C)

Repeated measures one-way ANOVA revealed effects of time ($F_{2,48}$= 41.81; p≤0.001/ p≤0.001 for all treatment groups) and the interaction of time and treatment($F_{4,48}$= 5.59; p=0.001). At baseline, both separated groups had higher levels of ACTH than the NON SEP group (HOME SEP: p=0.015, NOVEL SEP: p=0.012). At 15min, HOME SEP displayed lower levels than NON SEP (p=0.007) and NOVEL SEP (p=0.011).

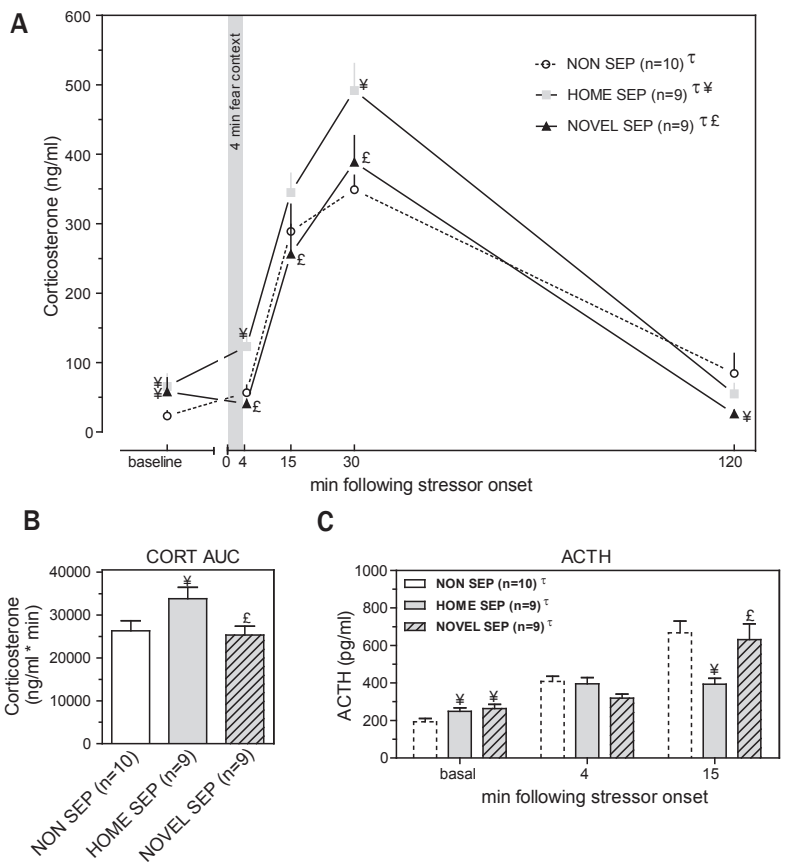

Figure 5. Endocrine conditioned fear response of the adult rat. Corticosterone in ng/ml (CORT; (A) blood plasma concentrations and area under the curve (AUC; B) and ACTH blood plasma concentrations in pg/ml (C). Blood samples were taken either at basal resting conditions (one week prior fear conditioning) or 4, 15, 30 or 120min after the onset of re-exposure to the fearful context. Early-life conditions: non separated (NON SEP) had no previous history of separation before weaning and maternally separated rats had experienced 3 times 8h-MS on pnd 3, 4 & 5 in home context (HOME SEP) and novel context (NOVEL SEP). Data are represented as MEAN±SEM. Significance level was set at p≤ 0.05. τ denotes overall time effect in this group, ¥ vs. NON SEP, £ vs. HOME SEP, Φ vs. NOVEL SEP. The exact number of rats used is indicated in the panels.

3.3.4 c-Fos mRNA in the Amygdala

In the adult rat we could subdivide the amygdale into three subnuclei.

Basolateral amygdala mRNA (Fig. 6): For the NON SEP individuals one-way ANOVA revealed a main effect of stress ($F_{1,7}$= 27.58; p=0.002). Conditioned emotional stress increases c-Fos expression. One-way ANOVA for the individuals that were exposed to conditioned emotional stress revealed a main effect of treatment ($F_{2,11}$= 13.14; p=0.002). The NOVEL SEP expressed more c-Fos than the controls (p=0.002) and the HOME SEP (p=0.009).

Central Amygdala mRNA (data not shown): For the NON SEP individuals one-way ANOVA revealed a main effect of time ($F_{1,7}$= 8.20; p=0.029). Conditioned emotional stress increases c-Fos expression. One-way ANOVA for the individuals that were exposed to conditioned emotional stress did not reveal a main effect of treatment.

Medial Amygdala mRNA (data not shown): For the NON SEP individuals one-way ANOVA did not reveal a main effect of time. One-way ANOVA for the individuals that were exposed to conditioned emotional stress revealed a main effect of treatment ($F_{2,11}$= 13.02; p=0.002). NOVEL SEP and HOME SEP expressed more c-Fos than the controls (for the HOME SEP: p=0.003, for the NOVEL SEP: p=0.004).

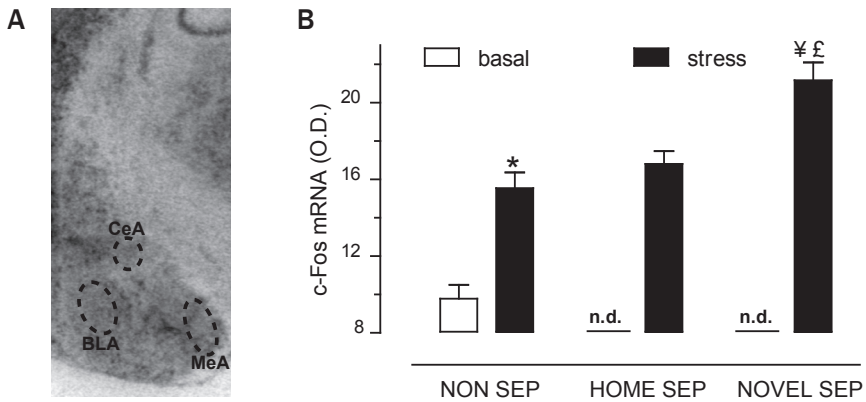

Figure 6. (A) Representative image of c-Fos in situ hybridization for basolateral amygdala (BLA), central amygdala (CeA) and medial amygdala (MeA). (B) c-Fos mRNA expression in BLA of adult rats. Measurements performed in brain sections at basal conditions (white bar) or 30min after the onset of re-exposure to the fear conditioning context. Early-life conditions: non separated (NON SEP) had no previous history of separation before weaning and maternally separated rats had experienced 3 times 8h-MS on pnd 3, 4 & 5 in home (HOME SEP) and novel context (NOVEL SEP). Data are represented as MEAN±SEM. Significance level was set at p≤ 0.05. * vs. basal, ¥ vs. NON SEP, £ vs. HOME SEP, "n.d." denotes not determined values. n=5 rats per time point of each treatment group.

3.3.5 Maternal care

Upon reunion, maternal care increases. Apparently, the dams try to compensate, especially for the lack of LG, in the post reunion periods. Separated pups (both HOME SEP and NOVEL SEP) experience on average less care (LG and AN) compared to NON

SEP pups, however there is no difference in the maternal behavior measures (data not shown).

Additionally we wanted to validate the magnitude of the maternal care deficit. Using two separate rat populations that were bred and were characterized for the maternal care they received, we were able to demonstrate the normal distribution of LG of our population according to previously described methodology [15, 26]. Interestingly, both NOVEL SEP and HOME SEP pups experience on average maternal care (LG) in the Low range of an undisturbed population (data not shown).

4. Discussion

We investigated the role of the environmental context pups experienced during maternal separation in the programming of a fearful phenotype. This study of the effect of environmental context was performed with the goal to link our previous findings on the repeated maternal separation paradigm with the recently described programming of the amygdala fear pathway [7]. In the present study we demonstrated that the stressful experience of peer deprivation in a novel cage during repeated MS (NOVEL SEP) rather than the maternal absence experience per se (HOME SEP) caused priming of the amygdala fear pathway with lasting consequences on behavior and stress-responsiveness. The NOVEL SEP pups displayed enhanced c-Fos amygdala activation and pituitary ACTH release in response to acute stressors that persisted into adulthood. These NOVEL SEP pups also showed several aspects of a behaviorally fearful phenotype given their reduced play fighting at adolescence and their enhanced freezing response during contextual fear conditioning.

Over the last 50 years, the rodent literature suggests that either experimentally induced or naturally occurring early-life adverse events can predict altered social behavior, enhanced psychoneuroendocrine response to stress, and impaired cognitive functioning, as well as alterations in working memory, social interaction, sensorimotor gating, and dopamine susceptibility [10, 21, 27-35]. Our study was designed to examine the importance of the non-shared early-life experience [36] on the development of behavioral and neuroendocrine characteristics by isolating the pups in a novel environment. These non-shared factors are in turn important since they can predict the development of stress-related pathologies in later-life [37, 38]. Another example can be found in twin studies, where important part of the variance in stress reactivity in childhood can be accounted by non-shared environmental factors between the siblings [39].

In high early-life adversity conditions realized by long-lasting and repeated maternal absence, our study is the first to our knowledge to address the short-term and long-lasting impact of non-shared early-life experiences. Already in the neonate, the

different separation conditions of experiencing maternal absence at home versus in a novel environment without peers had a profoundly different outcome. After daily 8h-MS on pnd 3 and 4, the NOVEL SEP pups, which had been kept in isolation away from the home cage and peers during MS, displayed enhanced stress induced amygdala c-Fos expression and ACTH release on pnd 5 (after a 24h post-reunion interval). This phenomenon was in fact already observed immediately after the first 8h-MS on pnd 3. Interestingly, these pups, while showing the central stress-induced activation, did not show a peripheral adrenocortical response to stress confirming the notion that the hyporesponsiveness of the adrenals actually accounts for the SHRP [5, 40, 41]. In contrast, the pups that stayed in the home cage during MS responded to the mild novelty-stressor on pnd 5 with a brisk CORT response since, as we showed before, in this HOME SEP condition the adrenal cortex gets primed for hyper-responsiveness [5].

The premature amygdala activation observed in NOVEL-SEP is reminiscent to the findings in the pioneering studies of Sullivan's group on odor-learning. The odor system is fully developed and functional the first week of life [8] and the dam's presence makes the impact of noxious stimuli towards the pups negligible while attachment to the care-giver is developing [42]. Until approximately pnd 10, pups exhibit preference to novel odors, even if they are paired with negative stimuli, by co-activation of the locus coeruleus - olfactory bulb pathway [43, 44]. In the post-sensitive period, odor-avoidance behavior appears and is associated with the activation of neural processes in amygdala and piriform cortex [44].

Interestingly, when the dam is away during the "sensitive" period, the odor aversion neuronal system can be activated prematurely and aversive memories can be formed as long as the CORT levels are elevated in blood and amygdala [4, 8]. 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, we found that the pups in both contexts experience high amounts of CORT. Hence after the first separation period under both conditions CORT priming of the amygdala is possible, but yet the outcome is profoundly diverging. This is possibly because in the NOVEL SEP the first CORT rise on pnd 3 occurs in the context of a more enhanced central stress system activity as reflected by the higher pituitary stress responsiveness.

The phenotype of the NOVEL SEP offspring persisted into adulthood in its enhanced amygdala c-Fos activation and ACTH release. The central stress response pattern matched with fear conditioning behavior, since enhanced ACTH and amygdala c-Fos expression corresponded with the retention of the freezing response observed in NOVEL SEP. Again, in contrast with the NOVEL SEP, the HOME SEP animals maintained their remarkable enhanced stress-induced CORT response into adulthood. In the adult

HOME SEP the enhanced CORT response occurred in the face of attenuated stress-induced ACTH release indicating a profound adrenal hyper-responsiveness.

That the NOVEL SEP individuals initiated less social play, than the other groups, can be interpreted as a precursor of the later fearful phenotype. However, this can be debated since the offspring of Low LG dams, which were previously shown to develop fearful phenotypes in adulthood, are at adolescence significantly more inclined to initiate play fighting behaviors [32]. This discrepancy could be more related to a difference in basal or stress-induced CORT levels between NOVEL SEP and Low LG rats. Veenema and Neumann [21] demonstrated that male rats going through 3h-MS on pnd 1-14 displayed an increase in offensive play behaviors. In their study, increased offensive social play was linked to increased basal CORT levels of the MS individuals. This finding is in agreement with our observation that HOME SEP characterized by CORT hyper-responsiveness displayed more social play than NOVEL SEP. Since in their experiment they exposed pups to a novel environment as a whole litter, this raises the interesting possibility that actually the presence or absence of peers during early-life adversity may predict if later social interactions would be increased or decreased.

The high stress-induced CORT secretion of HOME SEP might also explain the poor retention of the freezing/scanning response. It is known for long that CORT facilitates extinction of fear-motivated behavior [45]. In line with this, more recently, injections of CORT before retrieval reduced fear memory [46]. Furthermore, Brinks and colleagues [47] distinguished individuals engaged in scanning during fear acquisition from the rather passive freezing response. Increased scanning of the HOME SEP indicates a more active coping style towards the stressful electric shock that might prepare for the rapid extinction of the freezing response on retest. In a previous study we have also shown that HOME SEP and NOVEL SEP display differences in acoustic startle response and its prepulse inhibition, which indicates differential stimuli processing [48, 49].

In the peri-pubertal period, spontaneous alternation in a T-maze was measured. HOME SEP rats performed worse than 50% chance level and worse than NON SEP and NOVEL SEP, which performed above chance level. This together with the reduced retention of the freezing and scanning response in the contextual fear-conditioning as well as the increased stress-induced CORT output would imply that rats with a history of HOME SEP display actually a certain degree of cognitive impairment that involves hippocampus dysfunction.

An important issue in the programming of the fearful phenotype is the precise role of maternal care. Besides that the MS models is a laboratory model to mimic the effects of maternal absence, also the pattern of care is severely altered [5, 50, 51]. In our MS model, even though the dams of the repeatedly separated pups show enhanced

care upon reunion, this does not compensate. As a consequence the pups suffer from a reduced amount of care [5]. In this study we observed, by using a large undisturbed population of Wistar dams (run in parallel), that the LG levels of both HOME SEP and NOVEL SEP dams are in the Low LG range, in spite of the post-reunion rebounds of LG.

The reduction in maternal care alone does not seem to predict higher fear-conditioning in our Wistar population of rats, since NON SEP displayed similar contextual fear with the HOME SEP offspring, although the latter had a great overall deficit of care. HOME SEP and NOVEL SEP rats both experience a lack of LG, but they have much opposing phenotypes. However, Long Evans Low LG offspring did freeze more than High LG in contextual fear-conditioning paradigm [26, 52]. This difference raises the interesting possibility that naturally-occurring and experimentally induced maternal care deficits do not represent the same degree or quality of early-life experience. The maternal mediation hypothesis therefore has been extended with the notion that environmental adversity (e.g. peer deprivation) and the maternal repertoire may both contribute to the lasting alterations in the offspring's HPA-axis responses and behaviors [53]. The results presented here support this notion.

Overall, opposing phenotypes arise in pups during maternal absence and persist into adulthood as a consequence of a distinctly different environmental context during the separation. The NOVEL SEP has no deficit in cognitive performance and diminished social interaction in adolescence seems to be the precursor of the later more fearful phenotype. The most profound feature is however, the fearful phenotype which seems related to a putatively enhanced amygdale drive, enhanced pituitary ACTH release and adrenal hyporesponseiveness. The HOME SEP has mild cognitive impairments, and enhanced adrenocortical function with elevated stress-induced CORT. Stress-related mental diseases often display both these phenotypes and are also linked to adverse early-life events [54]. Further behavioral and neuro-endocrine characterization and pharmacologic validation of our animal model has to follow-up.

6. Conclusion

The present study substantiates the importance of early-life MS conditions and asks for standardization of experimental procedures aimed to test the outcome of mother-pup interactions. Beyond that, the findings disclose the amazing plasticity of the newborn brain. This became already apparent in previous studies demonstrating the rapid adaptation of the neonate to repeated absence of the dam, as if the pup has readily has the ability to predict reunion [5, 6]. In the present study, we discovered that the outcome is different if the pup remain in the home cage together or are put for 8 hours elsewhere in isolation during maternal absence. The latter conditions produce amygdala activation and enduring hyporesponsiveness of the adrenal, precipitating a

more fearful phenotype.

7. Acknowledgments

We would like to thank Servane Lachize and Maaike van der Mark for the technical assistance with RIA; Wesley Fung, Marjolein Koller and Jasper Laboyrie for help in brain cryosectioning and in situ hybridization; Wout Meelis for the technical assistance with animal sampling and decapitation; Ricardo Llorente and Stephanie Schnorr for help with maternal care observations. This work was supported by the Top-Institute Pharma T5 #209 (NPD), Marie Curie Foundation (DLC), Smartmix SenterNovem (DLC), NWO-IRTG (AD, MSO), EU-lifespan (SEFC), NWO-Aspasia (MSO), the Royal Netherlands Academy of Arts and Sciences (ERdK).

7. References

[1] Schmidt, M., Enthoven, L., van Woezik, J. H., Levine, S., de Kloet, E. R., Oitzl, M. S. The dynamics of the hypothalamic-pituitary-adrenal axis during maternal deprivation. Journal of neuroendocrinology. 2004,16:52-7.
[2] Levine, S., Huchton, D. M., Wiener, S. G., Rosenfeld, P. Time course of the effect of maternal deprivation on the hypothalamic-pituitary-adrenal axis in the infant rat. Developmental psychobiology. 1991,24:547-58.
[3] Fenoglio, K. A., Chen, Y., Baram, T. Z. Neuroplasticity of the hypothalamic-pituitary-adrenal axis early in life requires recurrent recruitment of stress-regulating brain regions. J Neurosci. 2006,26:2434-42.
[4] Moriceau, S., Wilson, D. A., Levine, S., Sullivan, R. M. Dual circuitry for odor-shock conditioning during infancy: corticosterone switches between fear and attraction via amygdala. J Neurosci. 2006,26:6737-48.
[5] Daskalakis, N. P., Claessens, S. E., Laboyrie, J. J., Enthoven, L., Oitzl, M. S., Champagne, D. L., et al. The newborn rat's stress system readily habituates to repeated and prolonged maternal separation, while continuing to respond to stressors in context dependent fashion. Hormones and behavior. 2011,60:165-76.
[6] Enthoven, L., Oitzl, M. S., Koning, N., van der Mark, M., de Kloet, E. R. Hypothalamic-pituitary-adrenal axis activity of newborn mice rapidly desensitizes to repeated maternal absence but becomes highly responsive to novelty. Endocrinology. 2008,149:6366-77.
[7] Sullivan, R. M., Holman, P. J. Transitions in sensitive period attachment learning in infancy: the role of corticosterone. Neuroscience and biobehavioral reviews. 2010,34:835-44.
[8] Moriceau, S., Sullivan, R. M. Maternal presence serves as a switch between learning fear and attraction in infancy. Nature neuroscience. 2006,9:1004-6.
[9] Sevelinges, Y., Moriceau, S., Holman, P., Miner, C., Muzny, K., Gervais, R., et al. Enduring effects of infant memories: infant odor-shock conditioning attenuates amygdala activity and adult fear conditioning. Biological psychiatry. 2007,62:1070-9.
[10] Oomen, C. A., Soeters, H., Audureau, N., Vermunt, L., van Hasselt, F. N., Manders, E. M., et al. Severe early life stress hampers spatial learning and neurogenesis, but improves hippocampal synaptic plasticity and emotional learning under high-stress conditions in adulthood. J Neurosci. 2010,30:6635-45.
[11] Moriceau, S., Raineki, C., Holman, J. D., Holman, J. G., Sullivan, R. M. Enduring neurobehavioral effects of early life trauma mediated through learning and corticosterone suppression. Frontiers in behavioral neuroscience. 2009,3:22.
[12] Sevelinges, Y., Mouly, A. M., Raineki, C., Moriceau, S., Forest, C., Sullivan, R. M. Adult depression-like behavior, amygdala and olfactory cortex functions are restored by odor previously paired with shock during infant's sensitive period attachment learning. Dev Cogn Neurosci. 2011,1:77-87.
[13] Paxinos, G., Watson, C. The Rat Brain in Stereotaxic Coordinates. 6th Edition. San Diego, Elsevier Academic Press. 2007.
[14] Karssen, A. M., Meijer, O. C., Berry, A., Sanjuan Pinol, R., de Kloet, E. R. Low doses of dexamethasone can produce a hypocorticosteroid state in the brain. Endocrinology. 2005,146:5587-95.
[15] Champagne, F. A., Francis, D. D., Mar, A., Meaney, M. J. Variations in maternal care in the rat as a mediating influence for the effects of environment on development. Physiology & behavior. 2003,79:359-71.

[16] Myers, M. M., Brunelli, S. A., Squire, J. M., Shindeldecker, R. D., Hofer, M. A. Maternal behavior of SHR rats and its relationship to offspring blood pressures. Developmental psychobiology. 1989,22:29-53.
[17] Vanderschuren, L. J., Niesink, R. J., Van Ree, J. M. The neurobiology of social play behavior in rats. Neuroscience and biobehavioral reviews. 1997,21:309-26.
[18] Niesink, R. J., Van Ree, J. M. Involvement of opioid and dopaminergic systems in isolation-induced pinning and social grooming of young rats. Neuropharmacology. 1989,28:411-8.
[19] Deacon, R. M. Burrowing in rodents: a sensitive method for detecting behavioral dysfunction. Nature protocols. 2006,1:118-21.
[20] Vanderschuren, L. J., Niesink, R. J., Spruijt, B. M., Van Ree, J. M. Effects of morphine on different aspects of social play in juvenile rats. Psychopharmacology. 1995,117:225-31.
[21] Veenema, A. H., Neumann, I. D. Maternal separation enhances offensive play-fighting, basal corticosterone and hypothalamic vasopressin mRNA expression in juvenile male rats. Psychoneuroendocrinology. 2009,34:463-7.
[22] Deacon, R. M., Rawlins, J. N. T-maze alternation in the rodent. Nature protocols. 2006,1:7-12.
[23] Roozendaal, B., McEwen, B. S., Chattarji, S. Stress, memory and the amygdala. Nature reviews. 2009,10:423-33.
[24] Rodrigues, S. M., LeDoux, J. E., Sapolsky, R. M. The influence of stress hormones on fear circuitry. Annu Rev Neurosci. 2009,32:289-313.
[25] Fluttert, M., Dalm, S., Oitzl, M. S. A refined method for sequential blood sampling by tail incision in rats. Laboratory animals. 2000,34:372-8.
[26] Champagne, D. L., Bagot, R. C., van Hasselt, F., Ramakers, G., Meaney, M. J., de Kloet, E. R., et al. Maternal care and hippocampal plasticity: evidence for experience-dependent structural plasticity, altered synaptic functioning, and differential responsiveness to glucocorticoids and stress. J Neurosci. 2008,28:6037-45.
[27] Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., et al. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science (New York, N.Y. 1997,277:1659-62.
[28] Plotsky, P. M., Thrivikraman, K. V., Nemeroff, C. B., Caldji, C., Sharma, S., Meaney, M. J. Long-term consequences of neonatal rearing on central corticotropin-releasing factor systems in adult male rat offspring. Neuropsychopharmacology. 2005,30:2192-204.
[29] Liu, D., Diorio, J., Day, J. C., Francis, D. D., Meaney, M. J. Maternal care, hippocampal synaptogenesis and cognitive development in rats. Nature neuroscience. 2000,3:799-806.
[30] Veenema, A. H., Blume, A., Niederle, D., Buwalda, B., Neumann, I. D. Effects of early life stress on adult male aggression and hypothalamic vasopressin and serotonin. The European journal of neuroscience. 2006,24:1711-20.
[31] Lukas, M., Bredewold, R., Landgraf, R., Neumann, I. D., Veenema, A. H. Early life stress impairs social recognition due to a blunted response of vasopressin release within the septum of adult male rats. Psychoneuroendocrinology. 2011,36:843-53.
[32] Parent, C. I., Meaney, M. J. The influence of natural variations in maternal care on play fighting in the rat. Developmental psychobiology. 2008,50:767-76.
[33] Zhang, T. Y., Chretien, P., Meaney, M. J., Gratton, A. Influence of naturally occurring variations in maternal care on prepulse inhibition of acoustic startle and the medial prefrontal cortical dopamine response to stress in adult rats. J Neurosci. 2005,25:1493-502.
[34] Ellenbroek, B. A., Cools, A. R. The long-term effects of maternal deprivation depend on the genetic background. Neuropsychopharmacology. 2000,23:99-106.
[35] Ellenbroek, B. A., van den Kroonenberg, P. T., Cools, A. R. The effects of an early stressful life event on sensorimotor gating in adult rats. Schizophrenia research. 1998,30:251-60.
[36] Claessens, S. E., Daskalakis, N. P., van der Veen, R., Oitzl, M. S., de Kloet, E. R., Champagne, D. L. Development of individual differences in stress responsiveness: an overview of factors mediating the outcome of early life experiences. Psychopharmacology. 2011,214:141-54.
[37] Feinberg, M. E., Neiderhiser, J. M., Simmens, S., Reiss, D., Hetherington, E. M. Sibling comparison of differential parental treatment in adolescence: gender, self-esteem, and emotionality as mediators of the parenting-adjustment association. Child Dev. 2000,71:1611-28.
[38] Feinberg, M. E., Hetherington, E. M. Sibling differentiation in adolescence: implications for behavioral genetic theory. Child Dev. 2000,71:1512-24.
[39] Ouellet-Morin, I., Boivin, M., Dionne, G., Lupien, S. J., Arseneault, L., Barr, R. G., et al. Variations in heritability of cortisol reactivity to stress as a function of early familial adversity among 19-month-old twins. Arch Gen Psychiatry. 2008,65:211-8.
[40] Okimoto, D. K., Blaus, A., Schmidt, M. V., Gordon, M. K., Dent, G. W., Levine, S. Differential expression of c-fos and tyrosine hydroxylase mRNA in the adrenal gland of the infant rat: evidence for an adrenal hyporesponsive period. Endocrinology. 2002,143:1717-25.
[41] Rosenfeld, P., Suchecki, D., Levine, S. Multifactorial regulation of the hypothalamic-pituitary-adrenal axis during development. Neuroscience and biobehavioral reviews. 1992,16:553-68.
[42] Raineki, C., Moriceau, S., Sullivan, R. M. Developing a neurobehavioral animal model of infant attachment to an abusive caregiver. Biological psychiatry. 2010,67:1137-45.
[43] Sullivan, R. M., Stackenwalt, G., Nasr, F., Lemon, C., Wilson, D. A. Association of an odor

with activation of olfactory bulb noradrenergic beta-receptors or locus coeruleus stimulation is sufficient to produce learned approach responses to that odor in neonatal rats. Behavioral neuroscience. 2000,114:957-62.
[44] Sullivan, R. M., Landers, M., Yeaman, B., Wilson, D. A. Good memories of bad events in infancy. Nature. 2000,407:38-9.
[45] Bohus, B., de Kloet, E. R. Adrenal steroids and extinction behavior: antagonism by progesterone, deoxycorticosterone and dexamethasone of a specific effect of corticosterone. Life sciences. 1981,28:433-40.
[46] Cai, W. H., Blundell, J., Han, J., Greene, R. W., Powell, C. M. Postreactivation glucocorticoids impair recall of established fear memory. J Neurosci. 2006,26:9560-6.
[47] Brinks, V., de Kloet, E. R., Oitzl, M. S. Strain specific fear behaviour and glucocorticoid response to aversive events: modelling PTSD in mice. Progress in brain research. 2008,167:257-61.
[48] Daskalakis, N., Diamantopoulou, A., Tjälve, M., Meelis, W., Oitzl, M. S., de Kloet, E. R. Early-life adversity load predicts psychosis-like behaviour in the rat. Chicago, IL: Society for Neuroscience, 2009. 2009:Online, Program No. 341.9/O18.
[49] Daskalakis, N. P., Oitzl, M. S., Schächinger, H., Champagne, D. L., de Kloet, E. R. Testing the cumulative stress and mismatch hypotheses of psychopathology in a rat model of early-life adversity. submitted. 2011.
[50] Pryce, C. R., Bettschen, D., Feldon, J. Comparison of the effects of early handling and early deprivation on maternal care in the rat. Developmental psychobiology. 2001,38:239-51.
[51] Macri, S., Mason, G. J., Wurbel, H. Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats. The European journal of neuroscience. 2004,20:1017-24.
[52] Bagot, R. C., van Hasselt, F. N., Champagne, D. L., Meaney, M. J., Krugers, H. J., Joels, M. Maternal care determines rapid effects of stress mediators on synaptic plasticity in adult rat hippocampal dentate gyrus. Neurobiology of learning and memory. 2009,92:292-300.
[53] Macri, S., Wurbel, H. Developmental plasticity of HPA and fear responses in rats: a critical review of the maternal mediation hypothesis. Hormones and behavior. 2006,50:667-80.
[54] Heim, C., Newport, D. J., Miller, A. H., Nemeroff, C. B. Long-term neuroendocrine effects of childhood maltreatment. JAMA. 2000,284:2321.