Severe Early Life Stress Hampers Spatial Learning and Neurogenesis, but Improves Hippocampal Synaptic Plasticity and Emotional Learning under High-Stress Conditions in Adulthood

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Early life stress increases the risk for developing stress-related pathologies later in life. Recent studies in rats suggest that mild early life stress, rather than being overall unfavorable, may program the hippocampus such that it is optimally adapted to a stressful context later in life. Here, we tested whether this principle of “adaptive programming” also holds under severely adverse early life conditions, i.e., 24 h of maternal deprivation (MD), a model for maternal neglect. In young adult male rats subjected to MD on postnatal day 3, we observed reduced levels of adult hippocampal neurogenesis as measured by cell proliferation, cell survival, and neuronal differentiation. Also, mature dentate granule cells showed a change in their dendritic morphology that was most noticeable in the proximal part of the dendritic tree. Lasting structural changes due to MD were paralleled by impaired water maze acquisition but did not affect long-term potentiation in the dentate gyrus. Importantly, in the presence of high levels of the stress hormone corticosterone, even long-term potentiation in the dentate gyrus of MD animals was facilitated. In addition to this, contextual learning in a high-stress environment was enhanced in MD rats. These morphological, electrophysiological, and behavioral observations show that even a severely adverse early life environment does not evolve into overall impaired hippocampal functionality later in life. Rather, adversity early in life can prepare the organism to perform optimally under conditions associated with high corticosteroid levels in adulthood.

Introduction

Epidemiological studies in humans have shown that adverse early life events can increase the risk for the development of psychopathology in adulthood (Heim and Nemeroff, 2001; McEwen, 2003). In rodents, effects of early life stress can be studied in a controlled manner by long-term removal of the dam, i.e., maternal separation or maternal deprivation (MD) during the first 2 weeks of life, which increases hypothalamic-pituitary-adrenal (HPA) axis activity in the offspring (Levine et al., 1991; Schmidt et al., 2004; Oomen et al., 2009). This rise in corticosterone (CORT) during the so-called stress hyporesponsive period is thought to exert long term programming effects on HPA axis parameters (Plotsky and Meaney, 1993; Penke et al., 2001; Workel et al., 2001; Lehmann et al., 2002).

When stress occurs during the first two postnatal weeks in rodents, it coincides with the development of the dentate gyrus (DG) of the hippocampus (Altman and Bayer, 1990a, 1990b), a brain region involved in learning and memory and sensitive to glucocorticoid exposure (McEwen and Magarinos, 2001; de Kloet et al., 2005). Emphasizing its persistent nature, MD in rodents was found to affect hippocampal structure in adulthood, resulting in lower hippocampal neuron and glia numbers (Leventopoulos et al., 2007; Fabricius et al., 2008) and reduced mossy fiber density (Huot et al., 2002). With respect to adult hippocampal neurogenesis, repeated maternal separation was shown to reduce only cell proliferation (Mirescu et al., 2004; Aisa et al., 2009) but not newborn cell survival in rats (Mirescu et al., 2004; Greisen et al., 2005; Petersen et al., 2008). These studies confirm the potentially unfavorable effects of early life stress.

Very recent studies, however, suggest that rather than being overall unfavorable, early life stress may program the hippocampus such that it optimally responds to stressful contexts encountered later in life (Champagne et al., 2008, 2009; Lyons et al., 2009). For example, the adult male offspring of low- versus high-caring mothers showed reduced synaptic plasticity when animals were studied under nonstress conditions, but when studied under conditions that mimic stress, low- versus high-care offspring exhibited enhanced synaptic plasticity (Champagne et al., 2008;

Received Jan. 15, 2010; revised March 23, 2010; accepted March 30, 2010.

P.J.L. was supported by the Nederlandse Hersenstichting, the European Union (Neurodegeneration in Alzheimer's Disease), ISAQ, and the Volkswagen Stiftung Germany. We thank Joop van Heerikhuizen (Netherlands Institute for Neuroscience, Amsterdam, The Netherlands) for assistance with the Neurodraw software; Els Velzing (Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands) for technical assistance, and Maaike van der Mark (Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, The Netherlands) for the analysis of plasma corticosterone levels.

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DOI:10.1523/JNEUROSCI.0247-10.2010

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Bagot et al., 2009). In agreement, offspring from low-caring mothers performed poorly in relatively low-stress learning conditions (Liu et al., 2000), but outperformed the high-care offspring in stressful learning tasks (Champagne et al., 2008; Bagot et al., 2009).

As differences in maternal care represent relatively small natural variations in the early life environment, we here asked whether the principle of “adaptive programming” also holds under severe early life stress conditions of prolonged (24 h) absence of the mother at postnatal day 3 (PND3). Therefore, we studied the consequences of early MD on different phases of adult hippocampal neurogenesis and granule cell morphology in males. In addition, we determined the functional consequences of maternal deprivation by investigating spatial and emotional learning and memory as well as synaptic plasticity in the dentate gyrus after MD, both under low-to-moderate stress conditions and in a high-stress emotional context.

Materials and Methods

Animals and breeding procedure. All animal procedures presented in this article were approved by the animal ethics committee of the University of Amsterdam (Amsterdam, The Netherlands). To minimize variation and avoid stress in the perinatal environment, all animals were bred in house. Wistar rats were purchased from Harlan CBP, kept under standard housing conditions (12/12 h dark/light phase, lights on at 8:00 A.M., humidity $55 \pm 15\%$, temperature $20\text{--}22^\circ\text{C}$), and habituated to the animal facilities for 10 d. For breeding, one male rat was put together with two females for a period of 1 week. After mating, females were pair housed until the beginning of the third gestational week. Then, females were individually housed with extra bedding material and monitored for birth each morning at 9:00 A.M. If a litter was found, the previous day was designated PND0, the day of birth. Dams with litters were left undisturbed until PND3 and then randomly assigned to one of the two experimental groups [MD or control (CON) procedure], making sure that litters from the same father were not in the same experimental group.

Maternal deprivation procedure. On the morning of PND3 (9:00 A.M.), litters were separated from their mother as a whole, handled shortly, culled to four males and four females, and placed back into their home cage. The cage was placed on a heating pad in another room to avoid disturbance by vocalization in the breeding room. The dam was housed in a novel cage and returned to the breeding room. Litters were kept at a constant temperature of 32°C during the 24 h deprivation period. Litters were, with the exception of two litters of six pups, larger than or equal to eight pups, with an average of 11 ± 2 pups. Due to large litter size, culling to four males and four females was usually possible. This study repeated the exact same experimental procedure as used in our previous study (Oomen et al., 2009). No pups were lost during the MD procedure. At 9:00 A.M. on PND4, cages were cleaned by replacing some of the sawdust, after which the dam was returned to the nest. Litters from the CON group were culled and partially cleaned at the moment of disturbance on PND3 (11:00 A.M.). Cages were put back into the breeding room and left undisturbed until weaning, with the exception of PND14, when some of the sawdust was replaced. On PND21, pups were weaned and housed in groups of four same-sex littermates for experiments I, II, III, and IV or in pairs for experiment V.

Experiment I: baseline and stress-induced corticosterone levels. To investigate the effect of early life stress on baseline corticosterone levels, 8- to 13-week-old CON ($n = 30$) and MD ($n = 23$) rats were killed by rapid decapitation between 9:00 and 10:00 A.M., trunk blood was collected in EDTA-covered tubes, centrifuged (5000 rpm for 20 min), and blood plasma was stored at -20°C until further processing. Stress-induced corticosterone levels were measured in a different cohort of CON ($n = 8$) and MD ($n = 9$) rats of 14–16 weeks of age. In the morning (between 9:00 and 10:00 A.M.), rats were brought to a novel room where a baseline blood sample was collected by tail bleeding from a small incision made halfway down the length of the tail (Flutterm et al., 2000). Blood was collected in an EDTA-covered capillary tube. Thirty minutes later, rats

were brought to the same room to collect a second sample by means of tail bleeding, which was considered the stress-induced blood sample. Blood was processed and stored as described above. Two weeks later, corticosterone levels were determined in the same animals during recovery from a swim stress. For this reason, rats were placed into a water maze (22°C , 150 cm in diameter, no platform present) for 2 min between 9:00 and 10:00 A.M. After this, rats were taken out of the water and placed back into their home cage. One hour after swim stress, rats were decapitated and trunk blood was collected and processed as described above. Plasma corticosterone concentrations from all three experiments were measured in duplicate using a commercially available radio immunoassay kit (MP Biochemicals).

Experiment II: adult neurogenesis and dentate gyrus architecture. To determine lasting effects of early MD on different phases of adult neurogenesis, eight CON and eight MD males (from four CON and four MD litters) were injected with bromodeoxyuridine (BrdU; 200 mg/kg, intraperitoneally; Sigma-Aldrich) on PND51 and killed 18 d later. On that day, animals were anesthetized in the morning by an injection of pentobarbital sodium salt (Nembutal; 1 mg/kg bodyweight; A.U.V. Cuijk) and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). To prevent pressure artifacts, brains were postfixed overnight in the skull at 4°C , after which they were carefully removed, washed, and cryoprotected by 20% sucrose in PBS. Frozen sections ($30\text{ }\mu\text{m}$ thick) were cut using a sliding microtome and collected in PB with azide.

Different stages of neurogenesis were studied as described previously (Mayer et al., 2006; Oomen et al., 2007). Immunohistochemistry for BrdU (1:2000; monoclonal murine anti-BrdU; Roche) was used to assess cell proliferation/newborn cell survival. The marker Ki-67, a cell cycle-related protein identifying all cells actively engaged in cell cycle (1:2000; polyclonal rabbit α -Ki-67; Novocastra), was used to assess cell proliferation. In addition, the number of young, differentiating neurons was identified with an antibody against the microtubule-associated protein doublecortin (DCX; 1:800; polyclonal goat anti-DCX; Santa Cruz Biotechnology). Amplification was performed with a biotinylated secondary antibody [sheep anti-mouse (1:200; GE Healthcare), goat anti-rabbit (1:200; Vector Laboratories), or donkey anti-goat (1:500; Jackson ImmunoResearch Laboratories)] and avidin-biotin complex (1:1000; Elite Vectastain ABC kit, Brunschwig Chemie) in combination with tyramide (1:500; 0.01% H_2O_2 ; kindly provided by Dr. I. Huitinga, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands). Subsequent chromogen development was done with diaminobenzidine (20 mg per 100 ml of Tris buffer, 0.01% H_2O_2).

All stereological quantification procedures described below were performed in every 10th coronal section along the entire rostrocaudal axis, in a total of 9 sections per animal. Total numbers of DG granule neurons and DCX-positive cells were quantified by systematic random sampling performed with the Stereo Investigator system (MicroBrightField). Stereo Investigator optical fractionator settings for the quantification of DCX were 140×80 grid size and 50×50 counting frame, which resulted in 300–500 markers per animal. StereoInvestigator settings for total granule cell count were 150×150 grid size and 15×15 counting frame, which resulted in 300–500 markers per animal.

We further distinguished morphologically different subtypes of the DCX-immunopositive cells, reflecting different stages of neuronal differentiation (Plumpe et al., 2006); the most mature DCX-positive cells were characterized by a primary dendrite that was orientated perpendicular to the subgranular zone and protruding into the molecular layer (categories E and F). DCX-positive cells without dendrites (category A), horizontally orientated dendrites, or dendrites growing into the granule cell layer but not into the molecular layer (categories B–D) are considered less mature and can still undergo cell division (Kronenberg et al., 2003; Plumpe et al., 2006; Walker et al., 2007).

Because of the relatively sparse occurrence and clustering of Ki-67-positive and BrdU-positive cells, these cells were counted manually by means of a modified stereological procedure using a Zeiss microscope ($200\times$ magnification) and multiplied by 10 to estimate the total number of Ki-67-positive and BrdU-positive cells in the DG (van Praag et al., 1999; Oomen et al., 2007). Dentate gyrus granule cell layer and molecular

layer surface area and volume measurements were performed according to Cavalieri's principle using the Stereo Investigator system (MicroBrightField).

Experiment III: granule cell morphology. Effects of maternal deprivation on granule cell morphology were determined by analyzing the dendritic tree of Golgi-stained neurons using three-dimensional (3D) reconstruction software. For this purpose, 8- to 13-week-old CON ($n = 7$) and MD ($n = 7$) animals were decapitated in the morning between 9:00 and 10:00 A.M. and trunk blood was collected in EDTA-covered tubes (experiment I). Immediately after decapitation, brains were rapidly removed and cut into two hemispheres. One hemisphere was used for Golgi–Cox impregnation and the other for electrophysiological recordings (experiment IV).

For Golgi–Cox impregnation, a similar procedure was used as described previously (Boekhoorn et al., 2006; Champagne et al., 2008; Bagot et al., 2009). Immediately after decapitation, brains were incubated in a Golgi–Cox solution (5% K_2CrO_4 , 5% $HgCl_2$, and 5% $K_2Cr_2O_7$) for 28 d, after which they were imbedded in celloidine and cut into 200- μ m-thick sections. From each animal, Z-stacks (step size 1 μ m) from 5–7 dentate granule cells were generated using a confocal microscope (LSM510, Zeiss) in bright-field mode (20 $\times$ objective) and reconstructed in ImagePro in combination with the NeuroDraw toolbox (kindly provided by G. Ramakers, J. van Heerikhuizen, and C. Pool, Netherlands Institute for Neuroscience). Criteria for inclusion were as follows: (1) only neurons from the suprapyramidal blade of the rostral dentate gyrus, -2.5 to -4.0 mm from bregma (Paxinos and Watson, 1986) were selected; (2) cells had to be evenly filled, without any severed dendrites exiting the section; (3) only cells from the middle third part of the granular cell layer were chosen to avoid relatively newborn granule cells residing in the inner part of the cell layer or cells formed early in ontogeny (embryonic days 21–22) that reside in the outer third part of the cell layer (Altman and Bayer, 1990a,b). Total dendritic length, number of branch points, and the number of primary dendrites were analyzed for every neuron. In addition, spine density was determined in two segments of dendrites at a distance of 90–110 μ m (proximal) and 190–210 μ m (distal) from the soma. Also, for each reconstructed neuron, a 3D Sholl analysis was performed using the free software package NeuronStudio (Wearne et al., 2005). Data from 5–7 neurons were averaged per animal and used in further statistical analysis.

Experiment IV: electrophysiological properties of the dentate gyrus. To determine effects of maternal deprivation on electrophysiological properties of the DG network, 42 8- to 13-week-old male rats were decapitated in the morning between 9:00 and 10:00 A.M. If possible, two measurements were performed in each animal: one under vehicle (VEH) conditions and one under corticosterone conditions. In total, this resulted in 67 long-term potentiation (LTP) recordings in the presence and absence of bicuculline. First, we studied LTP in the absence of the GABAergic antagonist bicuculline in 12 control and 9 MD animals. Because this did not induce significant LTP, further experiments were performed in the presence of bicuculline in 11 CON and 10 MD animals.

For these experiments, stress from individual housing was avoided by decapitating the last two animals from the same cage simultaneously. Immediately after decapitation, the brain was rapidly removed and cut into two hemispheres. The left hemisphere was collected for Golgi–Cox impregnation (experiment III) and the right hemisphere was collected for electrophysiological recordings in ice-cold artificial CSF (aCSF) containing 120 mM NaCl, 3.5 mM KCl, 1.3 mM $MgSO_4 \cdot 7H_2O$, 1.25 mM NaH_2PO_4 , 2.5 mM $CaCl_2 \cdot 2H_2O$, 10 mM glucose, and 25 mM $NaHCO_3$ and oxygenated with 95% O_2 and 5% CO_2 . Coronal slices (400 μ m) were cut using a microtome (Leica VT1000S) at 5°C and then kept in oxygenated aCSF at room temperature for at least 2 h before recording. Sections containing the rostral part (-2.5 to -4.0 mm from bregma) (Paxinos and Watson, 1986) of the hippocampal dentate gyrus were placed in a recording chamber maintained at 30–32°C with a constant flow of oxygenated aCSF. Field EPSPs (fEPSPs) were recorded as described previously (Pu et al., 2007; Bagot et al., 2009) in the absence or presence of the GABAergic antagonist bicuculline methiodide (10 μ M; Tebu-bio). fEPSPs were evoked using a stainless steel bipolar stimulation electrode (60 μ m diameter, insulated except for the tip) positioned in the medial per-

forant pathway and recorded through a glass electrode (2–5 M Ω impedance, filled with aCSF) positioned in the middle third of the molecular layer of the upper blade. A stimulus–response curve was generated by gradually increasing the stimulus intensity to define a level that generated the half-maximal response that was used for the remainder of the experiment. Once the input–output curve for each recording was established, baseline synaptic transmission was monitored (0.017 Hz) during 20 min. When recordings were stable, theta burst stimulation (four pulses of 100 Hz followed by a 200 ms interval, followed by another four pulses) was applied. This sequence was repeated 5 $\times$ with a 30 s interval. After theta burst stimulation, the degree of potentiation was determined by recording the fEPSP every minute during 1 h (0.017 Hz). The magnitude of the fEPSP was assessed by analyzing the slope of the signal. To determine whether the presence of the stress hormone corticosterone during stimulation modulates the degree of LTP, corticosterone (100 nM, dissolved in 0.01% ethanol; Sigma-Aldrich) or vehicle (0.01% ethanol) was added to the aCSF during the second half of baseline recordings ($t = -10$ to 0 min), coterminating with theta burst stimulation (Bagot et al., 2009). This concentration of corticosterone was shown before to occupy both mineralocorticoid receptor and glucocorticoid receptor and is therefore comparable and relevant to mimic *in vivo* stressful situations (Karst et al., 2000; Champagne et al., 2008).

Experiment V: behavior. One cohort of animals (10 CON and 10 MD rats) was used for three behavioral tasks described in the following section. Animals were housed in pairs. During testing, rats from the same home cage were tested on different days to avoid effects of acute stress. Before and in between tasks, rats were handled every other day, starting 2 weeks before the start of the first experiment. The order of testing was as described below, and the time in between tasks was at least 1 week. All behavioral tests were performed between 8:00 and 12:00 A.M.

To study potential changes in basal exploration and anxiety levels after maternal deprivation, CON ($n = 10$) and MD ($n = 10$) rats were tested in an elevated plus maze at the age of 11 weeks. For this, each rat was transferred from its home cage to the experimental setup in the adjacent room. In this testing room, at a light intensity of 60 lux, the animals were put on the center of an elevated plus maze (100 cm. from the ground) facing one of the two open arms. The plus maze was made of black plastic and shaped like a cross with two opposite open arms (10 $\times$ 40 cm) and two opposite closed arms (10 $\times$ 40 cm, 25 cm high walls) connected to an open center. Animals were placed in the center and allowed to freely explore the open and closed arms for 5 min. Exploration patterns were recorded by a video camera coupled to a computer and processed by EthoVision (Noldus). Total time, frequency and latency to first appearance in all compartments of the maze were analyzed to determine the exploration pattern.

To determine spatial learning ability of MD rats, animals were trained in a Morris water maze (Morris, 1984). CON ($n = 10$) and MD ($n = 10$) animals (13 weeks old) were trained in 2 d, during which they received 4 trials per day with an intertrial interval of 15 min. The water maze (150 cm in diameter) was situated in a room adjacent to where animals were housed and filled with opaque water (22°C, with added nontoxic paint). In one quadrant (northwest), a transparent platform (12 cm in diameter) was hidden 0.5 cm under the water surface. During training, trials were started in one of the three quadrants without platform (i.e., northeast, southwest, or southeast quadrant), and the starting position alternated between trials, rats, and days. At the start of each trial rats were placed in the water facing the wall of the pool. Animals were allowed to swim in the maze until they reached the platform or until a maximum time of 2 min was reached. When rats were unable to find the platform, they were guided there manually. Animals were left on the platform for an additional 20 s. Trials were recorded and analyzed for latency, swim distance, and time in the platform quadrant (EthoVision; Noldus).

To assess hippocampus and amygdala-dependent emotional memory, rats were subjected to contextual and cued fear conditioning (Phillips and LeDoux, 1992; Zhou et al., 2009). During fear conditioning, 15-week-old rats (CON = 10; MD = 10) learned to associate a fearful stimulus (footshock) with a context (conditioning box) and a cue (tone). The strength of fear-associated memory was determined by measuring the amount of freezing behavior. Scoring was done manually, every 2 s,

by two observers blinded to the experimental group. Rats were habituated to the experimental room starting 2 d before conditioning. Animals were trained by placing them in the fear-conditioning box (30 × 26 × 24 cm, walls made of black and transparent plastic with metal grid floor), which they were allowed to explore freely for 3 min. After that, a 30 s tone was played (100 dB, 2.8 kHz) that coterminated with a footshock (2 s, 0.4 mA). On the second day at the same time, animals were put into the conditioning box for 3 min under identical circumstances (i.e., the same cleaning solvent, lighting conditions, gloves, and lab coats). Contextual memory was evaluated by scoring freezing behavior. Two hours later, cued memory was tested by placing rats in a different box, with a solid white floor and light-colored walls under changed circumstances (i.e., different cleaning solvent, lighting conditions, gloves, and lab coat). Again, rats were allowed to explore the box freely for 3 min, after which the tone was presented for 30 s. Rats remained in the box for an additional 60 s to determine the behavioral response to the cue.

Statistical analysis. Statistical analysis was performed using SPSS16.0. All data are presented as average ± SEM. Differences between CON and MD animals concerning body weight, corticosterone levels, levels of neurogenesis (all expressed per hemisphere), morphological parameters, and water maze probe trial were tested for significance using a two-tailed Student's *t* test with a probability level of 5% after determining equality of variances using Levene's test (all data *p* > 0.05).

Differences in Sholl plots, acquisition of the water maze, and context-dependent and cue-dependent freezing were tested with a single-factor repeated-measures ANOVA using early life treatment as a between-subjects factor. The within-subjects factors used were as follows: interval from the soma (Sholl plot), trial (water maze), consecutive time interval (contextual fear conditioning), and before-tone versus after-tone interval (cued fear conditioning), respectively.

Effects of corticosterone treatment on baseline synaptic transmission were tested by comparing the average slope of the fEPSP of the first baseline (−20 to −10 min) with that of the second baseline (−10 to 0 min) using a three factor repeated-measures ANOVA (between-subjects factors: early life treatment and drug-treatment; within-subjects factor: baseline). To determine the effects of early life stress and application of the stress hormone corticosterone on the degree of LTP, a three-factor repeated-measures ANOVA was performed using early life treatment and corticosterone treatment as between-subject factors and pre-theta burst stimulation or post-theta burst stimulation as the within-subject factor. We compared the second baseline (−10 to 0 min) with early LTP; (0–30 min after theta burst stimulation) and total LTP (0–60 min after theta burst stimulation). When significant, a *post hoc* LSD test was performed to compare treatment groups.

Results

Experiment I: body weight and stress responsiveness

On PND51, MD animals had significantly (*p* = 0.01, both groups *n* = 8) lower bodyweights (263 ± 6 g) compared with CON animals (291 ± 8 g). As reported previously (Oomen et al., 2009), this difference in body weight after MD in adult animals was already present directly after deprivation, i.e., at PND4, and still discernable at PND21, which supports the idea that this effect is early in onset.

Baseline corticosterone levels were taken from animals decapitated in the morning between 9:00 and 10:00 A.M. These basal levels were not significantly affected by maternal deprivation [CON (*n* = 30), 8.24 ± 1.38 ng/ml; MD (*n* = 23), 13.07 ± 2.94 ng/ml; *p* = 0.12]. In addition, 30 min after novelty stress, corticosterone levels were comparable between CON (187 ± 48 ng/ml) and MD (154 ± 88 ng/ml) animals (*p* > 0.1). One hour after swimming in a water maze, both CON (427 ± 84 ng/ml) and MD (281 ± 46 ng/ml) rats showed elevated corticosterone levels, with a trend toward a lower plasma corticosterone levels in MD animals (*p* = 0.1) (both groups *n* = 9).

Figure 1. Effects of MD on hippocampal cell proliferation (Ki-67) and survival (BrdU). **A**, Photomicrograph of clustered Ki-67 labeled cells (arrow) in the subgranular zone of the dentate gyrus of 10-week-old rats. GCL, Granule cell layer; ML, molecular layer. **B**, Total numbers of Ki-67-positive cells in the subgranular zone (SGZ) and hilus per hemisphere (*n* = 8). MD treatment significantly reduced the number of Ki-67-positive cells in the subgranular zone (**p* = 0.03), but not the hilus (*p* = 0.2). **C**, Photomicrograph of BrdU labeled cells in the subgranular zone (arrowhead) and GCL (arrow). **D**, Total BrdU-positive cell numbers (*n* = 8) in the subgranular zone and granule cell layer per hemisphere were not affected by maternal deprivation (*p* = 0.34), but in the caudal part of the DG the number of BrdU-positive cells was significantly reduced (**p* = 0.03).

Experiment II: adult neurogenesis is reduced by maternal deprivation

Quantification of Ki-67-positive cells (Fig. 1A) revealed a significantly lower level of cell proliferation in the hippocampus of 10-week-old MD rats when compared with control rats (*p* = 0.03, both groups *n* = 8) (Fig. 1B). This effect was evenly distributed along the rostrocaudal axis (data not shown) and was specific for the subgranular zone and not present in the hilus (*p* = 0.2).

Overall cell proliferation/survival, as measured by the total number of BrdU-positive cells in the subgranular zone and granular cell layer (Fig. 1C), was not affected by MD (*p* = 0.34) (Fig. 1D). However, when examined along the rostrocaudal axis, a significant decrease was found in the caudal part (last four sections −4.5 to −6.7 mm from bregma) of the DG (*p* = 0.03) (Fig. 1D).

The total number of immature neurons was determined by DCX-positive cell numbers (Fig. 2A). Absolute DCX-positive cell numbers per hemisphere did not differ between CON and MD (*p* = 0.16) (Fig. 2B). Interestingly, we also observed here an effect along the rostrocaudal axis. MD males displayed lower ab-

Figure 2. Effects of MD on neuronal differentiation (doublecortin immunohistochemistry). **A**, Photomicrograph of the dentate gyrus of 10-week-old male rats, showing examples of DCX expression along the subgranular zone. **B**, Total numbers of DCX-positive cells in the subgranular zone per hemisphere were not different between the groups ($n = 8$); however, MD treatment caused a significantly lower number of DCX-positive cells in the caudal part of the dentate gyrus ($*p = 0.04$). **C**, Examples of relatively immature DCX-positive cells (arrow, category A; arrowhead, category B/C). **D**, An example of an intermediate DCX phenotype with outgrowing dendrites (category C). GCL, Granule cell layer; sgz, subgranular zone. **E**, Example representing a mature DCX phenotype (category F). ML, Molecular layer. **F**, The percentage of DCX-positive cells as a percentage of total granule cells was decreased in MD animals ($*p = 0.04$) because of a decline in the more immature DCX-positive cells (categories A–D); $\#p = 0.01$. Mature DCX cell types were unaffected (classification according to Plumpe et al., 2006).

Table 1. Effects of MD on granule cell morphology^a

Morphological parameter	CON	MD
Dendritic length (μm)	1602 $\pm$ 115	1608 $\pm$ 78
Number of branch points	8.1 $\pm$ 0.9	8.5 $\pm$ 0.5
Number of primary dendrites	2.96 $\pm$ 0.1	2.06 $\pm$ 0.2*
Maximum length of primary dendrites (μm)	253 $\pm$ 11	286 $\pm$ 7*
Spine density (number of spines/ μm)	0.5 $\pm$ 0.06	0.7 $\pm$ 0.06 [#]

^aMorphological features of granule cells in CON ($n = 7$) and MD ($n = 7$) animals are summarized. MD treatment resulted in a change in dendritic arrangement, most notably in the number and the maximum length of primary dendrites ($*p < 0.05$). In addition, a trend toward an increase in spine density was observed ($\#p < 0.1$).

solute DCX-positive cell numbers compared with control animals between -4.5 and -6.7 mm from bregma ($p = 0.04$) (Fig. 2B). If the total (along the entire rostro-to-caudal axis) DCX-positive cell number is expressed as a percentage of total granule neurons, MD animals showed a lower percentage of DCX-positive cells ($p = 0.04$, Fig. 2F). This decrease was mainly attributable to cells belonging to categories A–D (Plumpe et al., 2006), representing the more immature phenotype as determined by subsequent morphological characterization (Fig. 2C–E); MD males showed a lower number of the more immature DCX-

positive cells type A through D ($p = 0.01$) (Fig. 2F) compared with control, while no effect of MD was observed for cells from categories E and F.

The volumes of the granular cell layer (CON, 1.14 ± 0.03 mm³; MD, 1.07 ± 0.03 mm³; $p = 0.12$) and molecular cell layer (CON, 3.38 ± 0.08 mm³; MD, 3.31 ± 0.15 mm³; $p = 0.67$) of the dentate gyrus were not significantly changed by maternal deprivation. Also, total granule cell number was comparable (CON, $769,795 \pm 17,817$; MD, $805,349 \pm 33,316$; $p = 0.38$).

Experiment III: dendritic complexity is altered by maternal deprivation

Maternal deprivation affected the shape of the dendritic tree of granule neurons in adult males (Table 1). Representative examples of reconstructed Golgi-stained neurons are shown in Figure 3, A (CON) and B (MD). Although total dendritic length and the number of branch points were not changed, MD treatment decreased the number of primary dendrites ($p = 0.03$), which was paralleled by an increase in the maximum length of individual primary dendrites ($p = 0.04$). This resulted in a decreased dendritic mass in the proximal part of the dendritic tree ($1-85$ μm from the soma; repeated-measures ANOVA, main effect for treatment, $F_{(1,12)} = 7.82$; $p = 0.02$), as revealed by Sholl analysis (Fig. 3C). In addition, there was a trend for an increased total spine density in both the proximal and distal part of the dendritic tree ($p = 0.09$).

Experiment IV: synaptic plasticity in a stressful context is enhanced after

maternal deprivation

First, we examined whether LTP could be induced in the dentate gyrus using theta burst stimulation, but in neither CON ($n = 12$) nor MD ($n = 9$) animals was the DG network significantly potentiated. The presence of the stress hormone corticosterone did not change this (repeated-measures ANOVA baseline vs post-theta burst stimulation ($1-60$ min), $F_{(1,29)} = 0.03$; $p = 0.86$). This is not unprecedented, since GABAergic innervation is known to drive dentate granule cells *in vitro* away from their firing threshold (Wisden et al., 1992; Coulter and Carlson, 2007). Therefore, we applied theta burst stimulation in the presence of the GABAergic antagonist bicuculline methiodide, which resulted in an overall significant induction of LTP (repeated-measures ANOVA, $F_{(1,30)} = 18.75$; $p < 0.001$). Therefore, in all experiments described below, bicuculline was present.

There were no differences between CON ($n = 11$) and MD ($n = 10$) animals concerning the maximum response (CON, -2.49 ± 0.21 mV; MD, -2.66 ± 0.24 mV; $p = 0.60$), the half-maximum stimulation intensity (CON, 1.91 ± 0.02 arbitrary units; MD, 1.92 ± 0.02 arbitrary units; $p = 0.84$), or the half-

Figure 3. MD alters granule cell morphology in the dentate gyrus. *A, B*, Representative example of a Golgi-stained neuron and corresponding dendritic tree of a granule neuron in a control animal (*A*) and a MD animal (*B*). The 3D tracing of dendrites can be seen in blue, and the location of the proximal and distal part of the dendritic tree where spines were quantified is indicated in yellow ($n = 7$ animals per group). *C*, MD animals show a loss of dendritic mass in the proximal part of the dendritic tree (up to 85 μm from the soma) as revealed by Sholl analysis ($*p = 0.02$).

maximum response (CON, -1.51 ± 0.12 mV; MD, -1.49 ± 0.14 mV; $p = 0.93$), as determined from the input–output curve.

The effects of corticosterone on the degree of long-term potentiation was determined by perfusion of 100 nM corticosterone during the second half of baseline recordings, coterminating with the end of theta burst stimulation. Corticosterone application in

Figure 4. Effects of MD on synaptic plasticity. *A*, LTP measured under vehicle conditions (CON, $n = 10$; MD, $n = 10$). There was no effect of maternal deprivation on the degree of LTP in the dentate gyrus after theta burst stimulation. *B*, LTP measured after acute CORT application. In the presence of corticosterone, MD animals show a significantly greater LTP compared with CON ($*p = 0.01$). In CON animals ($n = 6$), no significant LTP ($p = 0.67$) was induced. All effects are significant over the first 30 min after theta burst stimulation.

itself had no effect on the magnitude of the signal during the second half of baseline recordings, as revealed by a three-way repeated-measures ANOVA comparing baseline 1 ($t = -20$ to -10 min) with baseline 2 ($t = -10$ to 0 min) (baseline $\times$ drug $\times$ group, $F_{(1,30)} = 0.03$, $p = 0.86$; drug $\times$ group, $F_{(1,30)} = 0.04$, $p = 0.84$). For LTP, we found a significant main effect of group (CON vs MD; $F_{(1,30)} = 5.60$, $p = 0.02$) and a significant interaction effect between group (CON vs MD) and treatment (VEH vs CORT; $F_{(1,30)} = 4.30$, $p = 0.04$), comparing the second half of baseline recordings with the first 30 min of after theta burst stimulation recordings. As can be seen from Figure 4*A*, there was no difference in the degree of LTP ($t = 0$ –30 min) between CON ($n = 10$) and MD ($n = 10$) recordings under VEH conditions (*post hoc* LSD, $p = 0.81$). However, as can be seen from Figure 4*B*, application of corticosterone prevented the induction of LTP in recordings from CON animals ($n = 6$) (repeated-measures ANOVA revealed no difference between after and after theta burst stimulation, $F_{(1,6)} = 0.22$, $p = 0.67$) and enhanced LTP in recordings from MD animals ($n = 8$) (*post hoc* MD VEH vs MD CORT, $p = 0.04$). This resulted in a significant difference in after theta burst stimulation recordings between CON and MD animals in the presence of CORT ($p = 0.01$).

Experiment V: spatial learning is impaired, but emotional memory is improved by maternal deprivation

Baseline anxiety, as determined by elevated plus maze exploration, was not affected by maternal deprivation; there were no

Figure 5. MD impairs Morris water maze learning. MD animals showed an overall impaired spatial acquisition (repeated measures ANOVA, main effect of early life treatment, $p < 0.05$) and required more travel distance to reach the hidden platform than CON animals. This effect was most pronounced on the second trial of both training days (trial 2 and trial 6, both $*p = 0.02$).

significant differences in latency to first appearance (CON, 51.7 ± 21.0 s; MD, 32.2 ± 10.7 s; $p = 0.33$), visiting frequency (CON, 7.8 ± 1.5 times; MD, 7.5 ± 1.7 times; $p = 0.89$), and/or percentage of time spent in the open arms of the maze (CON, $40.1 \pm 6.3\%$; MD, $45.3 \pm 6.5\%$; $p = 0.55$).

Spatial acquisition of the water maze test was significantly hampered by maternal deprivation (Fig. 5). MD animals required more time (repeated-measures ANOVA, $F_{(1,18)} = 5.83$, $p = 0.03$) and travel distance ($F_{(1,18)} = 4.45$; $p < 0.05$) to reach the hidden platform. This difference was most apparent on the second trial of both days ($p = 0.02$). However, both CON and MD animals did acquire the task equally well by the end of the training period ($p = 0.16$). Moreover, memory retention after 7 d, as measured by the total time spent in the platform quadrant during the probe trial, was not affected by MD treatment (data not shown, $p = 0.47$).

To examine emotional memory, animals were trained in a fear-conditioning paradigm using a single mild footshock (2 s, 0.4 mA) that was paired with a tone. During training, freezing behavior was comparable between control and maternally deprived animals both under baseline conditions ($p = 0.74$) and after the tone–footshock combination ($p = 0.96$). Twenty-four hours later, animals were placed in the same context for 3 min. Initially, both groups showed little freezing behavior. However, in MD animals freezing behavior significantly increased over time, while freezing in control animals remained at a constant rather low level (Fig. 6A) (group $\times$ interval; $F_{(5,125)} = 2.3$; $p < 0.05$). This resulted in an increased freezing response in the MD animals versus CON animals when measured over the last three intervals ($p = 0.02$). In the cued fear conditioning task, MD treatment resulted in a significant increase in freezing behavior in response to the tone ($p = 0.02$, Fig. 6B).

Discussion

We show that severe early life stress results in lower levels of neurogenesis, changes in granule cell morphology, and impaired spatial learning, yet leads to enhanced synaptic plasticity *in vitro* in the presence of corticosterone and to improved fear memory formation. Thus, extensive yet differential structural and functional adaptation occurs following maternal deprivation on PND3.

Figure 6. MD enhances contextual and cued fear conditioning. **A**, Maternal deprivation increased freezing behavior in response to the context (repeated-measures ANOVA, treatment $\times$ interval interaction, $p < 0.05$), resulting in an increased level of freezing when analyzed over the last three intervals ($*p = 0.02$). **B**, MD animals show increased freezing behavior in response to the tone when compared with controls ($*p = 0.02$).

Effects of maternal deprivation on adult neurogenesis

Adult hippocampal neurogenesis is reduced by stress (Cameron and Gould, 1994; Lucassen et al., 2010) and is usually reversible in adulthood (Heine et al., 2004). During early postnatal stress, the rise in corticosterone level coincides with a crucial moment in DG development. We showed previously that this causes an unexpected increased neurogenesis in 3-week-old male rats (Oomen et al., 2009). This increase in prepubertal animals appears to be transient, as 10-week-old males (this study) show a decrease in neurogenesis.

Postnatal experience can influence adult neurogenesis, and earlier studies using repeated MD have shown an inhibitory effect on cell proliferation (Mirescu et al., 2004; Aisa et al., 2009), whereas handling and high maternal care increase cell survival (Bredy et al., 2003; Lemaire et al., 2006). Since proliferation was reduced by MD, possibly resulting in less BrdU labeling, we cannot state here that survival of newborn cells is reduced. In fact, proliferation was reduced over the entire rostro-to-caudal axis, yet reduced BrdU labeling was observed caudally. This might

indicate that MD increases newborn cell survival in the rostral hippocampus. The caudal part largely contains the ventral hippocampus or temporal region of the septotemporal axis, which is anatomically and functionally distinct from the rostral part (Maggio and Segal, 2007) and associated with the amygdala (Kjelstrup et al., 2002; Bannerman et al., 2004). Changed neurogenesis after stress and antidepressant treatment are more pronounced in the ventral hippocampus (Banast et al., 2006; Sahay and Hen, 2007; Paizanis et al., 2009).

Effects of MD on dendritic morphology of granule neurons

MD altered morphology of granule neurons. Our results did not show overall atrophy, and fewer primary dendrites can be a sign of more immature neurons (Claiborne et al., 1990). However, the data rather points to dendritic reorganization after MD, since decreased dendritic material in the proximal part of the tree was paralleled by longer primary dendrites. This may result from the increase in DCX-positive cells on PND21 (Oomen et al., 2009), and changes are indicative of a reorganized neuroanatomical development of the DG. This fits with observations that early life factors can alter morphology by reducing dendritic complexity of DG, CA1, and CA3 neurons (Brunson et al., 2005; Champagne et al., 2008; Bagot et al., 2009).

The present study is not conclusive about the stress hormones involved in structural reorganization. Glucocorticoids are necessary for normal brain maturation (Meyer, 1983; Weinstock, 2001; Welberg and Seckl, 2001) and dendritic integrity depends on continuous mineralocorticoid receptor activation (Wossink et al., 2001), but glucocorticoid overexposure during development can delay CNS maturation (Huang et al., 2001; Alfarez et al., 2009; Lucassen et al., 2009). Differences in postnatal corticosteroid levels, as described after MD (Oomen et al., 2009), may therefore contribute to the morphological alterations in adulthood. However, altered corticotropin-releasing hormone levels early in life could also play a role, as this hormone too is involved in the effects of early life stress on dendritic morphology, particularly in CA3 cells (Brunson et al., 2005). Putative MD-induced shifts in corticosteroid level during adulthood seem less likely; we found no changes in basal corticosteroid levels after MD, and stress-induced corticosteroid levels even tended to be lower after MD, consistent with earlier findings showing age-dependent regulation of HPA axis reactivity by MD (Workel et al., 2001).

Effects of maternal deprivation on synaptic plasticity in the dentate gyrus

Low excitability of DG granule neurons *in vitro* is due to strong GABAergic inhibition and a hyperpolarized resting membrane potential (Wisden et al., 1992; Coulter and Carlson, 2007), making it difficult to induce LTP (Pu et al., 2007). Here, significant potentiation only occurred in the presence of the GABAergic antagonist bicuculline.

Early life adversity can impair LTP in CA3 (Brunson et al., 2005), DG (Bagot et al., 2009), and CA1 (Champagne et al., 2008), and MD causes an impairment of stress-induced late-phase LTP reinforcement in the DG (Gruss et al., 2008). Here, we found no evidence for reduced DG LTP due to MD treatment alone. Apparently, reduced neurogenesis and altered dendritic complexity do not necessarily affect the ability to elicit synaptic plasticity. Adult-born young neurons facilitate the induction of LTP (Schmidt-Hieber et al., 2004), but differences in neurogenesis may have been obscured by the presence of bicuculline, since newborn neurons lack inhibitory GABAergic input (Wang et al., 2000). Moreover, LTP recordings were performed in the dorsal

hippocampus, while MD effects on neurogenesis were most pronounced caudally.

Importantly, the presence of corticosterone during theta burst stimulation enhanced LTP in MD but not control animals. Thus, severe early life stress (24 h MD, PND3) replicates the synaptic plasticity phenotype seen earlier only in the DG of animals that received low amounts of maternal care (Bagot et al., 2009).

The biochemical basis for the effect of MD on hippocampal plasticity is presently unresolved. Brain-derived neurotrophic factor (BDNF) might be involved, as it affects hippocampal structure and plasticity (Cowansage et al., 2009) and is differently expressed in at least some models of early life stress (Kikusui et al., 2009). However, for the present model, it was shown before that 24 h MD at PND3 did not alter BDNF expression in the adult hippocampus (Schaaf et al., 2001).

Effects of maternal deprivation on learning and memory

We found that MD animals poorly acquire the hippocampus-dependent water maze. Previously, MD was found to impair water maze performance in adulthood (Oitzl et al., 2000; Uysal et al., 2005; Aisa et al., 2007; Garner et al., 2007). Many studies have found evidence for the involvement of neurogenesis in spatial acquisition (Gould et al., 1999; Clelland et al., 2009), and this type of learning seems mostly dependent on the dorsal hippocampus (Bannerman et al., 1999). A recent study, however, suggests that adult-born neurons in the ventral hippocampus are specifically activated in water maze learning (Snyder et al., 2009). Considering the overall decrease in proliferation, in combination with the effects on neurogenesis in the caudal part of the hippocampus, a reduced neurogenesis and changed neuronal morphology may have contributed to the cognitive impairment observed here.

MD animals showed enhanced contextual and cued fear conditioning. Basal anxiety as measured in the elevated plus maze and during fear training was unaffected. Studies using MD (although paradigms differ from the present study) report either no effect on contextual or cued fear conditioning (Stevenson et al., 2009) or a decreased performance (Lehmann et al., 1999; Kosten et al., 2006; Guijarro et al., 2007). However, Champagne et al. (2008), using a similar fear conditioning protocol, showed that animals with a history of low maternal care have a better memory of the fearful context. Possibly, amygdalar influences on DG network activity (Akirav and Richter-Levin, 1999; Kim and Diamond, 2002; Korz and Frey, 2005) are lastingly affected by early environment, amplified by differences in corticosterone responsiveness in the DG itself as found in our *in vitro* studies. In addition, differences in corticosterone responsiveness due to early experience in other subregions such as the CA1 can also be expected (Champagne et al., 2008). Moreover, changes in the DG, in view of its function as a filter for incoming information into the hippocampus (Hsu, 2007), may result in altered processing of relevant (stress-related) information downstream of the DG. Therefore, effects of early experience may involve functional changes in hippocampal regions other than the DG. Nevertheless, our observations at the DG cell and circuit level matched quite well with the behavioral observations, underlining that lasting consequences of early experience for DG structure and function are important for hippocampus-dependent behavioral output.

Mechanisms underlying differences in fear conditioning and the possible role for corticosterone remain to be determined. Changes in neurogenesis may explain the results on contextual

fear but not cue learning (Pham et al., 2005; Saxe et al., 2006; Kitamura et al., 2009; Revest et al., 2009). In fear conditioning, as in the water maze, better performance is associated with higher corticosterone levels (Cordero et al., 1998), although a ceiling effect exists (Pugh et al., 1997). Improved learning performance may therefore occur secondary to increased corticosteroid levels in response to stress. While this might be the case for low licking and grooming offspring, which are known to have higher stress-induced corticosterone levels (Liu et al., 1997), it certainly cannot explain the current observations, since MD animals, if anything, showed lower stress-induced corticosterone levels. Rather than differences in hormone level, altered responsiveness to corticosterone may explain why MD rats show stronger contextual fear conditioning and DG LTP in the presence of corticosterone. However, a role of stress hormones other than corticosterone cannot be excluded. Moreover, possible changes in (amygdala) dendritic complexity could play a role in enhanced fear memory.

The present morphological, electrophysiological, and behavioral observations demonstrate that even a severely adverse early life event in rats does not impair overall hippocampal functionality in adulthood. Rather, severe stress in early life might prepare animals to respond optimally under conditions associated with high corticosteroid levels during adulthood, i.e., when the adult and early life conditions closely match. Ultimately, interaction between the individual (epi)genetic profile and the early environment, such as MD, may amplify individual responsiveness in animals and can be involved in adaptive programming.

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