Programming of hippocampal structure and function by early-life stress: Opportunities for nutritional intervention

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Chapter 4

Supplementation of the maternal diet with essential micronutrients protects against early-life stress-induced cognitive impairments in offspring


Submitted
**ABSTRACT**

Early-life stress increases the risk to develop psychopathology later in life and is associated with lasting impairments in hippocampal integrity and function. The exact mechanisms involved in this early ‘programming’ remain elusive. Here we demonstrate that nutrition plays a pivotal role in programming cognition by early-life stress (ES).

By focusing on methyl donors, essential micronutrients for brain development and for the epigenetic machinery, we found ES exposure to specifically reduce levels of methionine in plasma and hippocampus of the mouse offspring, without affecting levels of homocysteine, vitamins B<sub>6</sub>, B<sub>12</sub>, B<sub>9</sub> (folic acid). Importantly, enrichment of the maternal diet with essential micronutrients during ES exposure restores methionine levels in plasma and brain of ES offspring and rescued ES-induced cognitive impairments in offspring object recognition performance and improved acquisition in the Morris water maze. We further show that these beneficial effects of the maternal diet do not involve changes in maternal care, hippocampal volume, hippocampal neurogenesis, global and *Nr3c1* specific DNA methylation or expression of key enzymes for DNA methylation (DNMT 1, 3a, 3b), but are mediated by preventing the ES-induced rise in corticosterone and adrenal gland hypertrophy in the offspring.

We show for the first time that a short, early nutritional supplementation, limited only to a specific group of essential micronutrients, can prevent lasting effects of ES on hippocampal functions in the offspring. This novel ‘window of opportunity’ allows modulating programming effects of ES exposure. As this creates new possibilities for early nutritional interventions, which is non-invasive and easily applicable, this may have important benefits for clinical practice.
INTRODUCTION

Early adversities impair hippocampal integrity and function, that manifests as deteriorated cognitive performance in adulthood, as established in clinical [1-3] and pre-clinical studies [4-7]. Because prevention of early-life stress (ES) exposure (e.g. impoverished environment, abuse, neglect) is in most cases not feasible, there is a high need for adequate intervention strategies [8]. The past 50 years have identified several key processes involved in programming of the brain, including: sensory stimuli from the mother [9-12] neuro-endocrine factors (e.g. stress-related hormones/neuropeptides [13-16], molecular mechanisms (e.g. those involved in neuronal plasticity [17-21] and epigenetic regulation [22-25] but a role for nutrition in this programming and as a potential tool for prevention has been largely ignored.

Indeed, nutrition might also be a crucial factor in programming ES effects, as: i), early brain development is a critical period with an extremely high demand for nutrients [26]; ii), there is an intense cross-talk between the stress and metabolic pathways [27-29], for instance ES exposure has been associated with altered eating behavior and metabolism [30,31]; iii) ES-exposure and early-malnutrition lead to strikingly similar cognitive outcomes [27,32,33]. Gaining further knowledge on the role of nutritional elements is thus important for our understanding of brain programming by ES exposure and could create novel opportunities for early nutritional interventions for vulnerable populations.

We here focus on a group of essential micronutrients including: methionine, homocysteine, vitamins B₆, B₁₂, B₉ (folic acid) and their metabolites. As these nutrients cannot be synthesized endogenously by most mammals (including humans and mice), an adequate dietary intake is essential. These nutrients, further referred to as methyl donors, are critical substrates and co-factors in the one-carbon (1-C) metabolism [34,35]. 1-C metabolism is crucial for brain function and development as it produces s-adenosyl methionine (SAM), the universal methyl donor \textit{in vivo} that is required for the methylation of DNA and histones, proteins, phospholipids and neurotransmitters. Thus, these nutrients are vital for several biological processes, including: dendritic branching, synapse formation, myelination [36], neurogenesis [37,38], as well as epigenetic mechanisms [39]. Accordingly, impaired 1-C metabolism has been associated with multiple neurological pathologies [40] and developmental anomalies, including neural-tube defects [41,42], increased susceptibility to autism [43], age-related neurodegenerative disorders [44] and (postpartum) depression [45,46].

Further supporting a possible involvement of these nutrients in the programming of the brain by ES, both clinical [47,48] and pre-clinical [49-51]
evidence demonstrates that an inadequate supply of methyl donors during critical developmental periods can lead to brain dysfunction and cognitive impairments throughout life. In addition, 1-C metabolites are modulated by exposure to acute stress in adulthood [52-54] and polymorphisms in the methylene-tetrahydrofolate reductase gene (a key enzyme in the 1-C cycle) are predictors for depressive symptomatology in the context of traumatic childhood events [55].

Altogether this evidence lead us to hypothesize that the availability of methyl donors is critically involved in the programming of cognitive functions by ES in mice. To test our hypothesis we assessed: i) whether peripheral (ingested milk and plasma) and central levels of methyl donors in the offspring are altered by ES exposure, ii) if restoring micronutrient availability improves the ES-induced lasting effects on cognitive performance in adult offspring and iii) which processes mediate the effects of this nutritional intervention.

The offspring's nutritional status during early postnatal life is solely dependent on maternal breast milk composition, which is highly sensitive to maternal diet, specifically in the case of water-soluble B-vitamins [56]. Therefore, our nutritional intervention consisted of enriching the maternal diet with the above-mentioned essential micronutrients during ES exposure. To gain further insight into the mechanisms underlying the effects of the nutritional intervention, it is crucial to acknowledge that nutritional factors and their actions are embedded in a complex network of environmental and endogenous elements, which synergistically affect the brain at multiple levels; from structural to molecular [27].

We therefore tested whether the effects of our nutritional intervention on cognition are mediated by: affecting maternal behavior, the offspring’s HPA-axis activity, hippocampal neurogenesis, hippocampal DNA methylation patterns (global and glucocorticoid receptor gene promoter specific) and hippocampal expression levels of epigenetic regulators (DNMT 1, 3a, 3b; the key enzymes for DNA methylation). We show that: i) methyl donor availability is a critical factor in programming of the brain by ES exposure, ii) maternal nutritional supplementation is a non-invasive intervention that ameliorates the lasting ES-induced impairments on hippocampus dependent cognitive functions, and iii) the beneficial effects of the diet are mediated by preventing ES-induced HPA axis hyperactivity.
MATERIALS & METHODS

ANIMALS
A total of 48 litters (13 Ctrl, 13 ES, 10 Ctrl-MD, 12 ES-MD), of five-six pups each, were used. All C57BL/6J mice were kept under standard housing conditions (temperature 20-22°C, 40-60% humidity, with food and water ad libitum). Animals were kept on a standard 12/12h light/dark schedule (lights on at 8 AM), except for the animals used for behavioral testing; they were housed in a room with a reversed light/dark cycle (lights on at 8 PM) from P120 onwards. All animals were fed Teklad global rodent diet 2018 (Harlan laboratories BV, Venray, the Netherlands) throughout their lifetime unless otherwise specified (see methyl donor supplementation). All experimental procedures were conducted under national law and European Union directives on animal experiments, and were approved by the animal welfare committee of the University of Amsterdam.

EARLY-LIFE STRESS PARADIGM AND MATERNAL BEHAVIOR OBSERVATIONS
To standardize the perinatal environment, mice were bred in house, as described previously [7]. The ES procedure consisted of the limited nesting/bedding-material model from postnatal day (P) 2-9, described previously in detail [7,57]. On the morning of P2, litters were randomly assigned to one of the following four conditions: control condition with standard diet (Ctl), early-life stress condition with standard diet (ES), control condition with methyl donor supplementation (Ctl-MD) and early-life stress condition with methyl donor supplementation (ES-MD). From P2-9 maternal behavior was observed daily in the dark phase (8.30 PM) during 48-minute observation sessions. On the morning of P9, dams and pups were moved to standard cages for long-term studies, or sacrificed for short-term studies as described below. For long-term studies, animal were weaned at P21 and housed in groups of the same sex and age (2-3 animals/cage), for these experiments only male offspring was used.

MATERNAL METHYLDONOR SUPPLEMENTATION
Ctl-MD and ES-MD dams received custom made methyl donor supplementation diet (SniFF, Soest Germany) from P2-9, consisting of standard diet supplemented with: 15g/kg choline, 15g/kg betaine, 15 mg/kg folic acid, 1.5 mg/kg vitamin B$_{12}$, 7.5 mg/kg L-methionine and 150 mg/kg zinc (from ZnSO$_4$7H$_2$O) (previously described by [58]) and in addition, drinking water was supplemented with 15.3 ug/mL vitamin B$_6$ (Pyridoxine-HCL P9755, Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). Both the standard and the MD diets had an energy density of 3.1 kcal/gram (24% of the calories from protein, 18% from fat, 58% from carbohydrate).
Behavioral Analyses

Four-month-old male mice (Ctl n=14, ES n=13, Ctl-MD n=9, ES-MD n=9) were tested in a behavioral test battery including the following tasks (in order of testing): elevated plus maze (EPM), object recognition task (ORT), object location task (OLT), Morris water maze (MWM) and T-maze. During testing, behaviors were recorded by a video camera connected to a computer with Ethovision software (Noldus, the Netherlands) and in addition manually scored by an experimenter that was blind to the condition of the animals. All behavioral testing was conducted during the dark (active) phase between 1 and 4 PM as described previously [7].

In the EPM, velocity, exploration time per arm and open/closed arm entries were scored to assess basal exploration patterns and anxiety-like behavior. In OLT and ORT, the ratio of novel versus familiar object exploration time (on day 2) was used as an index of memory; a ratio>1 indicates a preference for the novel object/location, and is associated with memory of the object/location on the acquisition day. Mice that spend less than 10 seconds exploring the objects during either the acquisition or the testing phase were excluded from the analysis.

To assess spatial learning and memory, mice were subsequently tested in the MWM. In short, a circular water maze (110 cm in diameter) was filled with opaque water (23 ± 1°C, with non-toxic paint). One day of cued learning in which the platform was visible for the animal in the center of the pool, was followed by a six-day acquisition phase. During the acquisition phase, the mice were subjected to two training trials per day with an inter-trial-interval of 10 minutes while the platform was hidden in the NW-quadrant of the pool one cm below the water surface. Geometric visual cues (22x22 cm) were placed on the walls surrounding the pool. Between trials starting points were varied between one of the three quadrants without the platform (i.e. NE, SW, SE) to prevent the animals from using an egocentric search strategy. At the start of each trial, the mouse was placed in the water facing the wall of the pool. Twenty-four hours after the last acquisition trial, the platform was removed from the pool for a single probe trial, in which the time spent in the target quadrant was recorded. For the MWM, one Ctl-MD animal was excluded from the analysis, as it didn't obtain an active search strategy.

After a 7 day interval, reference memory was tested by assessment of spontaneous choice alternations during two consecutive testing days (3 trials/day with an interval of 90 minutes) in a T-maze apparatus (dimensions arms 30 L x 10 W x 20 H), according to the spontaneous alternation protocol by [59]. Animals with latency to choice >120 seconds were excluded from the analyses.
Newborn cell survival
Cell fate and survival of adult-born neurons was assessed in the same cohort of animals that underwent the behavioral testing. To this end, the cell birth date marker 5-bromo-2’-deoxyuridine (BrdU, Sigma-Aldrich) was injected i.p. at a concentration of 100 mg/kg, 8 weeks after the final behavioral testing day, 3x/day for two consecutive days (at P234-235). BrdU solutions (10 mg/mL dissolved in sterile saline + 0.007M NaOH) were freshly made on the morning of the day of injection. Animals were sacrificed by transcardial perfusion four weeks after the last injection at P265.

Tissue collection
For short-term studies (assessment of nutritional status, neurogenesis, gene expression and DNA-methylation), animals were sacrificed on the morning of P9 (between 8.00-9.30 AM). For the long-term studies, animals were sacrificed on P120 (assessment of gene expression and DNA-methylation) or P265 (assessment of neurogenesis and DG volume). When brains were intended for histology (see below), animals underwent transcardial perfusion with 4% PFA and dissected brains were postfixed overnight. When brain samples were intended for biochemical analyses and/or nutritional composition, brains were quickly isolated after rapid decapitation and hippocampi of both hemispheres were dissected in ice-cold saline. For microdissection of DG tissue, hippocampi were sliced into 4-5 coronal sections; the DG was cut out of these sections under a dissecting microscope. Dissected brain tissue was immediately frozen on dry-ice and stored on -80°C until further use. Thymus and adrenal glands were dissected after decapitation and weighted.

Corticosterone measurements
Basal plasma corticosterone (CORT) level was assessed at P9 (Ctl n=13, ES n=16, Ctl-MD n=9, ES-MD n=11) and at P90 (Ctl n=4, ES n=5, Ctl-MD n=4, ES-MD n=5). For measurements at P9, pups were rapidly removed from their cage on the morning, weighted and decapitated within two minutes of their disturbance. Blood samples of adult mice were collected via a tail incision at the morning of P90 between 8 and 9 AM. Blood was collected in ice-cold EDTA-coated tubes (Sarstedt, Etten-leur, The Netherlands), placed on ice, centrifuged at 15000 x g for 15 minutes and stored till further use at -20°C.

For determination of CORT levels in stomach milk, the milk was removed from the stomach directly after decapitation of the P9 mice and stored at -80°C until further processing. Stomach milk samples were then diluted 1:1 with PBS, sonicated (6m/sec for 30 sec) and centrifuged for 10 min at 10000 x g. The supernatant was collected from below the lipid layer using a needle with syringe; milk extractions from (n=3-6) pups from the same litter were pooled (resulting in the following number of milk samples per condition: Ctl n=6, ES
n=6, Ctl-MD n=4, ES-MD n=4). CORT levels in pup plasma (1:20), adult plasma (1:100) and milk extractions (1:20) were measured using a commercially available radioimmunoassay kit (MP Biomedicals, Eindhoven, the Netherlands).

**IMMUNOHISTOCHEMISTRY AND QUANTIFICATION**

Stainings were performed as described previously [7]. In short, 40 μm thick coronal sections were mounted on glass (Superfrost Plus slides, Menzel, Braunschweig, Germany) and antigen retrieval was performed by heating the sections in 0.1 M citrate buffer (pH 6.0) in a standard microwave (Samsung M6235) to a temperature of approximately 95°C for 15 minutes. Section were incubated with primary antibody for one hour at room temperature followed by overnight incubation at 4°C. The following primary antibodies were used: Ki-67p (1:20,000, Novocastra NCL-L-Ki67_MM1), monoclonal rat anti-BrdU (1:200, Accurate Chemical & Scientific Corporation OBT0030), monoclonal mouse anti-NeuN (1:1,000, Millipore MAB 377). Subsequently, the following secondary antibodies were used for stainings using diaminobenzidine (DAB): goat anti-rabbit biotinylated secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA), in combination with subsequent incubation with avidin-biotin complex (ABC kit, Elite Vectastain Brunschwig Chemie, Amsterdam, 1:800) and chromogen development with DAB (20 mg per 100 mL 0.05M Tris, 0.01% H2O2). For fluorescent stainings, Alexa fluor-conjugated secondary antibodies (Invitrogen; 1:1000) were applied.

Quantification of immunohistochemistry was performed by an observer blind to the experimental conditions. For each animal, coronal sections of 8 matched anatomical levels along the rostro-caudal axis (Bregma levels -1.34, -1.70, -2.06, -2.46, -2.80, -3.16, -3.52, -3.80) were used for quantification of immunoreactivity in the DG of both hemispheres. Intersection distance was approximately 160 μm for pups (P9) and 240 μm for adults (P265). All stainings to assess neurogenesis (Ki67, BrdU, NeuN) were performed on parallel series derived from the same individuals.

Ki67+ cells were counted on a Zeiss Axiophot light microscope with Microfire camera (Coptronics) using StereoInvestigator software (MicroBrightField, Germany) by means of a modified stereological procedure, using a 20x objective (200x magnification). Quantification of BrdU+/NeuN+ cells was performed on a Leica DM 5500B fluorescent microscope (Leica Microsystems, Wetzlar, Germany) with a 40x lens as described previously by [7].

**LC-MS**

After preparation and purification of stomach milk, plasma and dissected hippocampus derived from the same individuals, liquid chromatography mass spectrometric (LC/MS) analysis was performed to measure levels of
methionine, homocysteine, vitamins B6 (pyridoxamine, pyridoxine, pyridoxal, pyridoxal-5P), B12, B9 (folic acid and its metabolite 5-MTHF) within a single run, as described previously by [60].

**LINE1 and Nr3c1 specific DNA methylation**
RNA and DNA of dissected hippocampal tissue was subsequently isolated using 500 μL TRIzol reagent per sample (Ambion Life Technologies) following manufacturer’s guidelines. RNA and DNA content and purity were quantified by spectrophotometry at 260/280 OD (Nanodrop 2000, Thermo Scientific, Wilmington USA). Mean value of 260/280 ratio was 2.19 ± 0.03 for RNA samples and 1.73 ± 0.07 for DNA samples.

A pyrosequencing assay targeting mouse LINE-1 repetitive promoter elements was used to determine global methylation levels, as described previously [61]. In short, genomic DNA (500 ng) was bisulfite-converted using the EZ DNA methylation gold kit, (Zymo Research, Leiden, The Netherlands) according to the manufacturer’s guidelines. Bisulfite-specific biotinylated primers were used (for LINE1: forward: 5’-TTTGGGTTAGGATTGGGTATAAG-3’, reverse: 5’-Biotin-CCACTCACAAAATCTTAAAATC-3’; for Nr3c1 forward: 5’-GGATTTTAAGTGGGTGAATAAG-3’, reverse: 5’-biotin-TCAACAATCCCCCCTTTTTTCATA-3’). After amplification by HotStarTaq Mastermix (Qiagen), the PCR product was purified using streptavidin Sepharose HP beads. Hybridization of the sequencing primer (for LINE1: 5’-GATTTGGGTATAAGTTTTT-3’, for Nr3c1: 5’-AAGTGGGTGGAATAAGA-3’) with the biotinylated PCR product was performed as described in the PyroMark Q24 vacuum workstation guide (Qiagen). The sequence-to-analyze was TTYGTTYGATTYGWGATTYGAGTTTYGGYTA for LINE1 and YGTYGTAGTYGGYGGYGGGGGAGAAYGYGYGYGGGAGAYGGGAAYGGYGYYGGGGGTGTGGTTTAGT for Nr3c1. Finally, the Pyromark Q24 software (Qiagen) was used to determine the methylation percentage of the individual CpG positions analyzed.

**Real-time qPCR**
250 ng of RNA was reverse transcribed using the Superscript II reverse transcriptase kit (Life Technologies). RT-qPCR experiments were performed on a Applied Biosystems 7500 real-time qPCR system using 10 μl reaction volume/well of containing: 1 μl cDNA template, 150 nM forward primer, 150 nM reverse primer and 1x HOT FIREpol EvaGreen mastermix (Solis BioDyne). The following primers were used: Nr3c1 forward: AGGTGCCAAGGGTCTGGAGAGG, reverse: TGGTCCCGTTGCTGTGGAGGA; Dnmt1 forward: AGGCGCGTCATGGGTGCTAC, reverse: GCCGCCGCCATGGCATT; Dnmt3a forward: GCCAAGAAACCCAGAAAGAGC, reverse: GTGACATTGAGGCTCCCACA; Dnmt3b forward: GCGTCAGTACCACCATGCTC;
**α-tubulin** forward: CCCTCGCCTTCTAACGCGTTGC, reverse: TGGTCTTGTCACTTGGCATCTGGC; *Tbp* forward: GTCATTTTCTCCGCAGTGCC, reverse: GCCTTGTTCTGGTCCATGAT; *Hprt1* forward: CTTCCTCCTCGACGAGG, reverse: CACTTTTTCCAAATCCTCGGCA. Cycling conditions comprised 15 minutes polymerase activation at 95°C and 40 cycles (15 seconds at 95°C, 20 seconds at 65°C and 35 seconds at 72°C). After completion of the amplification reaction melting curve analysis of all samples was conducted.

Relative quantification of gene expression was calculated using Qbase+ software (version 2.6.1, Biogazelle) by the ΔΔCt method corrected for amplification efficiencies (between 90-110%) and normalized for expression of a set of three housekeeping genes (*Tbp*, *α-tub* and *Hprt1*) used as internal controls. A GeNorm pilot experiment [62] was conducted to determine that this set of genes was suitable as housekeeping genes in the various cohorts of samples (M=0.39 CV=0.16 for pup tissue, M=0.28 CV=0.12 for adult tissue).

**Statistical analysis**

Data were analyzed using SPSS 20.0 (IBM software) and Graphpad Prism 5 (Graphpad software). All data are expressed as mean ± standard error of the mean (SEM). Data were considered statistically significant when p<0.05.

Maternal behavior was analyzed using repeated measure two-way ANOVA. Physiological, behavioral and immunohistochemical data were compared using two-way ANOVA with the fixed factors condition (Ctl vs. ES) and diet (standard vs. methyldonor-supplemented). Post hoc analyses were performed using Bonferroni multiple comparison tests. Animals from multiple litters were included in each experiment and nested under the condition factor (3-7 litters in each of the 4 groups). Litter effects were negligible for most molecular, histological and behavioral outcomes, with the exception of physiological data at P9, there litter was included as a random factor.

Nutrient levels were analyzed by a multivariate analysis of variance (MANOVA) with condition and diet as between-subject factors. If significant effects were detected, univariate F-tests were used to identify the variables (nutrients) that contributed to these effects. For the ORT and OLT, one-sample T-tests were used to compare the exploration ratio of novel/familiar to one (no discrimination). Learning and memory in the MWM was analyzed using a three-way repeated measures ANOVA, with two ‘between subjects’ factors (Ctl vs. ES and standard diet vs. MD diet) and one ‘within-subjects’ factor (training day). To analyze performance during the probe trial, the percentage of the total time in the target quadrant was calculated and one-sample T-tests were used to compare mean quadrant percentages to chance (25%). Relative gene-expression data was logarithmically transformed to meet assumptions of parametric statistics.
RESULTS

METHIONINE LEVELS IN PLASMA AND HIPPOCAMPUS ARE REDUCED BY CHRONIC EARLY-LIFE STRESS

Methionine status was reduced by ES at P9, both peripherally (in plasma) and centrally (in hippocampal tissue). In plasma samples of ES pups, methionine levels were 30.1% lower compared to Ctl plasma samples (post hoc analysis Ctl vs ES p<0.05, table 1). Accordingly, hippocampal methionine levels were 17.6% lower in ES animals than in Ctl animals (posthoc analysis Ctl vs ES p<0.05, table 1). ES did not affects levels of the other 1-C metabolism-associated nutrients in the hippocampus (p>0.05). On the contrary, nutrient content of stomach milk was not altered by ES exposure (table 1, MANOVA revealed no multivariate effect of condition HT=0.34, F_{7,37}=1.79 p=0.118).

MATERNAL METHYLDONOR SUPPLEMENTATION IS TRANSFERRED VIA MATERNAL MILK AND RESTORES METHIONINE LEVELS IN THE OFFSPRING

The supplemented diet was eaten and absorbed by the dam (supplementary figure 1). Maternal dietary MD supplementation was reflected in the nutrient content of ingested milk, indicating transfer from dam to offspring via maternal milk. Indeed, nutrient composition of the stomach milk of pups at P9 revealed an effect of diet (two factor MANOVA, HT=7.22 F_{7,37}=38.16 p<0.0001) for folic acid, 5-MTHF, pyridoxal and homocysteine (univariate F-tests, p<0.05, table 1). Accordingly, MD supplementation restored nutrient levels in the pup’s plasma and brain to control levels (figure 1). A two factor MANOVA for plasma nutrient levels revealed an effect of condition (HT=0.814 F_{5,47}=7.65 p<0.0001) and diet (HT=0.544 F_{5,47}=5.12.16 p=0.001) without interaction effect (HT=0.210 F_{5,47}= 1.98 p=0.099, table 1). Univariate F-tests revealed that plasma methionine which was reduced by condition (main effect of condition F_{1,51}=26.25 p<0.0001) was increased by MD diet (main effect of diet F_{1,51}=5.95 p=0.018), with no interaction of condition x diet (F_{1,51}=0.353 p=0.555, figure 1a).

Similarly, nutrient levels in hippocampal tissue revealed significant multivariate effects for condition (HT=0.345 F_{4,33}= 2.84 p=0.0039) and diet (HT=0.746 F_{4,33}= 6.156 p=0.001) on micronutrient content. In line with the plasma data, methionine levels were the main contributors to these multivariate effects; while ES significantly reduced hippocampal methionine (main effect of condition F_{1,35}=7.73 p=0.009), MD supplementation increased hippocampal methionine levels (main effect of diet F_{1,35}=7.77 p=0.009, no interaction effect). While ES exposure reduced methionine levels in both standard and MD enriched cohorts, MD diet restored methionine levels to Ctl levels (T-test Ctl (n=13) vs ES-MD (n=7), p=0.187).
### Nutrients in Milk

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Ctl</th>
<th>ES</th>
<th>Ctl-MD</th>
<th>ES-MD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folic Acid (pmol/g)</td>
<td>167 ± 9</td>
<td>148 ± 9</td>
<td>323 ± 13*</td>
<td>277 ± 14*</td>
</tr>
<tr>
<td>5-MTHF (pmol/g)</td>
<td>296 ± 30</td>
<td>287 ± 30</td>
<td>194 ± 42</td>
<td>178 ± 45</td>
</tr>
<tr>
<td>Vitamin B6 (pmol/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Pyridoxal</td>
<td>4675 ± 484</td>
<td>4755 ± 484</td>
<td>7779 ± 684*</td>
<td>8973 ± 731*</td>
</tr>
<tr>
<td>- P5P</td>
<td>4089 ± 207</td>
<td>4400 ± 207</td>
<td>3693 ± 293</td>
<td>3894 ± 313</td>
</tr>
<tr>
<td>- Pyridoxamine</td>
<td>180 ± 22</td>
<td>195 ± 22</td>
<td>159 ± 31</td>
<td>175 ± 33</td>
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<tr>
<td>Vitamin B12 (pmol/g)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Homocysteine (nmol/g)</td>
<td>2.4 ± 0.5</td>
<td>1.5 ± 0.5</td>
<td>8.2 ± 0.7*</td>
<td>7.0 ± 0.8*</td>
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<tr>
<td>Methionine (nmol/g)</td>
<td>25.2 ± 8.2</td>
<td>51.2 ± 8.2</td>
<td>17.4 ± 11.6</td>
<td>19.8 ± 12.4</td>
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### Nutrients in Plasma

<table>
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<tr>
<th>Nutrient</th>
<th>Ctl</th>
<th>ES</th>
<th>Ctl-MD</th>
<th>ES-MD</th>
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</thead>
<tbody>
<tr>
<td>Folic Acid (pmol/ul)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>5-MTHF (pmol/ul)</td>
<td>40.7 ± 4.2</td>
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<td>36.4 ± 5.0</td>
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<td>Vitamin B6 (pmol/ul)</td>
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<tr>
<td>- Pyridoxal</td>
<td>663 ± 96</td>
<td>605 ± 90</td>
<td>850 ± 101</td>
<td>577 ± 101</td>
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<tr>
<td>- P5P</td>
<td>126 ± 15</td>
<td>97.1 ± 14.0</td>
<td>92.5 ± 18.0</td>
<td>101 ± 18</td>
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<tr>
<td>- Pyridoxamine</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
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<tr>
<td>Vitamin B12 (pmol/ul)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homocysteine (nmol/ul)</td>
<td>5.6 ± 0.4</td>
<td>5.5 ± 0.3</td>
<td>5.3 ± 0.4</td>
<td>5.0 ± 0.4</td>
</tr>
<tr>
<td>Methionine (nmol/ul)</td>
<td>125 ± 6.4**</td>
<td>88.0 ± 5.9**</td>
<td>144 ± 6.8**</td>
<td>113 ± 6.8**</td>
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### Nutrients in Brain

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Ctl</th>
<th>ES</th>
<th>Ctl-MD</th>
<th>ES-MD</th>
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<tbody>
<tr>
<td>Folic Acid (pmol/g)</td>
<td></td>
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<td>5-MTHF (pmol/g)</td>
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<td>Vitamin B6 (pmol/g)</td>
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<td>- P5P</td>
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<td>1725 ± 79</td>
<td>1640 ± 107</td>
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<td>- Pyridoxamine</td>
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<td>Vitamin B12 (pmol/g)</td>
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<td>Homocysteine (nmol/g)</td>
<td>2.59 ± 0.14</td>
<td>2.4 ± 0.1</td>
<td>2.88 ± 0.19</td>
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<td>Methionine (nmol/g)</td>
<td>134 ± 5.5**</td>
<td>111 ± 5.5**</td>
<td>163 ± 7.5**</td>
<td>147 ± 7.5**</td>
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### Table 1.

**Nutrient Levels in Stomach Milk, Plasma and Brain of P9 Pups**

Nutrient composition of the stomach milk of pups at P9 was altered by diet (two factor MANOVA, HT=7.22 F(7,37)= 38.16 p<0.0001) for folic acid, 5-MTHF, pyridoxal and homocysteine (univariate F-tests, p<0.05) but not by ES (MANOVA revealed no multivariate effect of condition HT=0.34, F(7,37)=1.79 p=0.118).

MD supplementation restored nutrient levels in the pup’s plasma. A two factor MANOVA for plasma nutrient levels revealed main effects of condition (HT=0.814 F(5,47)= 7.65 p<0.0001) and diet (HT=0.544 F(5,47)= 5.12.16 p=0.001).
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**Effect**

**Increased by diet: F(1,43)= 7.7**

**Decreased by diet: F(1,43)= 8.0**

**Increased by diet: F(1,43)= 36.5**

N.S.
N.S.

**Increased by diet: F(1,43)= 79.3**

N.S.

**Increased by diet: F(1,51)= 26.25; decreased by ES: F(1,51)= 0.353**

**Increased by diet: F(1,35)=7.73; decreased by ES: F(1,35)=7.77**

---

**TABLE 1. Continued**

In line with the plasma data, nutrient levels in hippocampal tissue were affected by ES (HT=0.345 F(4,33)= 2.84 p=0.0039) and diet (HT=0.746 F(4,33)= 6.156 p=0.001). Hippocampal methionine levels were 17.6% lower in ES animals than in Ctl animals (posthoc analysis Ctl vs ES p<0.05). Levels of the other 1-C metabolism-associated nutrients detected in hippocampal tissue (pyridoxal, pyridoxal-5P and homocysteine) were not affected by ES (p>0.05).

**Abbreviations:** 5-MTHF: 5-methyltetrahydrofolate; P5P: pyridoxal-5-phosphate
Chapter 4

To test our hypothesis that ES-induced behavioral deficits can be prevented by MD-supplementation, mice were tested in five different behavioral tasks (elevated plus maze, t-maze, object recognition task (ORT), object location task (OLT) and Morris water maze (MWM)).

Anxiety-like behavior and exploratory behavior in adulthood was not affected by exposure to chronic ES (as previously found [7]), nor by MD supplementation according to the performance in the elevated plus maze (EPM). The amount of open arm entries relative to total arm entries were not different between the groups (condition: F1,39=0.0875, p=0.78; diet: F1,39=0.9710, p=0.331; interaction: F1,39=3.115, p=0.0854, data not shown).

Reference memory was not affected by ES or MD supplementation as tested by measurement of spontaneous alternations in the T-maze during 2 consecutive testing days. All animals tested in the T-maze performed well (average score: 75.4 ± 2.6% of spontaneous alternations) and there were no effects of condition: F1,18=0.2965, p=0.59; diet: F1,18=1.747, p=0.20 or interaction: F1,18=0.017, p=0.90 (data not shown).

ES exposure impaired the performance of mice in all three tasks (ORT, OLT, MWM) used to test the learning and memory function, confirming previous findings by us and others [7,57]. The ES-induced impairments in ORT were fully prevented by the MD diet: on the testing day, the ratio novel/familiar

FIGURE 1. ES REDUCES CENTRAL AND PERIPHERAL METHIONINE LEVELS AT P9.
(A) Methionine levels in pup plasma at P9 (in nmol/µL) are reduced by ES (#: F(1,51)=26.25, p<0.0001) and increased by MD diet (*:F(1,51)=5.95, p=0.018), with no interaction of stress x diet (F(1,51)=0.353, p=0.555). (B) Hippocampal methionine levels at P9 (in nmol/gram tissue) are reduced by ES (#: F(1,35)=7.73, p=0.009) and increased by MD supplementation (*:F(1,35)=7.77, p=0.009).
Data expressed as mean ± SEM.
Micronutrients prevent early-life stress effects

(A) In the object recognition task (ORT), ES reduces the ratio novel/familiar exploration time in the standard diet condition (interaction effect of stress x diet: F(1,41)=5.432, p=0.0248, posthoc Ctl vs ES: t=4.164 p<0.001*), this impairment was prevented by MD supplementation (Ctl-MD vs. ES-MD mice p>0.05).

(B) In the object location task (OLT), ES impairs performance (#: F(1,38)=14.24, p=0.0005), and this is not prevented by the MD supplementation (no effect of diet, F(1,38)=0.02, p=0.8853; no interaction of stress x diet (F(1,38)=0.02, p=0.645).

(C) Spatial acquisition in the Morris water maze. In Ctl animals, short-term spatial memory reflected by a short latency to find the hidden platform in each second training session of the day, is not affected by diet (F(1,21)=0.907, p=0.352). (D) In ES animals, the latency to find the platform during each second training session of the day is reduced by MD supplementation (F(1,20)=4.434, p=0.048). (E) Performance in the probe trial of the MWM is impaired by ES (#: F(1,40)=9.523, p=0.0037) but not restored by diet. ES and ES-MD mice showed no preference over chance (25%) for the target quadrant, while Ctl and Ctl-MD animals did (One-sample T-tests, Ctl (n=14) p=<0.001, Ctl-MD (n=8) p=<0.0010). Data expressed as mean ± SEM.
object exploration time (discrimination ratio) was significantly above 1 for all groups except for the ES condition (One-sample T-tests, Ctl: 1.96±0.16, N=14, P<0.0001 Ctl-MD: 2.03±0.07, N=9, p<0.00001; ES-MD: 1.94±0.18 N=9, p=0.0007; ES: 1.21±0.11, N=13, p=0.09). Comparison of these discrimination ratios between groups revealed an interaction of condition x diet (F 1,41=5.432, p=0.0248, figure 2a) and post hoc testing revealed a significant difference between Ctl and ES animals that received the standard diet (t=4.164 p<0.001), without significant difference between Ctl or Ctl-MD and ES-MD mice (p>0.05).

In contrast, ES-induced impairments in OLT persisted even with the MD diet. In fact, while both Ctl and Ctl-MD mice acquired the task, (One-sample T-tests, Ctl: 1.90±0.19 N=12, p=0.0005; Ctl-MD: 1.80±0.19, N=9, p=0.0029), neither ES or ES-MD mice were able to discriminate between the displaced object and the identical non-moved object (ES: 1.21±0.12 N=12, p=0.1096; ES-MD: 1.26±0.13, N=9, P=0.0748). Two-way ANOVA revealed effect of condition (F 1,38=14.24, p=0.0005), with no effect of diet (F 1,38=0.02, p=0.8853) and no interaction of condition x diet (F 1,38=0.22, p=0.645, figure 2b). All necessary assumptions for the ORT and OLT were met: during acquisition on day one, mice had no preference for either of the two identical objects and there was no difference in the total exploration time between groups.

Finally, MD diet improved some aspects of the acquisition of the MWM task as revealed by a strong statistical trend without preventing the ES-induced impairments in the testing phase of the task (probe trial). Indeed, all animals were able to learn the task within six days as evident by the significantly reduced latency to find the platform between the first and the last training day in all four groups (paired T-test day 1 versus day 6, p<0.05 in all groups, figure 2c-d).

Acquisition consists of two trials each day for six consecutive days, therefore the performance in the first trial/day represents a long-term (24h) memory recollection, while the second trial/day rather reflects properties of short term memory function. As expected during the different training days, latencies are shorter during the second trial compared to the first trial/day (latency first trial: 33.0 ± 1.8 sec, latency second trial: 27.0 ± 2.1 sec, p=0.031). Conventionally, an average of the latencies is reported, this was not significantly different between the four groups (condition: F1,40=0.789, p=0.38, diet: F 1,40=3.119, p=0.085, interaction: F 1,40=0.109, p=0.743, repeated measures ANOVA).

However, a careful separate analysis of all first and second trials revealed the following. While no differences in latencies during the first trial/day was revealed (condition: F 1,40=0.620, p=0.44, diet: F 1,40=1.1713, p=0.20, interaction: F 1,40=1.161, p=0.690), there was a strong trend towards a main effect of diet when
Micronutrients prevent early-life stress effects

analyzing all second trials/day (two-way repeated measures ANOVA, $F_{1,40}=3.88$, $p=0.056$, no effect of condition $F_{1,40}=0.766$, $p=0.387$), largely attributable to the shorter latencies of the ES-MD group. Even though no interaction effect was detected ($F_{1,40}=0.896$, $p=0.350$), comparing the performance between the ES and ES-MD animals during the second trials revealed a significant effect of diet (One-way repeated measures ANOVA, $F_{1,20}=4.434$, $p=0.048$), while there was no differences between Ctl and Ctl-MD animals (One-way repeated measures ANOVA, $F_{1,21}=0.907$, $p=0.352$, figure 2c-d). These results indicate that MD supplementation improved short-term memory of spatial learning in the ES condition during the acquisition phase of the task.

MD diet did not prevent the ES-induced impairment in the testing phase of the MWM (probe trial; Figure 2e-f). Indeed Two-way ANOVA revealed a main effect for condition ($F_{1,40}=9.523$, $p=0.0037$) but no effect for diet and no interaction effect. ES and ES-MD mice showed no preference over chance for the target quadrant (One-sample T-tests compared to chance level, ES (n=13) $p=0.34$, ES-MD (n=9) $p=0.16$) and were different from Ctl and Ctl-MD mice who spent significantly more time in the target quadrant (One-sample T-tests, Ctl (n=14) $p=<0.001$, Ctl-MD (n=8) $p=<0.0010$). Differences in performance in this task were not due to differences in swimming ability or motivation, as swim speed was not different between conditions (data not shown).

**The beneficial effects of maternal methyldonor supplementation are not mediated by alterations in maternal care**

Exposure to the limited nesting and bedding material cage from P2-9 resulted in fragmented maternal care in both the standard (in line with [7]) and the MD-supplemented conditions. ES dams in both diet groups showed more nest exits (two-way ANOVA, main effect of condition: $F_{1,22}=11.99$, $p=0.002$, no effects of diet or interaction figure 3a) and had more difficulty to keep all the pups in the nest area as was evident from increased time during which one or more pups were outside the nest area (two-way ANOVA, main effect of condition: $F_{1,22}=244.76$, $p<0.0001$, no effects of diet or interaction figure 3b). The total time spent nursing was not significantly different between the groups (figure 3c). Thus MD supplementation cannot exert its beneficial effect by preventing fragmentation of maternal care, induced by limiting nesting/bedding material.

**Methyldonor supplementation prevents the ES-induced raise in corticosterone and adrenal gland hypertrophy**

We tested if the enriched diet interferes with the ES-induced HPA axis hyper activation. Basal plasma corticosterone levels in ES pups (at P9) were elevated when compared to Ctl (confirming our previous findings [7]).
This increase was entirely prevented by MD supplementation (2-way ANOVA showed a main effect of condition (F1,45=4.516, p=0.039) a main effect of diet (F1,45=13.287, p=0.001) and a significant condition x diet interaction effect (F1,45=4.186, p=0.047). Post hoc analyses revealed that basal CORT levels were elevated after ES in the standard diet condition but not in the MD supplemented condition (p<0.01), indicating a repressive effect of MD-supplementation on HPA-axis activity (figure 4a).

While the relative weight of the adrenal glands at P9 was increased in ES pups (main effect of condition: F1,28=6.435, p=0.017, no effect of diet F1,28=0.234 p=0.632, interaction effect: F1,28=7.408 p=0.011) this ES-induced adrenal gland hypertrophy was prevented by the MD supplementation (post hoc analysis p<0.001, figure 4b) further supporting that MD diet prevents the ES-induced HPA-axis hyperactivity. Interestingly, CORT levels in pup stomach milk were increased by ES in MD supplemented animals but not in standard-diet animals (condition: F1,16=20.803, p<0.001, diet: F1,16=0.001, p=0.993, interaction: F1,16=11.654, p=0.004) as revealed by post hoc analysis (p<0.001, supplementary figure 2a). As CORT levels in the stomach milk are indicative for maternal CORT levels (in plasma and breast milk), this suggests that ES did elicit a CORT increase in the ES-MD dams (dam plasma CORT levels were not available).

The effects of ES and/or maternal MD-supplementation on basal HPA-axis activity were limited to early-life as analysis of basal plasma morning CORT levels in adult offspring revealed no lasting changes (no effect of condition: F1,14=0.087, p=0.772, no effect of diet: F1,14=0.277, p=0.607, no interaction effect: F1,14=0.070 p=0.985, data not shown).
While MD supplementation blunted the ES-induced raise in basal CORT levels and adrenal hypertrophy, it did not prevent the ES-induced reduction in relative thymus weight (main effect of condition F_{1,40}=10.875, p=0.002, no effect of diet F_{1,40}=2.882, p=0.097, no interaction effect F_{1,40}=2.745, p=0.105, supplementary figure 2b), nor the significant reduction in bodyweight gain from P2 till P9. Actually, MD supplementation further reduced bodyweight gain in both Ctl and ES pups (main effect of condition: F_{1,144}=28.095, p<0.001, main effect of diet: F_{1,144}=37.996, p<0.001, no interaction effect, supplementary figure 2c) and in the dams (supplementary figure 1c).

**FIGURE 4.** MD supplementation dampsens the ES-induced HPA-axis hyper activation.

(A) The ES-induced increase in basal plasma corticosterone levels in pups (at P9) is prevented by MD supplementation (significant stress x diet interaction effect (F(1,45)=4.186, p=0.047; post hoc Ctl vs ES * significant increase (p<0.01). (B) The ES-induced increase in relative weight of the adrenal glands at P9 is prevented by MD supplementation (significant stress x diet interaction effect: F(1,28)=7.408 p=0.011; post hoc Ctl vs ES * significant increase (p<0.001). Data expressed as mean ± SEM.

**The beneficial effects of methyl donor supplementation are not mediated by preventing the ES-induced alterations in dentate gyrus volume and neurogenesis**

ES exposure reduced volume of the granular zone of about 20% (GZ: granular cell layer + subgranular zone) at P9 (two-way ANOVA, main effect of condition F_{1,22}=31.70 p<0.0001, **figure 5a**), this reduction was not prevented by MD supplementation (no effect of diet F_{1,22}=1.147, p=0.2959, no interaction effect F_{1,22}=1.319 p=0.2631).

In adult offspring, the reduction in volume of the GZ was no longer apparent (two-way ANOVA, no effect of condition F_{1,29}=1.928 p=0.1756 no effect of diet F_{1,29}=0.172 p=0.6814, no interaction effect), but a trend towards an ES-induced reduction in the total volume of the dentate gyrus was detected (trend towards an effect of condition F_{1,29}=3.709 p=0.0640, no effect of diet F_{1,29}=0.6779 p=0.4170, no interaction effect, **figure 5b**).
FIGURE 5. The ES-induced alterations in neurogenesis, dentate gyrus volume reductions are not prevented by MD supplementation.

(A) ES reduces granular zone volume at P9 with ± 20% (#: effect of stress F(1,22)=31.70 \(p<0.0001\)), this was not prevented by MD supplementation (diet: F(1,22)=1.147, \(p=0.2959\), no interaction effect). (B) A trend towards an ES-induced reduction in the total volume of the dentate gyrus is also detected in adult offspring ($: F(1,29)=3.709 \(p=0.0640\), this was not affected by MD supplementation (diet: F(1,29)= 0.6779 \(p=0.4170\), no interaction effect). (C) At P9, ES increases the number of proliferating (Ki67+) cells in the GZ of the DG (expressed as numeric densities) (#: F(1,22)=20.85 \(p=0.002\)). This was not prevented by MD supplementation (diet: F(1,22)=2.733, \(p=0.1125\), no interaction effect). (D) In adulthood, the number of proliferating (Ki67+) cells is not affected by ES and/or MD supplementation (condition: F1,29 =2.43 \(P=0.130\), diet: F1,29 = 0.55 \(P=0.461\) no interaction effect). (E) Experimental timeline: animals were injected three weeks after the final behavioral test with 100 mg/kg BrdU three times per day for two consecutive days and killed by transcardial perfusion four weeks after the last injection. (F) ES lastingly reduces the survival of adult-born (BrdU+) neurons (#:F(1,41)=4.658 \(p=0.037\), this effect on adult neurogenesis is not prevented by MD supplementation (F(1,41)=0.008 \(p=0.930\), no interaction effect).
At P9, the number of proliferating (Ki67+) cells in the granular zone of the DG (expressed as numeric densities) was increased by ES exposure (two-way ANOVA, main effect of condition \( F_{1,22}=20.85 \) p=0.002, in line with [7]). This was not prevented by MD supplementation (no effect of diet \( F_{1,22}=2.733 \) p=0.1125, no interaction effect \( F_{1,22}=3.346 \) p=0.0809, figure 5c).

In adulthood, levels of proliferation were not affected by ES exposure and/or maternal MD supplementation (two-way ANOVA, no effect of condition \( F_{1,29}=2.43 \) p=0.130, no effect of diet \( F_{1,29}=0.55 \) p=0.461 no interaction effect, **figure 5d**), while 4 week survival of adult-born (BrdU+) neurons was reduced by ES exposure (**Figure 5e-f**, main effect of condition: \( F_{1,41}=4.658 \) p=0.037), in line with [7]. Similar to the increase in developmental neurogenesis, the ES-induced reduction in survival of adult-born cells was not prevented by MD supplementation (\( F_{1,41}=0.008 \) p=0.930, no interaction effect \( F_{1,41}=1.587 \) p=0.215). None of the above mentioned alterations were subregion-specific, neither between the supra- versus infra-pyramidal blade nor between the rostro- versus the caudal parts of the DG (data not shown).

**Global DNA methylation in dentate gyrus at P9 and in adulthood**

To determine if ES-exposure and/or MD-supplementation (and the associated alterations in central and peripheral methionine status) were accompanied by epigenetic dysregulation at P9, we first determined global DNA-methylation levels for postnatal DG using a LINE1 pyrosequencing assay. The methylation of five individual CpG’s within the LINE1 promoter was analyzed after bisulfite conversion and amplification by PCR. At P9, the average LINE1 methylation was increased after ES by \(<1\%\) in the standard condition, but reduced by \(<1\%\) in the MD-supplemented condition (interaction effect: \( F_{1,41}=4.730 \) p=0.035, no effect of condition \( F_{1,41}=0.803 \) p=0.375, no effect of diet \( F_{1,41}=3.284 \) p=0.077, **figure 6a**). Post hoc analysis revealed significantly higher methylation levels in ES compared to ES-MD. In adulthood there are no differences in levels of LINE1 methylation (no effect of condition \( F_{1,34}=0.021 \) p=0.885, no effect of diet \( F_{1,34}=0.013 \) p=0.911, no interaction effect \( F_{1,34}=0.072 \) p=0.789 **figure 6b**).

Gene expression levels of DNA methyltransferases (DNMTs), the enzymes required for the de novo methylation (DNMT3a and 3b) of CpG regions or its maintenance (DNMT1) were not altered at P9 and P120 by ES nor diet. For P9: DNMT1: no effect of condition \( F_{1,19}=0.031 \) p=0.86, no effect of diet \( F_{1,19}=0.736 \) p=0.40, no interaction effect \( F_{1,19}=0.001 \) p=0.99; DNMT3a: no effect of condition \( F_{1,19}=0.496 \) p=0.49, no effect of diet \( F_{1,19}=0.033 \) p=0.86, no interaction effect \( F_{1,19}=0.339 \) P=0.57; DNMT3b: no effect of condition \( F_{1,19}=2.109 \) p=0.17, no effect of diet \( F_{1,19}=1.360 \) p=0.26, no interaction effect \( F_{1,19}=1.057 \) p=0.32 (**figure 6c**); for P120: DNMT1: no effect of condition \( F_{1,28}=0.686 \) p=0.42, no effect of diet \( F_{1,28}=0.021 \) p=0.887, no interaction effect \( F_{1,28}=0.056 \) p=0.82; DNMT3a: no
effect of condition $F_{1,28}=0.123$ $p=0.73$, no effect of diet $F_{1,28}=0.505$ $p=0.48$, no interaction effect $F_{1,28}=0.205$ $p=0.65$; DNMT3b: no effect of condition $F_{1,19}=0.839$ $p=0.37$, no effect of diet $F_{1,19}=0.142$ $p=0.71$, no interaction effect $F_{1,19}=1.994$ $p=0.17$ (figure 6d).

**FIGURE 6.** Global hippocampal DNA methylation and DNMT expression

(A) At P9, average LINE-1 methylation is slightly higher in ES compared to ES-MD (interaction effect: $F(1,41)=4.730$ $p=0.035$, *posthoc test: significant difference between ES vs ES-MD). (B) In adulthood there are no differences in levels of LINE-1 methylation (no effect of condition $F(1,34)=0.021$ $p=0.885$, no effect of diet $F(1,34)=0.013$ $p=0.911$, no interaction effect $F(1,34)=0.072$ $p=0.789$). (C) Relative DNMT mRNA expression at P9 is unaffected by ES and/or diet (determined by qPCR, normalized to TBP, α-tubulin and HPRT). (D) At P120, relative DNMT expression levels were not altered by ES nor diet (determined by qPCR, normalized to TBP, α-tubulin and HPRT). Data expressed as mean ± SEM.

**Effects of chronic early-life stress on GR expression in the dentate gyrus at P9 and P120 and methylation of the NR3C1 promoter**

Next, we set out to investigate if ES and/or diet affect gene-expression and methylation status of the glucocorticoid receptor (GR). At P9, GR expression in the DG was affected by the MD diet in control animals only while not in the once exposed to ES. Two-way ANOVA revealed a significant interaction effect of condition $x$ diet ($F_{1,19}=12.707$ $p=0.02$, figure 7a). Post hoc analyses revealed that MD supplementation increased GR expression in control animals ($p<0.05$),
Micronutrients prevent early-life stress effects whereas in ES-animals, MD supplementation had no effect on GR expression (p>0.05).

**Figure 7. Hippocampal GR expression and methylation of its promoter at P9 and P120**

(A) At P9, hippocampal GR expression is affected by ES in a diet-dependent manner (interaction: F(1,19)=12.707 p=0.02). MD supplementation increased GR expression in control animals (#: p<0.05), but not in ES-animals (p>0.05). (B) At P9, average methylation status of 9 CpGs within the Nr3C1 promoter is not significantly different between the four groups (condition: HT= 0.329, F(9.31)=1.134 p=0.370), diet: HT= 0.170, F(9.31)=1.585 p=0.799), but a trend towards an interaction effect (HT= 0.579, F(9.31)=1.996 p=0.074) is observed. (C) Separate analysis of the individual CpGs reveals that the trend towards an interaction is largely attributable to changes in methylation of CpG 8 (interaction effect: F(1.39)=2.207 p=0.020), but posthoc analysis revealed no significant differences between the groups. (D) At P120, the alterations in hippocampal GR expression are no longer present (condition: F(1,28)=0.123 p=0.73, interaction: F(1,28)=0.205 p=0.65). (E) At P120, differences in levels of Nr3c1 methylation are not detected (condition: HT= 0.238, F(9.25)=0.662 p=0.783, interaction: HT= 0.646, F(9.25)=1.795 p=0.120).

As methylation status of CpG islands in promoter regions is associated with altered gene expression [63], we investigated if the alterations in GR expression were attributable to alterations in methylation patterns of exon1 of the gene encoding for the GR (nuclear receptor subfamily 3, group C, member 1 (Nr3c1). Multivariate analysis of the methylation status of 9 CpGs within the Nr3c1 promoter revealed no effect for condition (HT= 0.329, F(9,31)=1.134 p=0.370), no effect for diet (HT= 0.170, F(9,31)=1.585 p=0.799), and a trend towards an interaction effect of condition x diet (HT= 0.579, F(9,31)=1.996 p=0.074, figure 7b). Separate analysis of the individual CpGs by univariate F-tests revealed
that this trend towards an interaction was largely attributable to changes in methylation of CpG 8 (interaction effect condition x diet: $F_{1,39}=2.207, p=0.020$, figure 7c), however post hoc analysis revealed no significant differences between the groups.

The alterations in GR expression in the DG did not persist into adulthood (no effect of condition $F_{1,28}=0.123, p=0.73$, no effect of diet $F_{1,28}=0.505, p=0.48$, no interaction effect $F_{1,28}=0.205, p=0.65$). Similarly, levels of \( \text{Nr3c1} \) methylation in adulthood were not affected by ES ($HT=0.238, F_{9,25}=0.662, p=0.734$), diet ($HT=0.217, F_{9,25}=0.602, p=0.783$), or an interaction of condition x diet ($HT=0.646, F_{9,25}=1.795, p=0.120$).

**DISCUSSION**

Exposure to early-life stress (ES) has a life-long impact on cognitive functions. Here we describe evidence in support of our original hypothesis [27] that essential micronutrients implicated in the one carbon (1-C) metabolism are critically involved in programming cognitive function by ES. We demonstrate for the first time that \( i \) chronic ES exposure specifically reduces peripheral and central levels of the essential amino acid methionine, without affecting the other measured 1-C metabolism-associated nutrients. Importantly, we show that supplementing the maternal diet with donors during the ES period is able to: \( ii \) restore methionine levels in plasma and brain of the offspring, and to \( iii \) improve ES-affected cognitive function in object recognition and acquisition of spatial information in the MWM. Furthermore, we established that: \( iv \) these beneficial effects of the diet do not involve ES-induced alterations in maternal care, hippocampal volume, neurogenesis, nor, surprisingly, epigenetic modifications in the offspring, but \( v \) are largely mediated by preventing the ES-induced hyper-activation of the HPA axis.

**ARE REDUCED METHIONINE LEVELS IMPLICATED IN THE ES-INDUCED COGNITIVE IMPAIRMENTS?**

We show here that exposure to ES reduces specifically methionine levels in plasma and brain and that restoring methionine levels through MD-enriched diet ameliorates the ES-induced cognitive impairments. Methionine, an essential amino acid, which is not synthesized \textit{de novo} in most mammals can be the rate-limiting factor for protein synthesis [64] as it is encoded for by the most common start codon for protein translation (AUG). Furthermore it is needed for incorporation into proteins. Hence, a lack of methionine could affect brain development and function through modulating/inhibiting the many (developmental) processes that require protein formation (e.g. neurogenesis). In addition, methionine is key in 1-C metabolism as it is the
Micronutrients prevent early-life stress effects

precursor of s-adenosyl methionine (SAM), the universal methyl donor required for important epigenetic processes like histone and DNA methylation. While a reduction in methionine thus potentially hampers epigenetic processes, which have been implicated before in mediating brain programming by early-life experiences [14,15,24,65,66], in our current study, we did not find indications for drastic acute or lasting epigenetic alterations. This suggests that the reduced methionine levels do not result in an overall lack of methyl groups, that impede methylation reactions, possibly because central levels of the other measured 1-C associated nutrients are unaffected by ES.

Our finding that ES-exposure reduces methionine levels in plasma and hippocampus of 9-day-old pups however highlights the sensitivity of this essential micronutrient during critical developmental periods to the stress of the mother. Importantly, we here present for the first time evidence that restoring the ES-induced reduction in methionine levels (peripherally and centrally) via supplementation of the maternal diet during the ES period, has lasting benefits for her offspring. In fact, this dietary intervention counteracts the negative effects of ES at 5 month of age in object recognition performance and improved acquisition in the MWM. This indicates that the ES-induced lack of methionine during this critical phase of development might be a determinant factor in the cognitive impairments observed in the adult ES offspring.

Further supporting the critical role of 1-C metabolism associated nutrients for brain development and cognitive function, several studies have demonstrated lasting behavioral deficits due to a lack of essential micronutrients (induced by methyl donor deficient diets instead of ES) during early-life [49,50] or in adolescence [51,67]. In line with this preclinical evidence, clinical studies also show that, experimentally- or disease-induced alterations of the B vitamins are related to cognitive performance. For instance, subclinical vitamin B deficiencies are associated with impaired cognition [68] and low vitamin B levels (and the high homocysteine levels associated with these) in ageing men predict later cognitive decline [69].

While we are the first to show that a short nutritional intervention with a specific set of methyl donors administered to the lactating mother can prevent ES-induced cognitive impairments in the offspring, the efficacy of methionine supplementation during adulthood has been shown before. For instance, adult cognitive impairments induced by earlier folate deficiency can be prevented by prolonged dietary methionine supplementation [70]. Moreover, acute or prolonged supplementation/administration of methionine or SAM during adulthood rescued cognitive impairments due to earlier epilepsy, lead or cocaine exposure [71-74] and the same applies for adult anxiety-related behavior induced by low levels of maternal care [25,75].
Together, these studies support the powerful properties of these nutrients under specific conditions. Our current findings highlights the key role of these nutrients in long-term brain programming and further expands the ‘window of opportunity’ to provide nutritional interventions.

**What are the underlying mechanisms of the beneficial effect of methyldonor supplementation?**

Possible explanations for how early MD supplementation can exert its effects on brain function include a direct effect of essential nutrients at the neuronal level, thereby influencing brain structure and plasticity, and/or indirectly by modulation of other processes. We investigated several possible candidates based on literature, including: alterations in HPA axis activity [76], maternal care [77,78], hippocampal volume [79-81], neurogenesis [82] and epigenetic modifications [14,22,83]. MD supplementation prevented in particular the ES-induced elevation of basal plasma CORT levels at P9 and the increase in adrenal weight without affecting the other parameters. Thus, changes in early nutrient levels result in the absence of chronic HPA-axis activation, and this is likely involved in mediating, at least partly, the later cognitive rescue. Similarly, direct modulation of the HPA axis via other tools (e.g. GR antagonists or adrenalectomy) during ES exposure [84,85], or after adulthood stress [86-88], prevented the changes in several hippocampal parameters. Direct suppressive effects of methyldonors or amino acids on HPA-axis activity have not been described so far, and it remains unclear how exactly methyldonor supplementation affects HPA-axis activity.

We did not detect changes in GR expression in the DG due to ES exposure. This is in line with the recent observation that hippocampal GR expression was unaffected by maternal separation in mice [89]. This is in contrast with the well established literature on ES and limited maternal care exposure in rats where lasting alteration in hippocampal GR have been described [15,90,91] indicating a species specific effect. Thus in mice, chronic elevation of CORT does not seem to affect hippocampal GR. This is further supported by the fact that MD supplementation temporarily increased hippocampal GR expression in control pups even though there was no significant difference between the CORT levels in ES pups fed the MD diet and those fed the standard diet.

Even though we did not detect any major effect on GR expression it was still of interest to study if ES and/or methyldonor availability might affect epigenetic modifications of *Nr3c1* (the gene encoding the GR), possibly setting the tone of responsivity of this gene to later life stimuli. In fact GR has been identified as being specifically vulnerable to epigenetic modifications in early-life both in human [92-96] as well as rodents [15,89]. In our study we observed a subtle ES-induced hypermethylation (of in particular CpG8) in animals fed the standard
Micronutrients prevent early-life stress effects

diet which was absent in MD-fed pups. In any case, because no differences in GR expression were found within these groups, this methylation status was not sufficient to modulate gene expression levels. There is evidence that effects of ES and methionine administration are brain region and/or gene specific. For example similar to our finding, maternal deprivation in mice did not alter hippocampal Nr3c1 methylation [89] and central methionine infusion in adult rats left the majority of genes unaffected [75], and only modified a set of specific genes. Further research is needed to investigate how the selective vulnerability of specific brain regions and genes to early-life stress is regulated.

Regarding levels of global DNA methylation, we found a subtle interaction effect of ES and MD diet in the 9-day-old offspring and this effect was not lasting into adulthood. Interestingly, in contrast with our original hypothesis that reduced methionine availability would hamper methylation processes, we observed slightly higher methylation levels in the ES mice fed the standard diet, compared to the ES-MD mice, indicating that the process of DNA methylation is tightly regulated and global methylation levels remain relatively stable even when methyl donor input varies [97,98]. In fact, the subtle difference in global hippocampal (LINE1) DNA-methylation between ES and ES-MD animals comprised less than 1% (on an average of 81.2% methylation) and therefore its biological relevance might be limited. Furthermore, no alterations were found in DNMT expression levels. Although global 5-mC levels in the prefrontal cortex were found to be altered by dietary methyl donor supplementation [74], our findings are consistent with studies showing that neither maternal care, nor methionine treatment, nor acute methionine administration in a rat model of epilepsy, lastingly altered global DNA methylation in the hippocampus [25,71].

**Early dietary intervention prevents some but not all ES-induced cognitive impairments**

The current dietary intervention prevented some, but not all, ES-induced cognitive deficits indicating that the beneficial effects of MD supplementation were directed towards specific aspects of adult cognitive function. A possible explanation for this is that MD diet repressed the ES-induced rise in CORT, but did not further modulate ES-induced changes in maternal care. An additional important implication of this observation is that neither alterations in maternal care [90,91,99,100], nor rise in CORT alone are sufficient to explain all ES-induced cognitive impairments supporting the notion that the ES-induced phenotype is the result of synergistic actions of multiple processes [27].

Thus, interventions that act at multiple levels and restore levels of maternal tactile stimulation next to modulating CORT might have additional beneficial effects. In fact, stroking rat pups to mimic maternal sensory stimulation, prevented maternal deprivation-induced deficits only in combination with
food administration [101].

As to how the MD diet specifically improves ORT and MWM performance without improving OLT performance, a possible explanation lies in the fact that the MD diet was unable to restore the ES-induced reduction in survival of adult born neurons. Indeed, we have reported that ES reduces cell survival of adult born neurons and that the ES-induced impairments in OLT and MWM probe performance are partly dependent on these reductions in adult neurogenesis, while ORT performance, is not [7].

The fact that MD-supplementation did not prevent the ES-induced changes in the levels of neurogenesis seems somewhat contrasting with the strong association between micronutrient availability and neurogenesis, described by others. Deficiencies in maternal folic acid [102], vitamin B₁₂ [49], choline [103,104] or zinc [105] during the perinatal period have been associated with decreased neurogenesis and increased neuronal apoptosis in the offspring, while MD-supplementation during preconception and gestation was associated with increased proliferation and differentiation of neural progenitors [106]. Here we did not observe such effects, possibly because the period during which maternal diet was enriched with MD was much shorter than in previous papers. Next we should also consider that ES increases developmental neurogenesis at P9 [7] and a (further) MD-induced increase in developmental neurogenesis may have no more additional beneficial effects and is therefore possibly prevented or compensated for.

Other forms of hippocampal plasticity like spine density, dendritic length/complexity and electrophysiological properties, have not been studied here, but ES and possibly MD diet likely has also affected these measures [5,21], even though we showed that the ES-induced reduction in DG volume was not prevented by MD supplementation. Up to now, sensory stimuli by the mother and glucocorticoids have been held responsible for many of the ES-related lasting effects on brain structure and function. Here for the first time, we provide evidence for a critical role of early nutrition in programming of the brain.

**Supplementation to the Lactating Dam, a Clinically Relevant Strategy**

It remains to be elucidated how exactly ES exerts its effect on methionine status of the pups. So far, ES-induced alterations in nutrient content could not be detected in the (ingested) stomach milk. Possibly ES diminishes the net intake of maternal milk, thereby reducing total methionine consumption and/or impairs methionine uptake and bioavailability, for instance by affecting gastrointestinal (GI) tract functioning. As stress is known to affect various physiological functions of the GI tract (see [107] for an overview) and the gut
is an important site of methionine metabolism, that utilizes itself a significant proportion (about 25-40%) of the dietary sulfur amino acid intake [108], this warrants further investigation. Interestingly, several clinical studies show a positive association between high glucocorticoid levels and high homocysteine levels (a hallmark of low MD-status) in healthy adults [109] and in patients with Cushing’s disease [110,111].

Our approach to supplement the diet of the ES-exposed lactating dam with a set of methyl donors is based on the idea that this enriches breast milk composition under this vulnerable circumstance and thereby prevents possible deficits of these essential nutrients in the offspring. Maternal intake of particularly water-soluble B vitamins directly affects milk composition, while milk levels of other nutrients (e.g. zinc) are relatively unaffected by maternal intake [56,112]. It has not been studied how other environmental factors than diet (e.g. stress) affect breast milk composition in terms of nutrients (e.g. methyl donors), cytokines and hormones (e.g. glucocorticoids). Here we show that our MD supplementation increased levels of several micronutrients (including folic acid, 5-MTHF and vitamin B6) in stomach milk. We found MD-diet to increase methionine levels in plasma and hippocampus of the offspring, without altering stomach milk methionine levels, possibly because stomach milk content changes after ingestion by metabolic processes (and therefore not entirely reflects the exact breast milk composition), and thus peripheral and central levels of methyl donors reflect not only nutritional intake of the offspring but also metabolic processes [113]. Interestingly, maternal MD supplementation dampens HPA-axis activity in pups, despite the presence of CORT in maternal milk in the MD-ES condition. Such dissociations between stomach milk and serum CORT levels during the early postnatal phase has been reported before [114]). In line with previous studies [58,115,116] no detectable adverse effects of the MD diet were found, we only observed a reduced bodyweight gain in dams and pups in MD conditions, probably due to the high intake of B vitamins, which also play a role in energy expenditure [117]).

In conclusion, our findings show that improving methyl donor status during lactation via dietary supplementation of the dam is a promising tool to prevent lasting effect of ES in her offspring. Therefore we anticipate this approach will have interesting benefits in clinical practice in preventing the lasting effects of chronic ES on brain structure and function.
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SUPPLEMENTARY MATERIAL

**FIGURE S1. THE MD SUPPLEMENTED DIET IS TAKEN IN BY THE DAMS.**

(A) During P2-9, ES-exposed dams eat more than control dams (#: main effect of stress: F(1,42)=8.855, p=0.005). MD-supplementation also increases food intake (*: main effect of diet: F(1,42)=11.774, p=0.001, no interaction effect: F(1,42)=1.365, p=0.249). (B) Water intake is increased by MD supplementation (*: main effect of diet: F(1,42)=28.993, p=0.000, no effect of condition, no interaction effect). (C) Bodyweight gain of dams is reduced by MD supplementation (*: main effect of diet: F(1,42)=11.774, p=0.001), but not affected by ES (no effect of ES: F(1,42)=0.039, p=0.844, no interaction effect: F(1,42)=1.365, p=0.249). Data expressed as mean ± SEM.

**FIGURE S2.**

(A) CORT levels in pup stomach milk are significantly increased by ES in MD supplemented animals and not in standard-diet animals (stress x diet interaction effect: F(1,16)=11.654, p=0.004; post hoc Ctl-MD vs ES-MD p<0.001*). (B) The ES-induced reduction in relative thymus weight is not prevented by MD supplementation (#: main of condition F(1,40)=10.875, p=0.002, no effect of diet F(1,40)=2.882, p=0.097, no interaction effect F(1,40)=2.745, p=0.105). (C) Bodyweight gain from P2 till P9 is reduced by ES (#: F(1,144)=28.095, p<0.001) and MD supplementation (* F(1,144)=37.996, p<0.001, no interaction effect)