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ABSTRACT: Early-life stress (ES) impairs cognition later in life. Because ES prevention is problematic, intervention is needed, yet the mechanisms that underlie ES remain largely unknown. So far, the role of early nutrition in brain programming has been largely ignored. Here, we demonstrate that essential 1-carbon metabolism–associated micronutrients (1-CMAMs; *i.e.,* methionine and B vitamins) early in life are crucial in programming later cognition by ES. ES was induced in male C57Bl/6 mice from postnatal d (P)2–9. 1-CMAM levels were measured centrally and peripherally by using liquid chromatography-mass spectroscopy. Next, we supplemented the maternal diet with 1-CMAM only during the ES period and studied cognitive, neuroendocrine, neurogenic, transcriptional, and epigenetic changes in adult offspring. We demonstrate that ES specifically reduces methionine in offspring plasma and brain. Of note, dietary 1-CMAM enrichment during P2–9 restored methionine levels and rescued ES-induced adult cognitive impairments. Beneficial effects of this early dietary enrichment were associated with prevention of the ES-induced rise in corticosterone and adrenal gland hypertrophy did not involve changes in maternal care, hippocampal volume, neurogenesis, or global/*Nr3c1*-specific DNA methylation. In summary, nutrition is important in brain programming by ES. A short, early supplementation with essential micronutrients can already prevent lasting effects of ES. This concept opens new avenues for nutritional intervention.—Naninck, E. F. G., Oosterink, J. E., Yam, K.-Y., de Vries, L. P., Schierbeek, H., van Goudoever, J. B., Verkaik-Schakel, R.-N., Plantinga, J. A., Plosch, T., Lucassen, P. J., Korosi, A. Early micronutrient supplementation protects against early stress–induced cognitive impairments. FASEB J. 31, 505–518 (2017). www.fasebj.org

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Adversities early in life are strongly associated with decreased cognitive performance in adulthood, an increased vulnerability to developing psychopathology, and impaired hippocampal integrity and function, as established in several clinical (1, 2) and preclinical studies (3). As a result of a lack of insight into the key factors that contribute to these detrimental effects of early-life stress (ES), there are currently no strategies available to solve this problem. The past 50 yr have already identified some key processes that are involved in brain programming, including maternal care (4, 5)

and neuroendocrine factors [*e.g.,* stress-related hormones/ neuropeptides (6–9)], as well as some molecular mechanisms [*e.g.,* those involved in neuronal plasticity (10, 11) and epigenetic regulation (12–14)]. Recently, inflammation (15, 16) and microbiota (17) have also attracted considerable attention, but the role of early nutrition in brain programming has so far been poorly studied.

Nutrition, however, is potentially very important in early programming, as: *1)* brain development comes with an extremely high demand for nutrients (18), *2)* there is an intense cross-talk between the stress and metabolic pathways (19, 20), and *3)* ES and early malnutrition generally lead to similar cognitive deficits (21). Thus, a better understanding of the role of nutrition in ES effects on brain programming could create novel opportunities for early intervention in vulnerable populations.

Here, we focus on a group of essential micronutrients (as their availability fully depends on dietary intake), including methionine, homocysteine, vitamins B_6, B_{12}, B_9 (folic acid), and their metabolites. These micronutrients are notably critical in the 1-carbon (1-C) metabolism (22),

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which is required for methylation and for synthesis of proteins, phospholipids, and neurotransmitters. In this paper, we further refer to them as 1-C metabolism–associated micronutrients (1-CMAMs).

1-CMAMs are vital for brain function and development as they are involved in many biological processes, including neurogenesis (23, 24) and epigenetic mechanisms (25). Accordingly, impaired 1-C metabolism has been associated with neurological pathologies (26) and developmental anomalies [*e.g.,* neural-tube defects (27), neurodegeneration (28), and depression (29)]. In addition, a deficit in 1-CMAMs during critical developmental periods leads to brain dysfunction and lasting cognitive impairments, which suggests the involvement of 1-CMAMs in brain programming (30–33). Finally, 1-CMAMs are modulated by stress in adulthood (34–36), and polymorphisms in the methylene-tetrahydrofolate reductase gene relate to depression when studied in the context of early adversity (37).

This led us to hypothesize that 1-CMAMs are critical for programming cognition by ES. To this end, we assessed in mice: *1*) whether peripheral and central levels of 1-CMAMs are altered by ES, *2*) if restoring early micronutrient availability ameliorates ES-induced cognitive deficits in adulthood, and *3*) which processes mediate the effects of this nutritional intervention. We took a multilevel approach at 3 different ages, that is, postnatal day (P)9, P120, and P265, and studied possible changes in maternal behavior, hypothalamic-pituitary-adrenal (HPA) axis activity of the offspring, neurogenesis in and volume of the hippocampal dentate gyrus (DG), hippocampal DNA methylation [globally and glucocorticoid receptor (GR)-specific], and hippocampal expression of key epigenetic regulators [DNA methyltransferases (DNMTs), the enzymes required for *de novo* DNA methylation (DNMT3a/3b) or its maintenance (DNMT1)].

We demonstrate that: *1*) 1-CMAMs are critical in brain programming by ES, *2*) maternal nutritional supplementation with 1-CMAMs ameliorates several lasting ES-induced cognitive impairments in offspring, and *3*) mechanistically, the beneficial effects of the diet are mediated by preventing the ES-induced rise in corticosterone and adrenal gland hypertrophy.

MATERIALS AND METHODS

Animals

A total of 48 litters [13 control (Ctl), 13 ES, 10 Ctl-supplemented, 12 ES-supplemented] of 5–6 C57Bl/6j mice each were generated; only male offspring were used in these experiments. All procedures were conducted under European Union directives on animal experiments and approved by the animal welfare committee of the University of Amsterdam.

ES paradigm

The ES procedure consisted of the limited nesting/bedding material model from P2 to P9 as previously described (38, 39). On the morning of P2, litters were randomly assigned to one of the following conditions: control condition with standard diet (Ctl), ES condition with standard diet (ES), control condition with

1-CMAM supplementation (Ctl-supplemented), or ES condition with 1-CMAM supplementation (ES-supplemented). On P9, pups were killed for short-term studies or moved to standard cages for long-term studies and weaned at P21.

Micronutrient supplementation

Considering that offspring nutrition during early postnatal life depends on breastmilk, the composition of which is sensitive to maternal diet, specifically regarding water-soluble B vitamins (40), we enriched the maternal diet with 1-CMAMs during ES exposure to achieve supplementation of the offspring diet.

Throughout their lifetime, all animals were fed Teklad global rodent diet 2018 (Harlan Laboratories, Venray, The Netherlands), which contained 1200 mg/kg choline, 4 mg/kg folic acid, 0.08 mg/kg vitamin B_{12} (cyanocobalamin), 0.4% methionine, 70 mg/kg zinc, and 18 mg/kg vitamin B_6 (pyridoxine). From P2–9, Ctl-supplemented and ES-supplemented dams received a 1-CMAM–supplemented diet (Ssniff, Soest, Germany) that was 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 $ZnSO_4 7H_2O$; according to Wolff *et al.* (41)], as well as drinking water that was supplemented with 15.3 µg/ml vitamin B_6 (pyridoxine-HCL P9755; Sigma-Aldrich, Zwijndrecht, The Netherlands). Both standard and 1-CMAM diets had an energy density of 3.1 kcal/g (24% of calories from protein, 18% from fat, 58% from carbohydrate).

Behavioral analyses

Four-month-old mice (Ctl $n = 14$; ES $n = 13$; Ctl-supplemented $n = 9$; ES-supplemented $n = 9$) were tested in the elevated plus maze (EPM), object recognition task (ORT), object location task (OLT), Morris water maze (MWM), and T maze, as prevously described (39). In the EPM, velocity, exploration time, and open/closed arm entries were scored to assess anxiety-like behavior.

In OLT and ORT, the ratio of novel *vs.* familiar object exploration time (on d 2) was used as an index of memory; a ratio >1 indicates a preference for the novel object/location and is thus associated with good memory of the object/location. Mice that spent <10 s exploring the objects were excluded from analysis. All necessary assumptions for the ORT and OLT were met: during acquisition, mice had no object preference and there was no difference in the total exploration time between groups.

MWM was used to assess spatial learning and memory, a 6-d acquisition phase (two 60-s trials/d) was followed by a single probe trial, as previously described (39). For MWM, one Ctl-supplemented animal was excluded from analysis, as it did not obtain an active search strategy.

Reference memory was tested by assessment of spontaneous choice alternations during 2 consecutive testing days (3 trials/d with an interval of 90 min) in a T-maze apparatus (dimensions arms $30 L \times 10 W \times 20 H$), as previously described (42). Animals with latency to choice of >120 s were excluded from analyses.

Eight weeks after the final behavioral test, 100 mg/kg 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich; dissolved in sterile saline + 0.007 M NaOH) was injected 3×/d, i.p. at P234 and P235 to assess survival of adult-born neurons. Animals were killed at P265.

Tissue collection

For short-term studies (assessment of nutritional status, neurogenesis, gene expression, and DNA methylation), animals were

killed on P9 (between 8:00 and 9:30 AM). For long-term studies, animals were killed on P120 (assessment of gene expression and DNA methylation) or P265 (assessment of neurogenesis and DG volume). When brains were intended for histology, animals underwent transcardial perfusion with 4% paraformaldehyde. 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 at −80°C until further use. Thymus and adrenal glands were dissected after decapitation and weighed.

Corticosterone measurements

For corticosterone measurements at P9, pups (Ctl $n = 13$; ES $n = 16$; Ctl-supplemented D $n = 9$; ES-supplemented $n = 11$) were rapidly removed from their cage between 8 and 9 AM, weighed, and decapitated within 2 min of their disturbance. Blood samples of adult mice (Ctl $n = 4$; ES $n = 5$; Ctl-supplemented $n = 4$; ES-supplemented $n = 5$; 1:100) were collected *via* a tail incision on 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 15,000 g for 15 min, and stored till further use at −20°C. Plasma corticosterone level was assessed by using a radioimmunoassay kit (MP Biomedicals, Eindhoven, The Netherlands) for which pup samples were diluted 1:20 and adult samples were diluted 1:100.

Immunohistochemistry and quantification

Immunohistochemistry for Ki-67, BrdU, and NeuN was performed and quantified as previously described (39). The following primary antibodies were used: Ki-67p (Novocastra 1:20,000; NCL-L-Ki67_MM1l; Leica, Buffalo Grove, IL, USA), monoclonal rat anti-BrdU (1:200; OBT0030; Accurate Chemical and Scientific Corp., Westbury, NY, USA), and monoclonal mouse anti-NeuN (1:1000; MAB 377; Millipore, Billerica, MA, USA).

Quantification was performed by an observer who was blinded to the experimental conditions. For each animal, coronal sections of 8 matched anatomic levels along the rostrocaudal 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 were performed on parallel series that were derived from the same individuals.

Ki-67$^+$ cells were counted on a Zeiss Axiophot light microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) with Microfire camera (Optronics, Goleta, CA, USA) using StereoInvestigator software (MicroBrightField, Magdeburg, Germany) by means of a modified stereologic procedure using a ×20 objective (×200 magnification). Quantification of BrdU$^+$/NeuN$^+$ cells was performed on a Leica DM 5500B fluorescent microscope (Leica Microsystems, Wetzlar, Germany) with a ×40 lens as previously described (39).

Micronutrient status assessment

Liquid chromatography-mass spectrometry was performed to measure 1-CMAMs in stomach milk, plasma, and hippocampus that were derived from the same individuals, as previously described (43).

Real-time quantitative PCR

RNA of P9 and P120 hippocampal tissue was isolated using 500 µl Trizol reagent/sample (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer guidelines. Subsequently, 250 ng of RNA was reverse transcribed using the Superscript II reverse transcriptase kit (Thermo Fisher Scientific). Real-time quantitative PCR experiments were performed on an Applied Biosystems 7500 real-time quantitative PCR system (Thermo Fisher Scientific) using 10 µl reaction volume/well that contained 1 µl cDNA template, 150 nM forward primer, 150 nM reverse primer, and 1× Hot FirePol EvaGreen Mastermix (Solis BioDyne, Tartu, Estonia). The following primers were used: *Nr3c1* [nuclear receptor subfamily 3, group C, member 1 (GR gene)] forward: 5′–AGGTGCCAAGGGTCTGGAGAGG–3′, reverse: 5′–TGGTCCCGTTGCTGTGGAGGA–3′; *Dnmt1* forward: 5′–AGGCGCGTCATGGGTGCTAC–3′, reverse: 5′–GGCGGC-GCTTCATGGCATTC–3′; *Dnmt3a* forward: 5′–GCCAAGA-AACCCAGAAAGAGC–3′, reverse: 5′–GTGACATTGAGGCT-CCCACA–3′; *Dnmt3b* forward: 5′–GCGTCAGTACCCCA-TCAGTT–3′, reverse: 5′–ATCTTTCCCCACACGAGGTC–3′; *α-Tub* forward: 5′–CCCTCGCCTTCTAACGCGTTGC–3′, reverse: 5′–TGGTCTTGTCACTTGGCATCTGGC–3′; *Tbp* forward: 5′–GTCATTTTCTCCGCAGTGCC–3′, reverse: 5′–GCCTTGT-TCTGGTCCATGAT–3′; and *Hprt1* forward: 5′–CTTCCTCC-TCAGACCGCTTT–3′, reverse: 5′–CACTTTTTCCAAATCCT-CGGCA–3′. Cycling conditions comprised 15 min polymerase activation at 95°C and 40 cycles (15 s at 95°C, 20 s at 65°C, and 35 s at 72°C).

Relative quantification of gene expression was calculated using Qbase+ software (version 2.6.1; Biogazelle, Ghent, Belgium) by the $\Delta\Delta C_t$ method corrected for amplification efficiencies (90–110%) and normalized for expression of a set of 3 housekeeping genes (*Tbp*, *α-tubulin*, and *Hprt1*).

Long interspersed nuclear element-1 and *Nr3c1*-specific DNA methylation

For methylation analyses, DNA was isolated using the NucleoSpin Tissue Kit (Macherey-Nagel, Duren, Germany). Subsequently, genomic DNA (500 ng) was bisulfite-converted by using the EZ DNA methylation gold kit, (Zymo Research, Leiden, The Netherlands) according to manufacturer guidelines. Bisulfite-specific biotinylated primers were used [for long interspersed nuclear element-1 (LINE1): forward: 5′–TTTGGGGTTAG-GATTTGGGGTATAAG–3′, reverse: 5′–biotin-CCACTCACCAA-AAATCTTAAAATC–3′; for *Nr3c1* forward: 5′–GGATTTTA-AGTGGGTGGAATAAG–3′, reverse: 5′–biotin-TCAACAA-TCCCCCCCTCTTTTCCATA–3′]. After amplification by HotStarTaq Mastermix (Qiagen, Valencia, CA, USA), PCR product was purified by using streptavidin Sepharose HP beads. Hybridization of the sequencing primer (for LINE1: 5′–GATTTGGGGTATAAGTTTTT–3′, for *Nr3c1*: 5′–AAGTG-GGTGGAATAAGA–3′) with the biotinylated PCR product was performed as described in the PyroMark Q24 vacuum workstation guide (Qiagen). The sequence-to-analyze was TTYGT-TYGATTYGWGATTYGAGTTTYGGYTA for LINE1 and YGTYGTAGTYGGGYGGYGYGGYGTYGGGAYGGGAGA-AYGYGYGYGGGGAGAYGGGAGYGGYGYGGGGGTTYGG-TTTGTTAGT for *Nr3c1* (44). Finally, the Pyromark Q24 software was used to determine the methylation percentage of the individual CpG (5′–C-phosphate-G–3′) positions analyzed.

Statistics

All data were analyzed by using SPSS 20.0 (SPSS, Chicago, IL, USA) and Prism 5 (GraphPad Software, La Jolla, CA, USA) and

were considered statistically significant at $P < 0.05$. *Post hoc* analyses were performed by using Bonferroni multiple comparisons tests. Animals from multiple litters were included in each experiment and nested under the condition factor (3–7 litters/group), when appropriate. Relative gene expression data were logarithmically transformed to meet assumptions of parametric statistics.

RESULTS

### ES reduces methionine levels, which are restored by 1-CMAM supplementation of the maternal diet

At P9, ES reduced methionine status both peripherally (30.1% reduction in plasma; $P < 0.05$) and centrally (17.6% reduction in hippocampal tissue; $P < 0.05$; **Table 1**), but did not affect levels of the other 1-CMAM nutrients ($P > 0.05$).

During P2–9, ES-exposed dams ate more than control dams did (condition: $F_{1,42} = 8.855$; $P = 0.005$), and supplementation of the dam diet with 1-CMAM further increased food intake (diet: $F_{1,42} = 11.774$; $P = 0.001$), which confirmed that the diet was eaten by the dams. In addition, 1-CMAM supplementation reduced body-weight gain in pups (diet: $F_{1,144} = 37.996$; $P < 0.001$), but without affecting body weight in adulthood (diet: $F_{1,29} = 0.8287$; $P = 0.370$). Supplementation of the dam diet with 1-CMAM restored methionine levels in her pup's plasma and brain (Table 1). Plasma methionine was reduced by ES (univariable F test $F_{1,51} = 26.25$; $P < 0.0001$), but was increased by 1-CMAM diet ($F_{1,51} = 5.95$; $P = 0.018$, no interaction $F_{1,51} = 0.353$; $P = 0.555$; **Fig. 1A**). Similarly, whereas ES reduced hippocampal methionine ($F_{1,35} = 7.73$; $P = 0.009$), 1-CMAM supplementation increased hippocampal methionine levels ($F_{1,35} = 7.77$; $P = 0.009$, no interaction; Fig. 1B) and restored them

TABLE 1. *Nutrient levels in stomach milk, plasma, and brain of P9 pups*

Nutrient	Ctl	ES	Ctl-supplemented	ES-supplemented	Effect
In milk					
Folic acid (pmol/g)	167.4 ± 9.4	148.1 ± 9.4	323.2 ± 13.2[a]	277.4 ± 14.2[a]	Increase by diet: $F_{1,43} = 7.7$
5-MTHF (pmol/g)	295.7 ± 29.8	287.0 ± 29.8	193.7 ± 42.1	177.9 ± 45.0	Decrease by diet: $F_{1,43} = 8.0$
Vitamin B_6 (pmol/g)					
Pyridoxal	4675 ± 484	4755 ± 484	7779 ± 684[a]	8973 ± 731[a]	Increase by diet: $F_{1,43} = 36.5$
P5P	4089 ± 207	4400 ± 207	3693 ± 293	3894 ± 313	n.s.
Pyridoxamine	179.7 ± 21.7	195.3 ± 21.7	159.4 ± 30.7	174.9 ± 32.9	n.s.
Vitamin B_{12} (pmol/g)	Undetectable	Undetectable	Undetectable	Undetectable	
Homocysteine (nmol/g)	2.38 ± 0.50	1.47 ± 0.50	8.15 ± 0.71[a]	6.94 ± 0.76[a]	Increase by diet: $F_{1,43} = 79.3$
Methionine (nmol/g)	25.2 ± 8.2	51.2 ± 8.2	17.4 ± 11.6	19.79 ± 12.4	n.s.
In plasma					
Folic acid (μM)	Undetectable	Undetectable	Undetectable	Undetectable	
5-MTHF (μM)	40.71 ± 4.17	39.64 ± 3.93	36.40 ± 5.03	49.14 ± 5.27	n.s.
Vitamin B_6 (μM)					
Pyridoxal	663.4 ± 95.5	605.3 ± 90.1	850.3 ± 101.4	577.7 ± 101.4	n.s.
P5P	126.3 ± 14.9	97.1 ± 14.0	92.5 ± 18.0	101.4 ± 18.8	n.s.
Pyridoxamine	<10	<10	<10	<10	—
Vitamin B_{12} (μM)	Undetectable	Undetectable	Undetectable	Undetectable	
Homocysteine (mM)	5.64 ± 0.36	5.51 ± 0.33	5.25 ± 0.38	5.02 ± 0.38	n.s.
Methionine (mM)	125.9 ± 6.4[b]	88.0 ± 5.9[b]	143.7 ± 6.8[b]	112.5 ± 6.8[b]	Increase by diet: $F_{1,51} = 26.25$; decrease by ES: $F_{1,51} = 0.353$
In brain					
Folic acid (pmol/g)	Undetectable	Undetectable	Undetectable	Undetectable	
5-MTHF (pmol/g)	Undetectable	Undetectable	Undetectable	Undetectable	
Vitamin B_6 (pmol/g)					
Pyridoxal	268.7 ± 16.5	305.7 ± 16.5	323.1 ± 22.5	277.1 ± 16.5	
P5P	1803 ± 79	1725 ± 79	1640 ± 107	1450 ± 107	n.s.
Pyridoxamine	Undetectable	Undetectable	Undetectable	Undetectable	n.s.
Vitamin B_{12} (pmol/g)	Undetectable	Undetectable	Undetectable	Undetectable	
Homocysteine (nmol/g)	2.59 ± 0.14	2.43 ± 0.14	2.88 ± 0.19	2.78 ± 0.19	n.s.
Methionine (nmol/g)	134.7 ± 5.5[b]	110.9 ± 5.5[b]	162.7 ± 7.5[b]	147.3 ± 7.5[b]	Increase by diet: $F_{1,35} = 7.73$; decrease by ES: $F_{1,35} = 7.77$

Data (represented as means ± SEM) were analyzed by a 2-factor multivariable ANOVA (MANOVA), with condition and diet as between-subject factors. If significant effects were detected, univariable F tests were used to identify the variables (nutrients) that contributed to these effects. Nutrient composition of the stomach milk of pups at P9 was altered by diet (2-factor MANOVA, HT = 7.22; $F_{7,37} = 38.16$; $P < 0.0001$) for folic acid, 5-methyltetrahydrofolate (5-MTHF), pyridoxal, and homocysteine (univariable F tests; $P < 0.05$). Conversely, nutrient content of stomach milk was not altered by ES exposure (MANOVA revealed no multivariable effect of condition HT = 0.34; $F_{7,37} = 1.79$; $P = 0.118$). In plasma samples of ES pups, 1-CMAM supplementation restored nutrient levels in the pup's plasma. A 2-factor MANOVA for plasma nutrient levels revealed a significant effect of condition (HT = 0.814; $F_{5,47} = 7.65$; $P < 0.0001$) and diet (HT = 0.544; $F_{5,47} = 512.16$; $P = 0.001$) without interaction effect (HT = 0.210; $F_{5,47} = 1.98$; $P = 0.099$). In line with plasma data, nutrient levels in hippocampal tissue revealed significant multivariable 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. Hippocampal methionine levels were 17.6% lower in ES animals than in Ctl animals (*post hoc* analysis Ctl *vs.* ES; $P < 0.05$). Levels of the other 1-CMAM nutrients detected in hippocampal tissue [pyridoxal, pyridoxal-5-phosphate (P5P), and homocysteine] were not affected by ES; $P > 0.05$. n.s., not significant. [a]Main effect for either diet or condition. [b]Significant effect for both condition and diet.

A Methionine (Plasma)

B Methionine (Hippocampus)
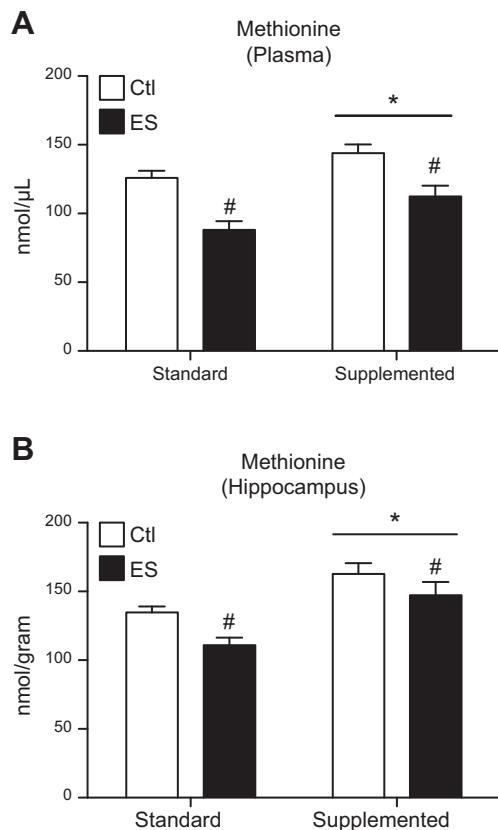
Figure 1. ES reduces central and peripheral methionine levels at P9. *A)* Methionine levels (mM) in pup plasma at P9 are reduced by ES ([#]$F_{1,51} = 26.25$; $P < 0.0001$) and increased by 1-CMAM diet (*$F_{1,51} = 5.95$; $P = 0.018$), with no interaction of stress $\times$ diet ($F_{1,51} = 0.353$; $P = 0.555$). *B)* Hippocampal methionine levels (nmol/g tissue) at P9 are reduced by ES ([#]$F_{1,35} = 7.73$; $P = 0.009$) and increased by 1-CMAM supplementation (*$F_{1,35} = 7.77$; $P = 0.009$). Data are expressed as means $\pm$ SEM.

to control levels [Student's *t* test, Ctl ($n = 13$) *vs.* ES-supplemented ($n = 7$); $P = 0.187$], whereas ES still affected them.

1-CMAM supplementation partly prevents ES-induced cognitive impairments

ES impaired performance in ORT, OLT, and MWM, which confirmed previous findings (38, 39). 1-CMAM supplementation prevented this in ORT and MWM, but not in OLT.

ES-induced impairments in ORT were fully prevented by the 1-CMAM diet. Two-way ANOVA revealed an interaction effect ($F_{1,41} = 5.432$; $P = 0.0248$; **Fig. 2A**), and *post hoc* testing showed impaired object recognition in ES animals compared with Ctl ($t = 4164$; $P < 0.001$), without difference between Ctl and Ctl-supplemented or ES-supplemented mice ($P > 0.05$). Similarly, 1-CMAM diet improved acquisition in MWM. Acquisition consisted of 2 trials/d for 6 consecutive days. Whereas performance in the first trial on the day represents long-term (24 h) memory, the second trial of the day reflects properties of short-term (15 min) memory function.

As expected, latencies were shorter during the second (27.0 ± 2.1 s) compared with the first trial of the day (33.0 ± 1.8 s; $P = 0.031$). A separate analysis of all first and second trials of the acquisition days revealed no differences in latencies during the first trial of the day (condition: $F_{1,40} = 0.620$; $P = 0.44$; diet: $F_{1,40} = 1.1713$; $P = 0.20$; interaction: $F_{1,40} = 1.161$; $P = 0.690$), but when all second trials were compared, there was a strong trend toward an effect of diet ($F_{1,40} = 3.88$; $P = 0.056$; no condition effect: $F_{1,40} = 0.766$; $P = 0.387$; no interaction: $F_{1,40} = 0.896$; $P = 0.350$), which was largely attributable to shorter latencies of the ES-supplemented group (Fig. 2C, D). This suggests that 1-CMAM supplementation improved short-term memory in ES mice during task acquisition, but did not prevent ES-induced impairment in the testing phase of MWM (probe trial, Fig. 2E, F). Finally, ES-induced impairments in OLT persisted despite 1-CMAM supplementation (condition: $F_{1,38} = 14.24$; $P = 0.0005$; diet: $F_{1,38} = 0.02$; $P = 0.8853$; interaction: $F_{1,38} = 0.22$; $P = 0.645$; Fig. 2B).

Anxiety-like behavior reflected by the relative amount of open arm entries in the EPM was not affected by ES ($F_{1,39} = 0.0875$; $P = 0.78$), as found before (39), nor by diet ($F_{1,39} = 0.9710$; $P = 0.331$) or interaction ($F_{1,39} = 3.115$; $P = 0.0854$; data not shown).

Reference memory in the T maze (on average $75.4 \pm 2.6\%$ of spontaneous alternations) was unaffected by condition ($F_{1,18} = 0.2965$; $P = 0.59$), diet ($F_{1,18} = 1.747$; $P = 0.20$), or interaction ($F_{1,18} = 0.017$; $P = 0.90$; data not shown).

1-CMAM supplementation of the dam prevents ES-induced HPA axis hyperactivity in her offspring

Basal plasma corticosterone levels at P9 were elevated by ES, which confirmed previous results (39). This was entirely prevented by 1-CMAM supplementation (interaction: $F_{1,45} = 4.186$; $P = 0.047$; *post hoc* ES *vs.* ES-supplemented; $P < 0.01$; **Fig. 3A**), Similarly, 1-CMAM supplementation prevented ES-induced adrenal gland hypertrophy (interaction: $F_{1,28} = 7.408$; $P = 0.011$; *post hoc* Ctl *vs.* ES; $P < 0.001$; Fig. 3B), which indicated an overall repressive effect of 1-CMAM supplementation on HPA axis activity and corticosterone production.

Neither ES, nor 1-CMAM supplementation led to lasting alterations in HPA axis activity (corticosterone at P90, condition: $F_{1,14} = 0.087$; $P = 0.772$; diet: $F_{1,14} = 0.277$; $P = 0.607$; interaction: $F_{1,14} = 0.070$; $P = 0.985$). 1-CMAM supplementation did not prevent the ES-induced reduction in pup thymus weight and body-weight gain (Fig. 3C, D).

Beneficial effects of 1-CMAM supplementation are not mediated by normalizing maternal care, nor by preventing ES-induced alterations in offspring DG volume, neurogenesis, GR expression, or DNA methylation

Exposure to limited nesting and bedding material from P2 to P9 resulted in fragmented maternal care, which confirmed previous findings (38, 39), independent of diet
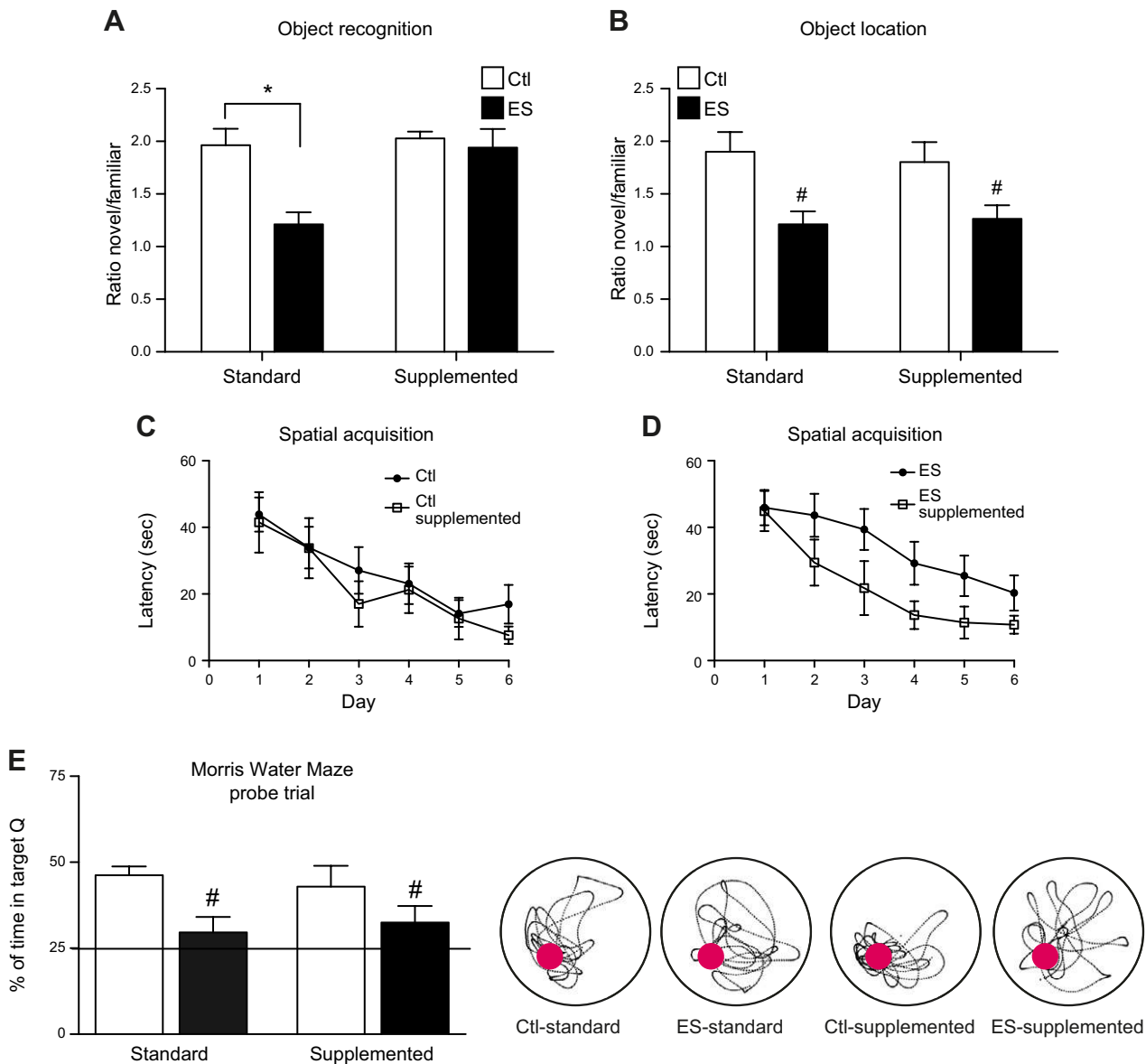
Figure 2. ES-induced cognitive impairments are prevented, in part, by 1-CMAM supplementation. *A*) In ORT, the ratio of novel to familiar was >1 for all but the ES condition (1-sample Student's *t* tests, Ctl: 1.96 ± 0.16; *n* = 14; *P* < 0.0001; Ctl-supplemented: 2.03 ± 0.07; *n* = 9; *P* < 0.00001; ES-supplemented: 1.94 ± 0.18; *n* = 9; *P* = 0.0007; ES: 1.21 ± 0.11; *n* = 13; *P* = 0.09). ES reduces the ratio of novel to familiar exploration time in the standard diet condition (interaction effect of stress × diet: $F_{1,41}$ = 5.432; *P* = 0.0248; *post hoc* Ctl *vs.* ES: *t* = 4164; **P* < 0.001). This impairment was prevented by 1-CMAM supplementation (Ctl-supplemented *vs.* ES-supplemented mice; *P* > 0.05). *B*) In OLT, ES impairs performance ([#]$F_{1,38}$ = 14.24; *P* = 0.0005), and this is not prevented by 1-CMAM supplementation (no effect of diet, $F_{1,38}$ = 0.02; *P* = 0.8853; no interaction of stress × diet ($F_{1,38}$ = 0.22; *P* = 0.645). In fact, whereas both Ctl and Ctl-supplemented mice acquired the task (1-sample Student's *t* tests, Ctl: 1.90 ± 0.19; *n* = 12; *P* = 0.0005; Ctl-supplemented: 1.80 ± 0.19; *n* = 9; *P* = 0.0029), neither ES, nor ES-supplemented mice were able to discriminate between the objects (ES: 1.21 ± 0.12; *n* = 12; *P* = 0.1096; ES-supplemented: 1.26 ± 0.13l *n* = 9; *P* = 0.0748). *C*) Spatial acquisition in MWM. All animals were able to learn the task within 6 d as evident by the significantly reduced latency to find the platform between the first and the last training day in all 4 groups (paired Student's *t* test d 1 *vs.* d 6, *P* < 0.05 in all groups). In Ctl animals, short-term spatial memory that was reflected by a short latency to find the hidden platform in each second training session of the day was not affected by diet (1-way repeated measures ANOVA, $F_{1,21}$ = 0.907; *P* = 0.352). *D*) In ES animals, latency to find the platform during each second training session of the day was reduced by 1-CMAM supplementation (1-way repeated measures ANOVA, $F_{1,20}$ = 4.434; *P* = 0.048). *E*) Performance in the probe trial of MWM was impaired by ES ([#]$F_{1,40}$ = 9.523; *P* = 0.0037) but not restored by diet. ES and ES-supplemented mice showed no preference over chance (25%) for the target quadrant [1-sample Student's *t* tests compared with chance level, ES (*n* = 13) *P* = 0.34; ES-supplemented (*n* = 9); *P* = 0.16] and were significantly different from Ctl and Ctl-supplemented mice that spent significantly more time in the target quadrant [1-sample Student's *t* tests, Ctl (*n* = 14) *P* ≤ 0.001; Ctl-supplemented (*n* = 8) *P* ≤ 0.0010]. Differences in the performance of this task were not a result of differences in swimming ability or motivation, as swim speed was not different between conditions (data not shown). Data are expressed as means ± SEM.
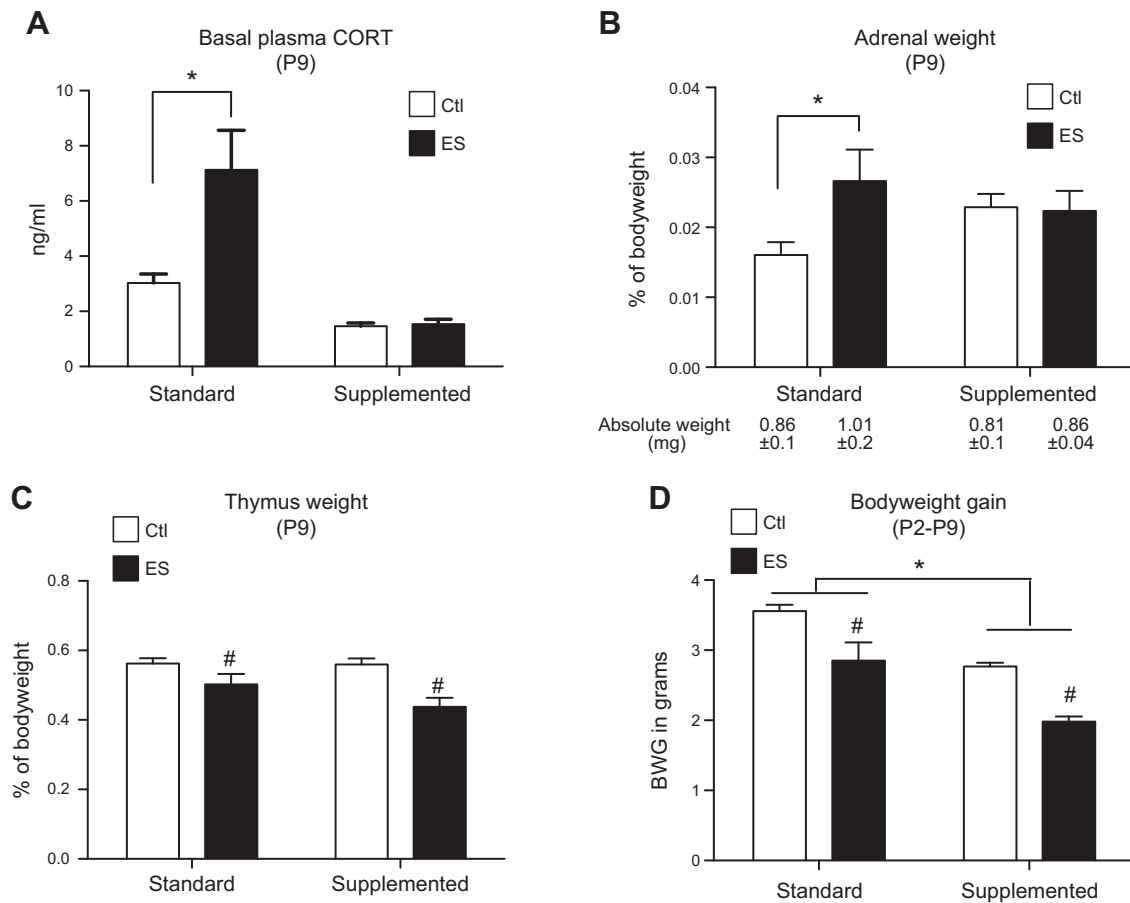
Figure 3. 1-CMAM supplementation dampens ES-induced HPA axis hyperactivation. *A)* ES-induced increase in basal plasma corticosterone (CORT) levels in pups (at P9) is prevented by 1-CMAM supplementation (2-way ANOVA, main effect of condition: $F_{1,45} = 4.516$; $P = 0.039$; main effect of diet: $F_{1,45} = 13.287$; $P = 0.001$; interaction effect: $F_{1,45} = 4.186$; $P = 0.047$). *Post hoc* analyses revealed that basal corticosterone levels were elevated after ES in the standard diet condition but not in the 1-CMAM–supplemented condition (*post hoc* Ctl *vs.* ES significant increase, *$P < 0.01$). *B)* ES-induced increase in relative weight of the adrenal glands at P9 is prevented by 1-CMAM supplementation (significant stress × diet interaction effect: $F_{1,28} = 7.408$; $P = 0.011$; *post hoc* Ctl *vs.* ES significant increase; *$P < 0.001$). *C)* ES-induced reduction in relative thymus weight is not prevented by 1-CMAM supplementation (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: $F_{1,40} = 2.745$; $P = 0.105$). *D)* Body-weight gain from P2 to -9 is reduced by ES (main effect of condition: #$F_{1,144} = 28.095$; $P < 0.001$). 1-CMAM supplementation further reduced body-weight gain in both Ctl and ES pups (main effect of diet: *$F_{1,144} = 37.996$; $P < 0.001$, no interaction effect). Data are expressed as means ± SEM.

(**Fig. 4**); therefore, 1-CMAM supplementation did not exert its beneficial effects by normalizing maternal care.

We next investigated whether 1-CMAM supplementation exerted its effect *via* modulating neurobiologic processes in the offspring's hippocampus. 1-CMAM supplementation did not prevent ES-induced alterations in DG volume (**Fig. 5***A*) and neurogenesis at P9 and P120 (Fig. 5*B*–*E*). Indeed, ES reduced the volume of the granular zone, defined as granular cell layer + subgranular zone, by approximately 20% at P9 (condition: $F_{1,22} = 31.70$; $P < 0.0001$; Fig. 5*A*), and this reduction was not prevented by 1-CMAM supplementation (diet: $F_{1,22} = 1.147$; $P = 0.2959$; no interaction: $F_{1,22} = 1.319$; $P = 0.2631$).

Similarly, at P9, the number of proliferating (Ki-67^{+}) cells in the granular cell layer + subgranular zone was increased by ES (condition: $F_{1,22} = 20.85$; $P = 0.002$), which is in agreement with previous findings (39), and this was not prevented by 1-CMAM supplementation (diet: $F_{1,22} = 2.733$; $P = 0.1125$; no interaction: $F_{1,22} = 3.346$; $P = 0.0809$;

Fig. 5*B*). In adulthood, proliferation was not affected by ES and/or 1-CMAM supplementation (condition: $F_{1,29} = 2.43$; $P = 0.130$; diet: $F_{1,29} = 0.55$; $P = 0.461$; no interaction; Fig. 5*C*), but the 4-wk survival of adult-born (BrdU^{+}) neurons was reduced by ES (condition: $F_{1,41} = 4.658$; $P = 0.037$; Fig. 5*D, E*), which was in line with prior results (39). This ES-induced reduction in survival of adult-born cells was not prevented by 1-CMAM supplementation ($F_{1,41} = 0.008$; $P = 0.930$; no interaction: $F_{1,41} = 1.587$; $P = 0.215$). None of the above mentioned alterations was specific for only the supra-/infrapyramidal blade or the rostro/caudal parts of the DG (data not shown).

We then investigated whether 1-CMAM supplementation exerted its effect *via* modulation of the epigenetic machinery in the hippocampus. Neither ES exposure, nor 1-CMAM supplementation—and the associated alterations in methionine status—were accompanied by clear indications of epigenetic dysregulation. In fact, even though global hippocampal DNA methylation levels at
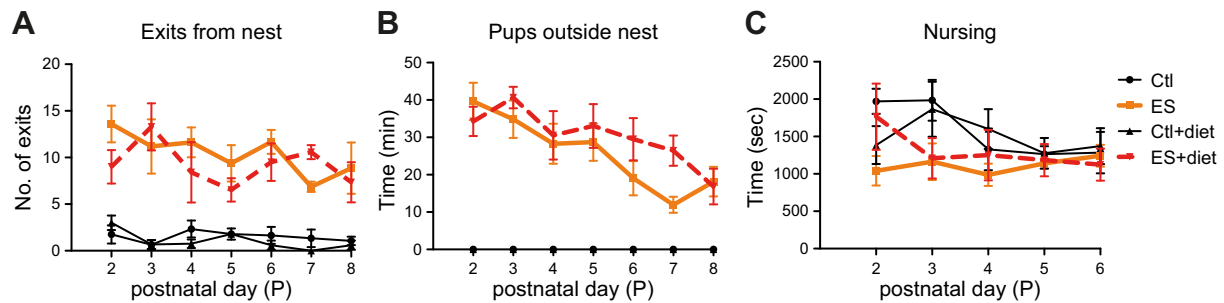
Figure 4. Effects of ES and of micronutrient supplementation on maternal behavior. *A*) Exposure to the limited nesting and bedding material cage from P2 to P9 resulted in fragmented maternal care in both the standard and 1-CMAM–supplemented conditions. ES increases the number of nest exits in both diet groups (2-way ANOVA, main effect of condition: $F_{1,22} = 11.99$; $P = 0.002$). *B*) ES drastically increases the time during which ≥ 1 pups are outside the nest area (2-way ANOVA, main effect of condition: $F_{1,22} = 244.76$; $P < 0.0001$), but this is not affected by the diet. *C*) Dams from all 4 groups spent, on average, equal amounts of time on nursing behavior. Data are expressed as means $\pm$ SEM.

P9, which were determined by a LINE1 pyrosequencing assay, were increased after ES by <1% in the standard condition and reduced by <1% after 1-CMAM supplementation (interaction: $F_{1,41} = 4.730$; $P = 0.035$; *post hoc* ES *vs.* ES-supplemented $P < 0.05$; **Fig. 6A**), given the small effect size, we considered them biologically negligible. These effects were not lasting in adulthood (condition: $F_{1,34} = 0.021$; $P = 0.885$; diet: $F_{1,34} = 0.013$; $P = 0.911$; interaction: $F_{1,34} = 0.072$; $P = 0.789$; Fig. 6B). In addition, gene expression levels of Dnmts were not altered at P9 and P120 by ES or diet ($P > 0.05$; Fig. 6C, D).

Finally, we investigated whether ES and/or diet affected expression and methylation status of GR gene (*Nr3c1*). At P9, hippocampal GR expression was increased by 1-CMAM diet in Ctl, but not ES, animals (interaction: $F_{1,19} = 12.707$; $P = 0.02$; *post hoc* Ctl *vs*. Ctl-supplemented, $P < 0.05$; Fig. 6E). These alterations were not attributable to altered methylation patterns on exon1 of the *Nr3c1* gene. Two-way multiviariable ANOVA of 9 CpGs within the *Nr3c1* promoter revealed no effects for condition (HT = 0.329; $F_{9,31} = 1.134$; $P = 0.370$) and diet (HT = 0.170; $F_{9,31} = 1.585$; $P = 0.799$), even though a trend toward an interaction effect was present (HT = 0.579; $F_{9,31} = 1.996$; $P = 0.074$; Fig. 6F). Separate analysis of individual CpGs revealed that this trend was largely attributable to changes in CpG8 methylation (univariable F tests, interaction: $F_{1,39} = 2.207$; $P = 0.020$; but *post hoc* $P > 0.05$; Fig. 6G).

These alterations in hippocampal GR expression at P9 did not persist into adulthood (condition: $F_{1,28} = 0.123$; $P = 0.73$; diet: $F_{1,28} = 0.505$; $P = 0.48$; interaction: $F_{1,28} = 0.205$; $P = 0.65$; Fig. 6H). Similarly, levels of *Nr3c1* methylation in adulthood were not affected by ES (HT = 0.238; $F_{9,25} = 0.662$; $P = 0.734$) or diet (HT = 0.217; $F_{9,25} = 0.602$; $P = 0.783$), and no interaction was present (HT = 0.646; $F_{9,25} = 1.795$; $P = 0.120$; Fig. 6I).

DISCUSSION

ES exposure has a lifelong impact on cognition. Here, we present evidence to suggest that restoring the ES-induced reduction in methionine levels, peripherally and centrally,

via supplementation of the dam's diet, and selectively during the ES period, has lasting benefits for her offspring. In fact, this short dietary intervention counteracted negative effects of ES in object recognition performance and improved acquisition in MWM. This indicates that the ES-induced lack of methionine during this critical phase might be a determinant factor in cognitive impairments observed in adult ES offspring. This supports our hypothesis (19) that essential micronutrients that have been implicated in 1-C metabolism might contribute to the programming effects of ES on later cognitive function. Furthermore, we established that the diet does not involve alterations in maternal care, hippocampal volume, neurogenesis, or epigenetic modifications, but prevents ES-induced hyperactivation of the HPA axis, which suggests that this might be implicated in its beneficial effects on later cognitive function.

Early nutritional intervention ameliorates ES-induced cognitive impairments

Our finding that ES reduces levels of the essential amino acid methionine in the plasma and hippocampus of P9 pups highlights the stress sensitivity of this micronutrient during critical developmental periods. Methionine, other than being an important substrate of 1-C metabolism, is also a necessary amino acid for protein synthesis (45). Hence, a lack of methionine could impair these processes and thereby affect brain development and function. As such, it could be a critical factor for specific cognitive functions later in life. ES-induced alterations in nutrient content could not be detected in the (ingested) stomach milk. This might be a result of the fact that stomach milk is an accumulated amount and its content after ingestion may have been further altered by metabolic processes; therefore, it may not be entirely representative of exact breast milk composition). Hence, measurement in ingested stomach milk reflects not only nutritional intake of the offspring but, at least to some extent, also ongoing metabolic processes. ES-induced reduction in methionine can thus be either a result of a reduction of intake of maternal milk and/or impairing methionine uptake and bioavailability, for
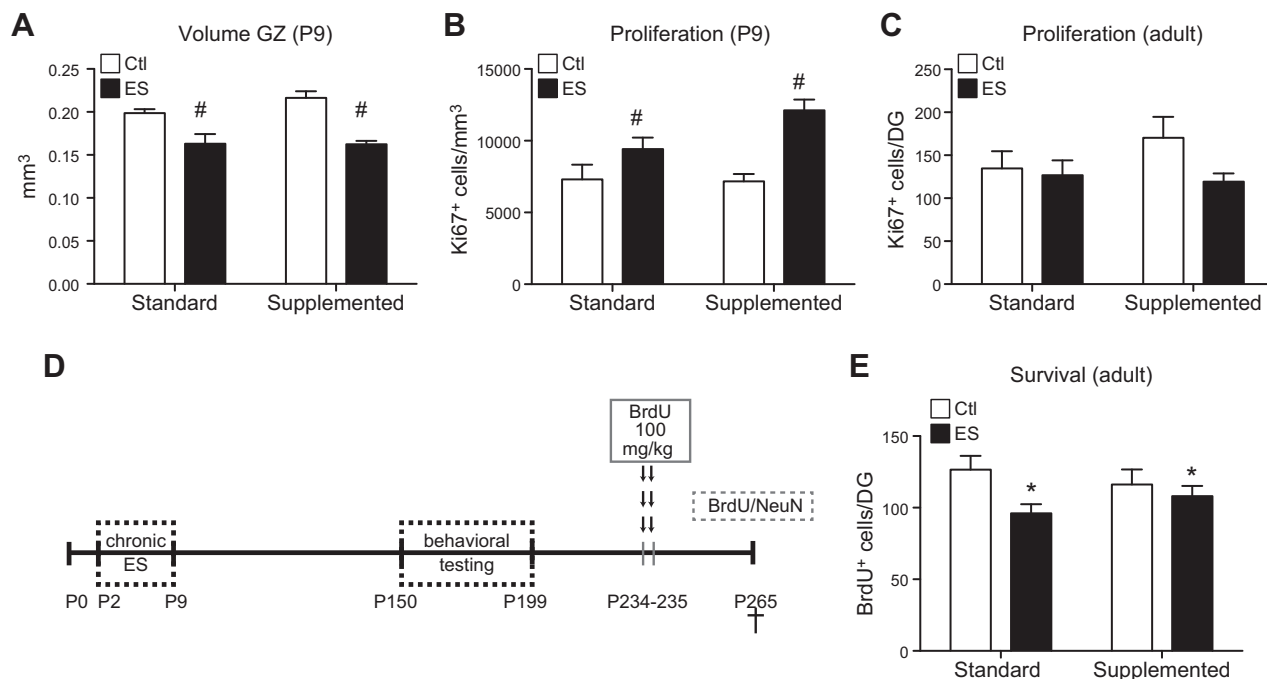
Figure 5. ES-induced alterations in neurogenesis and DG volume reductions are not prevented by micronutrient supplementation. *A*) ES exposure reduces volume of the granular zone (GZ; granular cell layer + subgranular zone) at P9 with ± 20% (2-way ANOVA, main effect of condition: [#]$F_{1,22} = 31.70$; $P < 0.0001$). This reduction was not prevented by 1-CMAM supplementation (no effect of diet: $F_{1,22} = 1.147$; $P = 0.2959$; no interaction effect: $F_{1,22} = 1.319$; $P = 0.2631$). *B*) At P9, ES increases the number of proliferating (Ki-67^{+}) cells in the GZ of the DG (expressed as numeric densities; 2-way ANOVA, main effect of condition: [#]$F_{1,22} = 20.85$; $P = 0.002$). This was not prevented by 1-CMAM supplementation (no effect of diet: $F_{1,22} = 2.733$; $P = 0.1125$; no interaction effect: $F_{1,22} = 3.346$; $P = 0.0809$.). *C*) In adulthood, the number of proliferating (Ki-67^{+}) cells was not affected by ES exposure and/or maternal 1-CMAM supplementation (2-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). *D*) Experimental timeline: to study survival of newborn cells in adulthood, animals were injected 3 wk after the final behavioral test with 100 mg/kg BrdU 3 times/d for 2 consecutive days and were killed by transcardial perfusion 4 wk after the last injection. *E*) ES lastingly reduces the survival of adult-born (BrdU^{+}) neurons (2-way ANOVA, main effect of condition: [#]$F_{1,41} = 4.658$; $P = 0.037$). This effect on adult neurogenesis is not prevented by 1-CMAM 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 was subregion specific, neither between the supra- *vs.* infrapyramidal blade, nor between the rostro *vs.* caudal parts of the DG. Data are expressed as means ± SEM.

instance, by affecting gastrointestinal tract functioning. As stress is known to affect various physiologic functions of the gastrointestinal tract and the gut is an important site of methionine metabolism itself, this warrants further investigation.

Of importance, supplementation of the maternal diet during the ES period restored methionine to Ctl levels and ameliorated some of the negative effects of ES on cognition, fully restoring performance in ORT. Because the prefrontal cortex is also involved in ORT performance, our behavioral data indicate that, in addition to the hippocampus, other brain regions (*e.g.,* prefrontal cortex) are likely involved in the beneficial effects of 1-CMAM supplementation on cognitive functioning.

In line with our findings, several preclinical studies in which fully 1-CMAM–deficient diets were administered during early life (30, 32) or adolescence (33, 46) reported lasting behavioral impairments, which further supports a critical role for 1-CMAM during early life for later cognitive function. This indicates that methionine levels need to reach a certain threshold to contribute to optimal adult brain functioning and points to early nutritional intervention as a promising tool to prevent lasting ES effects.

Here, we show that a short and early nutritional intervention with 1-CMAM can prevent lasting cognitive impairments induced by ES. Of interest, effects of methionine supplementation during adulthood have been reported before, although these were administered for a prolonged period of time under depletion conditions—for instance, severe adult folate deficiency induced cognitive impairments that could be prevented by a prolonged dietary methionine supplementation (47). In addition, also highlighting the powerful properties of these nutrients under specific conditions, acute or prolonged administration of methionine or the universal methyl donor *S*-adenosyl methionine during adulthood rescued cognitive impairments that were a result of earlier epilepsy or lead or cocaine exposure (12, 48–52) and improved drug-seeking behaviors (53). Our results show the sensitivity of specific nutrients to ES exposure and highlight a key role for them during early life in long-term brain programming, as well as the efficacy of early nutritional interventions to prevent later ES-induced effects on cognition. Because prevention of ES exposure (*e.g.,* during conditions of hospitalization, abuse, neglect) itself is often not feasible, such adequate intervention strategies are
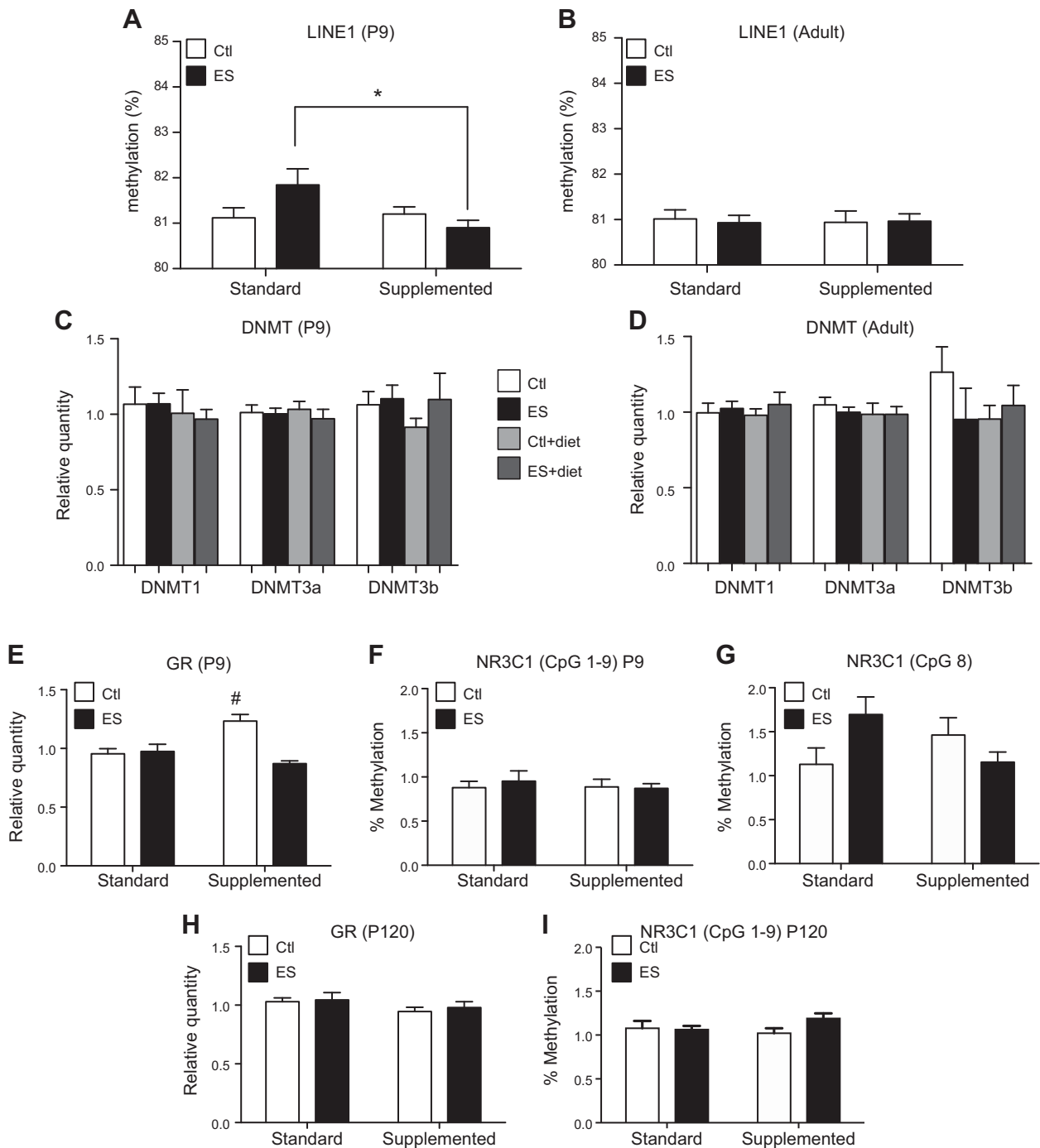
Figure 6. Global hippocampal DNA methylation and DNMT expression. *A*) At P9, average LINE-1 methylation is slightly higher in ES compared with ES-supplemented (no effect of condition: $F_{1,41} = 0.803$; $P = 0.375$; no effect of diet: $F_{1,41} = 3.284$; $P = 0.077$; interaction effect: $F_{1,41} = 4.730$; $P = 0.035$; *post hoc* test ES *vs.* ES-supplemented, $P < 0.05$). *B*) In adulthood, there was no difference 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 quantitative PCR, normalized to TATA-box binding protein (TBP), α-tubulin, and hypoxanthine phosphoribosyltransferase (HPRT); 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: $F_{1,19} = 1.057$; $P = 0.32$]. *D*) At P120, relative DNMT expression levels were not altered by ES or diet (determined by quantitative PCR, normalized to TBP, α-tubulin, and HPRT; 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: $F_{1,19} = 1.994$; $P = 0.17$). *E*) At P9, hippocampal GR expression is affected by ES exposure in a diet-dependent manner (2-way ANOVA,

(*continued on next page*)

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needed. As nutritional intervention may not be easily administered in all stressful environments, future studies that address additional windows of opportunity will aid in the development of practical intervention strategies.

Which processes are involved?

Early 1-CMAM supplementation can exert its effects on later brain function either *via* a direct effect at the neuronal level (*e.g.,* by influencing brain structure and plasticity) or by indirect modulation of other processes. Here, we investigated maternal care (4), the neuroendocrine stress system (54), hippocampal volume (2, 55, 56), neurogenesis (57, 58), and epigenetic modifications (13, 59, 60). 1-CMAM supplementation abolished ES-induced chronic HPA axis hyperactivation without affecting any of the other biologic processes we investigated. Hyperactivation of the stress axis is a likely candidate in determining long-term consequences of ES, as it is not only a common phenotype of ES exposure, but it has also been associated with later cognitive impairments. Indeed, a direct modulation of the HPA axis [*e.g., via* GR antagonists or adrenalectomy during ES (61, 62) or by exposure to stress at adulthood (63, 64)] can prevent ES-induced changes in several hippocampal parameters.

Our data demonstrate that 1-CMAM supplementation exerts a direct suppressive effect on ES-induced increase in basal plasma corticosterone and associated adrenal gland hypertrophy. We did not detect ES-induced changes in hippocampal GR expression, which was consistent with recent observations that hippocampal GR expression was unaffected by maternal separation in mice (65). Thus, in mice, chronic corticosterone elevation in early life does not seem to affect hippocampal GR, which is in contrast with well-established, lasting alterations in hippocampal GR that have been observed after ES exposure and limited maternal care in rats (4, 6, 66), which indicates a species-specific effect.

Furthermore, because methionine is a key factor in 1-C metabolism, which is crucial for epigenetic machinery, ES-induced reduction in methionine levels could potentially hamper epigenetic processes, which have been implicated in brain programming by early life experiences (6, 13, 67) and learning and memory (68). However, in contrast to our expectations, we did not find drastic alterations in global DNA methylation, which suggested that this process is tightly regulated and that global methylation levels remain relatively stable, even when

methionine input varies (69). In fact, the subtle differences in global hippocampal (LINE1) DNA methylation between ES and ES-supplemented animals comprised <1% (on average 81.2% methylation), and, therefore, its biologic relevance might be limited. Our finding of no alterations in global DNA methylation levels might be a result of the fact that central levels of the other measured 1-CMAM are unaffected by ES. These are further supported by evidence that both maternal care and acute methionine administration in a model of epilepsy failed to alter global DNA methylation in the hippocampus (12, 52).

In addition, ES exposure in mice did not alter any hippocampal DNMT levels, although others have recently described altered DNMT1 levels in neuronal progenitors after maternal separation—but this was in rats, which again points to a possible species-specific effect (70). However, clearly, methodologic differences could have also contributed to these discrepancies.

As the GR had been identified as being specifically vulnerable to epigenetic modifications in early life, both in human (71, 72) and rodents (6, 65), it was of considerable interest to study methylation of the *Nr3c1* promoter. We observed a subtle ES-induced hypermethylation of CpG8, in particular, in ES, which was not prevented in our ES-supplemented pups. These changes, however, were not sufficient to modulate GR gene expression levels. Similarly, maternal deprivation in mice failed to alter hippocampal *Nr3c1* methylation (65). In addition, central methionine infusion in adult rats left the majority of genes unaffected and modified only specific genes (48). Thus, effects of ES and methionine administration seem brain region and/or gene specific, and further research is needed to investigate if and how ES in mice leads to selective vulnerability of specific brain regions and genes.

Early nutritional intervention as a clinically relevant tool

The current maternal dietary supplementation prevented several ES-induced cognitive deficits in offspring, at least part, because 1-CMAM supplementation repressed the ES-induced rise in corticosterone. 1-CAM supplementation did not have any detectable adverse effects, which was in agreement with previous studies that administered this diet (41). We have observed reduced body-weight gain in 1-CMAM–supplemented pups—probably as a result of the high intake of B vitamins, which also plays a role in energy expenditure (73)—that did not, however, lead to any lasting alteration in body weight (data not shown).

interaction of condition × diet, $F_{1,19} = 12.707$; $P = 0.02$). 1-CMAM supplementation increased GR expression in control animals (*post hoc*: [#]$P < 0.05$), whereas in ES-animals, 1-CMAM supplementation had no effect on GR expression (*post hoc: P > 0.05*). *F*) At P9, average methylation status of 9 CpGs within the Nr3C1 promoter was not significantly different between the 4 groups (no effect for condition: HT = 0.329; $F_{9,31} = 1.134$; $P = 0.370$; no effect for diet: HT = 0.170; $F_{9,31} = 1.585$; $P = 0.799$), but a trend toward an interaction effect of condition × diet (HT = 0.579; $F_{9,31} = 1.996$; $P = 0.074$) was observed. *G*) Separate analysis of the individual CpGs revealed that the trend toward an interaction is largely attributable to changes in methylation of CpG8 (interaction effect condition × diet: $F_{1,39} = 2.207$; $P = 0.020$), but *post hoc* analysis revealed no significant differences between the groups. *H*) At P120, alterations in hippocampal GR expression were no longer present (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: $F_{1,28} = 0.205$; $P = 0.65$). *I*) At P120, differences in levels of Nr3c1 methylation were not detected (no effect of condition: HT = 0.238; $F_{9,25} = 0.662$; $P = 0.734$; no effect of diet: HT = 0.217; $F_{9,25} = 0.602$; $P = 0.783$; no interaction: HT = 0.646; $F_{9,25} = 1.795$; $P = 0.120$). Data are expressed as means ± SEM.

Of interest, this supplementation did not modulate ES-induced changes in maternal care, which was in line with our hypothesis that ES-induced phenotype is likely the result of synergistic actions of multiple processes, including sensory stimuli, hormone levels, and nutritional status (19). A lack of effect on maternal care could be a possible explanation for why not all cognitive readouts were rescued. In support of this, stroking rat pups to mimic maternal sensory stimulation was previously shown to prevent ES-induced HPA axis derangements only when it was combined with feeding (74).

Our finding that supplementation of maternal diet with 1-CMAM did not prevent ES-induced changes in neurogenesis is consistent with the observed DG volumes, which were reduced by ES but not altered by 1-CMAM supplementation. These results, however, contrast with earlier described alterations in neurogenesis that were observed upon prolonged deficiencies in maternal folic acid (75), vitamin B_{12} (30), or choline (76, 77) and upon prolonged protein and 1-CMAM supplementation throughout gestation and lactation (78). This is likely explained by the shorter duration of our intervention compared with previous studies.

In summary, until now, maternal care and glucocorticoids have often been held responsible for many ES-related lasting effects on brain structure and function. Here, we provide evidence for an additional, critical role of early nutrition in modulating ES-induced programming of adult brain function. Our findings open new avenues of early nutritional intervention to prevent lasting effects of ES in offspring, an approach that may have important implications for clinical practice. Ⓕ︎Ⓙ︎

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AUTHOR CONTRIBUTIONS

E. F. G. Naninck and A. Korosi designed the research; E. F. G. Naninck, J. E. Oosterink, K.-Y. Yam, L. P. de Vries, J. A. Platinga, and R. N. Verkaik-Schakel performed research; H. Schierbeek and J. B. van Goudoever contributed reagents/analytic tools; E. F. G. Naninck and T. Plosch analyzed data; and E. F. G. Naninck, P. J. Lucassen, and A. Korosi wrote the paper.

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Early micronutrient supplementation protects against early stress−induced cognitive impairments

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