Programming of hippocampal structure and function by early-life stress: Opportunities for nutritional intervention
Naninck, E.F.G.

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Chapter 5

Effects of chronic early-life stress on neurogenesis in female mice and its response to early nutrition and adult exercise.

A preliminary report.

E.F.G. Naninck, M. Abbink, L. de Vries, P.J. Lucassen and A. Korosi
ABSTRACT

Early-life stress (ES) exerts life-long adverse effects on hippocampal structure and cognition. One form of plasticity modulated by ES, is adult hippocampal neurogenesis (AHN) that refers to the generation of new neurons in the adult hippocampal dentate gyrus, a process well regulated by various factors (e.g. age, running, stress and nutrition). Previously we had reported that ES-exposed male mice show impaired cognitive functions associated with reduced levels of AHN, whereas ES exposed female mice seemed more resilient and exhibited only mild cognitive changes and no ES-induced reductions in AHN. We here questioned if the responsiveness of female mice to positive stimuli of AHN (early and in late adulthood) is altered by ES exposure. We had recently demonstrated that early micronutrient supplementation ameliorated ES-induced cognitive impairments in male mice, without affecting AHN in control or ES exposed offspring at 4 months of age. However, whether such nutritional intervention affects neurogenesis of adult female mice remained elusive. In addition, it was unknown whether ES modifies the response to well-known stimuli of neurogenesis like exercise, in adult female mice.

Chronic ES was induced in C57Bl/6j mice by limiting bedding/nesting material from postnatal day (P) 2-9. During this period, dams received control or methyl donor supplemented diet (MD-diet). At 8 months of age, female offspring had access to a functional, or fixed, running wheel for a period of six weeks. Afterwards, levels of AHN were assessed using immunohistochemistry for proliferating (Ki67') and differentiating (DCX') new-born cells.

In this thesis chapter, the presented data are still preliminary and final conclusions await the completion of ongoing experiments. So far, i) a trend suggests a positive effect of early MD-supplementation on AHN in 8-month-old female Ctl/ES mice; ii) while chronic ES does not affect neurogenesis under basal conditions, it appears to reduce the neurogenic response to stimulating conditions in adult female mice; iii) the response to pro-neurogenic stimuli appears to reach a ceiling effect. The current data enhance our understanding of hippocampal plasticity over the life span, and after programming by early-life experiences. They provide novel insights in the time windows and applicability of environmental intervention strategies to reverse and/or prevent the lasting consequences of ES exposure.
INTRODUCTION

Substantial clinical evidence shows that exposure to early-life stress (ES) has a life-long impact on mental health [1-4], and is associated with lasting hippocampal volume reductions [5,6] and cognitive impairments [7-9]. Similar lasting consequences of ES exposure on later hippocampal structure and function have also been established in animal models [10-14].

One of the structural parameters that is strongly affected by ES, is adult hippocampal neurogenesis (AHN) [15-17], a unique form of hippocampal plasticity that occurs in the subgranular zone of the dentate gyrus (DG). It comprises the proliferation of neuronal progenitor cells, their subsequent migration and differentiation into fully functional neurons, and their integration into the existing hippocampal circuitry [18]. These newly generated neurons have been implicated in hippocampal functioning, including learning and memory [19,20] and stress regulation [21]. They are broadly recruited following the neuronal activation that is associated with hippocampus-dependent tasks (e.g. Morris water maze (MWM) and novel environment exploration [22]). AHN is dynamically regulated by various behavioural, hormonal and environmental factors. Physical exercise is one of the best known positive stimuli for AHN [23-25], whereas ageing [26-28] and exposure to acute or chronic stress in adulthood [29-32] are potent inhibitors of AHN. Importantly, previous exposure to early-life stress can persistently alter AHN under basal conditions [17].

Nutrition is another important factor that affects AHN; next to caloric intake, meal frequency and food texture, particularly dietary content has been implicated in modulating levels of AHN [33]. For instance, dietary micronutrient deficiencies (e.g. in zinc [34,35] or folic acid [36,37]) in adulthood are associated with reduced proliferation and survival of new-born neurons. There is also some evidence that deficiencies of essential dietary micronutrients during critical developmental periods are associated with impairments in brain development and later cognitive functions [38-40].

In this context, methyl donors (including methionine, homocysteine, vitamins B₆, B₁₂, folic acid and their metabolites) are an interesting group of essential micronutrients as they are critical for the one-carbon (1-C) metabolism [41,42], the biochemical pathway required for the methylation of DNA, proteins, phospholipids and neurotransmitters. As such, methyl donors are not only key for epigenetic mechanisms [43], but also essential for brain development and neurogenesis [37,44]. In addition, the essential amino-acid methionine, required for protein synthesis initiation [45] and incorporation into protein, can be the rate-limiting factor for processes that require protein formation,
like neurogenesis. Thus, reduced methionine levels might have direct effects on brain development. Indeed, several clinical [46,47] and pre-clinical [48-50] studies show that inadequate MD-supply during critical developmental periods can lead to impaired brain development, neurogenesis and cognition throughout life [48,51-53]. Also, short and early nutritional MD-supplementation during ES exposure can ameliorate ES-induced cognitive impairments in male mice [54], without affecting levels of AHN. However, so far, it remains unknown if early MD supplementation also affects AHN in the female brain and if ES-exposed female mice will also benefit from early dietary intervention.

Interestingly, a sex-specific vulnerability exists towards the lasting effects of early-life experiences on cognition and several clinical studies show pronounced effects of ES exposure on disease susceptibility [55], anxiety [56] and hippocampal white matter volume reductions [57] in males compared to females. Similarly, various preclinical studies have reported sex-dependent effects of ES [16,58,59], suggesting an increased resilience of female offspring [60,61]. In line with these findings, we have shown recently that female mice seem more resilient to the effects of chronic ES [13]. While adult male mice exposed to chronic ES exhibit reduced survival of adult-born neurons, associated with impaired hippocampus-dependent learning and memory, adult female mice showed no ES-induced changes in AHN and less prominent cognitive deficits [13]. Here we question if ES exposure might affect AHN in the seemingly resilient female mice. Alternatively, ES-effects on AHN might just not be apparent under basal conditions, and could misleading us in our interpretation of resilience. However, a possible deficit in AHN might only become visible under stimulatory conditions. Therefore, we study AHN in 8-month-old female mice, in response to early MD dietary supplementation and/or voluntary exercise.

Our findings provide preliminary evidence in female mice for: i) a possible positive effect of early MD supplementation on AHN, ii) an ES-induced resilience to the positive effects of running on AHN, and iii) a possible ceiling effect of (combined) environmental stimuli on AHN. The data presented here are preliminary and final conclusions thus await the completion of additional experiments.

**METHODS**

**ETHICS STATEMENT**

All experimental procedures were conducted under national law and European Union directives on animal experiments and approved by the animal welfare committee of the University of Amsterdam.
ANIMALS
In total, 39 female C57Bl/6J mice were used in this study (8 Ctl, 8 ES, 9 Ctl-MD, 9 ES-MD, see table 1). All animals were kept under standard housing conditions with a temperature of 20-22°C, humidity of 40-60% and on a reversed 12/12h light/dark schedule (lights on at 8 PM). Throughout their lifetime, all animals were fed ad libitum with Teklad global rodent diet 2018 (Harlan laboratories BV, Venray, the Netherlands), unless specified differently (see nutritional intervention).

<table>
<thead>
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<th>CONDITION</th>
<th>DIET</th>
<th>WHEEL</th>
<th>ANIMALS/GROUP</th>
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<td>Locked (SED)</td>
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<td></td>
<td></td>
<td>Wheel (RUN)</td>
<td>N=9</td>
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TABLE 1.
OVERVIEW OF THE EXPERIMENTAL GROUPS AND THE SAMPLE SIZES

EARLY-LIFE STRESS PARADIGM AND EARLY NUTRITIONAL INTERVENTION
To standardize the perinatal environment, mice were bred in house; the breeding procedure was described previously [13]. On postnatal day 2 (P2), the dam and five to six pups per litter (including both sexes) were weighted and 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).

ES was induced by housing dam and pups, from P2 till P9, in a cage with a limited amount of nesting/bedding material, known to induce fragmented maternal care (as described previously by [13,62]). Throughout all early-life procedures, manipulation of pups was kept to a minimum to avoid handling effects.

From P2 till P9, Ctl-MD and ES-MD dams received custom made methyl-donor supplemented diet (SniFF, Soest Germany), consisting of Teklad global rodent diet 2018 supplemented with: 15g/kg Choline, 15g/kg Betaine, 15 mg/kg Folic acid, 1.5 mg/kg Vitamin B12, 7.5 mg/kg L-Methionine and 150 mg/kg Zinc (from ZnSO47H2O) (previously described by [63]) and in addition drinking water
supplemented with 15.3 ug/mL vitamin B₆ (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).

On the morning of P9, dams and pups were weighted and moved to standard cages equipped with normal amounts of bedding material and standard food and water ad libitum. Offspring was weaned at P21 and housed in groups of the same sex with 2-3 animals/cage, for the experiments described here, only female offspring was used. Body weights of the animals were measured at P9, P21 and P120.

**Voluntary wheel running**
At 8 months of age, running (RUN N=27) and sedentary (SED N=12) mice were moved to a larger cage (type III, Technilab-BMI, Someren, the Netherlands) with a functioning (RUN) or locked (SED) plastic running wheel (160 mm diameter, Ferplast, Italy) to control for cage enrichment. Running mice received access to the running wheel for 6 weeks. To avoid possible adverse effects of social isolation [64], animals remained housed with 2-3 animals per cage, therefore individual wheel running distances were not available. Voluntary physical activity in each cage was monitored daily by focal animal observations. Two RUN animals were excluded from the analysis, as they did not actively engage in running (this was also apparent from excess bodyweight gain > 5 grams).

**Experimental design**

**FIGURE 1. TIMELINE**
Chronic ES was induced from P2 till P9, during the same period, dams of Ctl-MD and ES-MD mice received custom made methyl-donor supplemented diet. At 8 months of age, mice received 6-week access to a functioning (RUN) or locked (SED) running wheel. At the end of the running wheel exposure (P280) mice were sacrificed.
Tissue collection
By the end of the 6 weeks running wheel exposure (at P280, see figure 1), mice were anaesthetized by an i.p. injection of pentobarbital (Euthasol® 120 mg/kg) and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB 0.1M, pH 7.4). The estrous cycle stage at time of perfusion was determined by vaginal smear cytology [65].

Brains were removed and postfixed overnight in PFA/0.1M PB at 4°C and stored in PB with 0.01% sodium azide at 4°C until further processing. After an overnight cryoprotection in 30% sucrose/0.1M PB, frozen brains were cut in 40 μm thick coronal sections using a sliding microtome, divided over 6 parallel series and stored in a cryoprotective solution (30% Ethylene glycol, 20% Glycerol, 50% 0.05M PBS) at -20°C until processing.

Immunohistochemistry
Immunocytochemistry was used to identify proliferating (Ki67⁺) cells and young, differentiating neurons (doublecortin, DCX⁺) cells. All stainings were performed on a one-in-six parallel series of sections (240 μm apart). Prior to Ki67 staining, sections were mounted on glass slides (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.

For both stainings, pre-mounted (Ki67⁺) or free-floating (DCX⁺) sections were incubated with 0.3% H2O2 for 15 minutes to block endogenous peroxidase activity. After 3x5 min washing with 0.05M TBS sections were incubated for 30 minutes with blocking solution (2% milk powder in TBS) and subsequently incubated with primary antibody in Supermix (0.5% Triton X-100, 0.25% gelatine in 0.05M TBS) for one hour at room temperature followed by overnight incubation at 4°C. The following primary antibodies were used: polyclonal rabbit anti-Ki67 (Novocastra NCL-L-Ki67_MM1, 1:20,000), polyclonal goat anti-DCX (SantaCruz Biotechnology sc-8066; 1:800). Next, sections were rinsed 5x5 minutes with 0.05M TBS and incubated with biotinylated secondary antibodies in Supermix (1:200 goat anti-rabbit, Vector Laboratories or 1:200 donkey anti-goat, Jackson Laboratories) for two hours at room temperature, followed by a 90 minute incubation with avidin-biotin complex (ABC kit, Elite Vectastain Brunschwig Chemie, Amsterdam, 1:800), an additional amplification with tyramide (1:500, 0.01% H2O2, once, for 30 minutes) and a second 90 minute incubation with avidin-biotin complex. Subsequent chromogen development was performed with diaminobenzidine (20 mg per 100 mL 0.05M Tris, 0.01% H2O2).
**IMAGING AND QUANTIFICATION**

Quantification procedures were performed on a Zeiss Axiophot light microscope with Microfire camera (Coptronics) by a researcher blind to the experimental conditions. Per animal, eight coronal sections of matched anatomical levels along the rostro-caudal axis were used for analysis.

To obtain volume estimations of the dentate granular zone (granular cell layer + sub granular zone) in mm\(^3\), the Cavalieri principle was applied. The number of Ki67\(^+\) and DCX\(^+\) immuno-reactive cells was counted using a 40x objective (400x magnification), and multiplied by 6 to obtain an estimation of the total number of immunoreactive cells per DG. Discrimination was made between the various anatomical (and functional) subregions of the dentate gyrus to determine possible differences between immunoreactivity between the supra- versus infrapyramidal blade and rostral versus caudal DG. Cell counts are represented as total number of immunoreactive cells per unilateral DG (subgranular zone + granular cell layer).

**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. Immunohistochemical data was analyzed using three-way univariate F-tests with the fixed factors: condition (Ctl vs. ES), diet (standard vs. MD) and/or exercise (RUN vs SED). Post-hoc analyses were performed using Bonferroni multiple comparison tests. Sample size estimations for future studies were calculated by: \(N/\text{group} = (Z_{\alpha/2}^2 + Z_{\beta}^2) \cdot (s_1^2 + s_2^2)/(\mu_1 - \mu_2)^2\) with \(\alpha=0.05, \beta=0.2\).

**RESULTS**

**BODYWEIGHT THROUGHOUT LIFE**

Exposure to the limited nesting and bedding cage induced physiological signs of chronic ES in the pups, including a reduced bodyweight gain (BWG) from P2-P9 (main effect of stress: \(F_{1,35}=21.58, p<0.0001\), [figure 2a]), as described previously [13]. MD-supplementation during the ES period further reduced bodyweight gain in Ctl and ES female pups (main effect of diet: \(F_{1,35}=12.65, p=0.0011\), no interaction effect: \(F_{1,35}=0.14 p=0.71\), confirming our previous findings in male pups (see [54]). At weaning (P21), bodyweight differences are no longer present (no effect of stress: \(F_{1,35}=0.71, p=0.4052\), no effect of diet: \(F_{1,35}=1.99, p=0.167\), no interaction effect: \(F_{1,35}=2.27 p=0.141\), [figure 2b]), indicating a catch-up growth in ES and MD animals.
Effects of early nutrition and exercise on neurogenesis in adult ES-exposed female mice

In adulthood, previous to running wheel exposure, bodyweights of ES-exposed females were slightly lower than those of controls, this effect was prevented by the MD-diet (interaction effect: $F_{1,35}= 6.51$ $p=0.015$, posthoc analysis reveals a significant difference between $Ctl$ and ES ($p<0.05$) but not $Ctl$-MD and ES-MD, data not shown). Overall, bodyweight gain during the wheel running period was lower in the running animals compared to sedentary animals ($2.3 \pm 0.3$ grams in RUN ($n=25$) vs. $2.9 \pm 0.3$ grams in SED ($n=12$), but this effect was not found to be significant (T-test, $p=0.307$), as the effect of running on bodyweight gain varied between the experimental groups (univariate F tests revealed a significant 3-way interaction of condition x diet x exercise on bodyweight gain ($F_{1,29}= 5.61$ $p=0.025$, data not shown).

Characterization of adult neurogenesis in 8-month-old female mice

Cell proliferation was evaluated by counting the number of Ki67$^+$ cells in the hippocampal DG. As expected, Ki67$^+$ cells were generally clustered and located in the SGZ of the DG, and the number of Ki67$^+$ cells was fairly limited at this age (see figure 3a). Doublecortin (DCX) was used as marker for differentiation. DCX is a brain-specific microtubule associated protein that is expressed in transiently amplifying progenitor cells (type 2b/3 cells) until they reach the post-mitotic immature granule cell stage [66], therefore DCX$^+$ cells are more abundant than Ki67$^+$ cells (see figure 3b). Quantification and qualitative classification of the DCX$^+$ cells (i.e. proliferative stage, intermediate stage or post mitotic stage (based on [11,67])) is currently ongoing.

Modulation of neurogenesis by ES, early nutrition and running

Comparison between all eight experimental groups revealed no significant effects on proliferation yet (Three-way ANOVA revealed no effect of stress: $F_{1,29}=0.58$, $p=0.453$, no effect of diet: $F_{1,29}=1.44$, $p=0.240$, no effect of exercise: $F_{1,29}=0.24$ $p=0.628$ and no significant interactions) due to limited sample size. A

**FIGURE 2. Effects of ES and MD supplementation on bodyweight**

(A) Bodyweight gain in pups (P2-P9) is reduced by chronic ES (*) and MD-supplementation (#). (B) Bodyweight at weaning (P21) is no longer different between the groups.

<table>
<thead>
<tr>
<th>A. P2-P9</th>
<th>B. P21</th>
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<tr>
<td><img src="image1" alt="Bodyweight gain" /></td>
<td><img src="image2" alt="Bodyweight gain" /></td>
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<tr>
<td><strong>STANDARD</strong></td>
<td><strong>STANDARD</strong></td>
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<td><strong>MD DIET</strong></td>
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<td><strong>Ctl</strong></td>
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power analysis based on the detected effect size indicates that the sample size needs to be increased to n=7-10/group to reveal a significant difference in the amount of Ki67+ cells between the groups. The experiments to increase the number of samples are currently ongoing. However, below we consider the trends pointing towards possible effects of MD supplementation and running on proliferation separately, in interaction with ES exposure.

**FIGURE 3.**
Representative images of immunohistochemical stainings in the dentate gyrus
(A) Nuclear Ki67 staining, indicating proliferating cells. Right panel: 100X magnification, left panel: 400X magnification. (B) Cytoplasmic Doublecortin (DCX) staining, indicating newborn immature neurons with dendrites extending to the molecular. Right panel: 100X magnification, left panel: 400X magnification.
Early MD-supplementation seems to increase proliferation in adult female mice and this is not affected by ES exposure

MD supplementation appears to enhance levels of proliferation in female mice. A two-way ANOVA comparing only the four sedentary groups revealed a trend towards a main effect of diet ($F_{1,8}=4.72$, $p=0.062$). In contrast, the same diet did not affect number of Ki67+ cells in 8-month-old male mice [54]. Interestingly, ES did not seem to influence this effect of MD-supplementation (Two-way ANOVA: no effect of stress: $F_{1,8}=0.31$, $p=0.591$ and no interaction effect: $F_{1,8}=0.027$, $p=0.874$, figure 4a).

ES seems to reduce the responsiveness to voluntary wheel running in adult female mice

ES does not affect levels of proliferation in standard (sedentary) conditions (T-test Ctl vs ES, $p=0.609$), coherent with previous findings in 5-month-old females [13] and 8-month-old males [54]. Here we also questioned if effects of ES on proliferation might become apparent under stimulating conditions (e.g. prolonged wheel running).

In line with the large body of literature establishing the pro-neurogenic effect of voluntary wheel running in female C57Bl6j mice [68-70], the number of proliferating cells in Ctl RUN (94.5 ± 9.9), appears to be higher than the number of proliferating cells in Ctl SED (67.5 ± 4.5). Interestingly, ES prevented this increase, and in fact the number of proliferating cells was similar between ES SED (63.0± 6.0) and ES RUN (71.5±10.0) mice (figure 4b). This suggests that exposure to ES reduces the neurogenic response to running in female mice. However, due to the small sample size, we were yet unable to determine if this differences is statistically significant (Two-way ANOVA comparing only the four standard diet groups reveals no effect of stress: $F_{1,12}=1.12$, $p=0.310$, no effect of exercise: $F_{1,12}=1.87$, $p=0.196$ and no interaction effect $F_{1,12}=0.51$, $p=0.489$).

A further increase in proliferation upon voluntary exercise in adulthood is not observed in mice exposed to MD-supplementation.

Interestingly, running did not further increase proliferation in animals that had been exposed to MD-supplementation (figure 4c). A two-way ANOVA comparing all four MD-supplemented groups revealed no effect of exercise ($F_{1,17}=0.39$, $p=0.543$), no effect of stress ($F_{1,17}=0.01$, $p=0.939$) and no interaction effect ($F_{1,12}=0.29$, $p=0.596$). This might indicate the possible existence of a ceiling effect of environmental stimuli on levels of AHN in 8-month-old females.
Figure 4.

**Proliferation and DG volume in adult female mice**

(A) MD supplementation possibly enhances proliferation in female mice (§ trend towards a main effect of diet: F₁,₈ = 4.72 p=0.062). ES does not seem to influence this effect of MD-supplementation. (B) ES possibly reduces the responsiveness to voluntary wheel running in female mice. The number of proliferating cells in Ctl RUN, appears to be higher than the number of proliferating cells in Ctl SED, while the number of proliferating cells was similar between ES SED and ES RUN mice. This effect did not reach significance. (C) Comparison between all eight experimental groups reveals that running does not further increase proliferation in animals that have been exposed to MD-supplementation. (D) Comparison between all eight experimental groups revealed a trend towards a running-induced increase of dentate granular zone volume, but no effect of stress or diet and no significant interactions.

**The volume of the dentate gyrus granular zone is increased by running and this effects is independent of ES and/or diet.**

Comparisons between all eight experimental groups revealed a trend towards a running-induced increase of dentate granular zone volume (Three-way ANOVA revealed a trend of exercise: F₁,₂₈ =3.37, p=0.077, but no effect of stress: F₁,₂₈ = 0.21, p=0.653, or diet: F₁,₂₈ =0.001 p=0.981 and no significant interactions, figure 4d).
DISCUSSION

While the final completion of the full dataset is still ongoing, our results so far suggest: i) a potential positive effect of early MD supplementation on the later levels of proliferating (Ki67+) cells in female mice; ii) a potential ES-induced inhibition of the running induced stimulation of proliferation at an adult age; and iii) no additive effect of early nutritional intervention combined with later life exercise on hippocampal proliferation. Considering the preliminary nature of this data, definite conclusions await additional experiments, that are required to obtain the statistical power needed to detect significant differences, and to determine differences in the various stages of neuronal differentiation (DCX morphologies). These experiments are currently ongoing. Increasing sample size will also enable the necessary correction for potential litter-to-litter variation [71] and for possible effects of the oestrous cycle phase (described previously by others [72]). Below we will discuss our initial findings.

**Early MD supplementation seems to increase AHN in female mice**

Early nutritional intervention seems to increase proliferation in both Ctl and ES females. This suggests that adult neurogenic capacity could be programmed by early nutrition, consistent with literature showing that MD status affects later levels of neurogenesis and apoptosis. Most of this knowledge however, is obtained through selective deficiency studies, e.g. in folic acid [52], vitamin B₁₂ [48], choline [51,53] or zinc [73]. Dietary micronutrient deficiencies in adulthood, e.g. zinc deficiency in rats [34,35] or folic acid deficiency in mice [36,37] are also associated with reductions in proliferation and survival of newborn neurons. Also, maternal MD-supplementation during preconception and gestation increased proliferation at weaning, as well as in vitro proliferation and differentiation of progenitors isolated from foetal hippocampi [74].

How early MD-supplementation exactly exerts its effect on AHN is unknown and its effect can be either direct or indirect. Considering the importance of methyl donors for epigenetic mechanisms [75-78], protein synthesis and (possibly) modulation of the HPA-axis [54], we hypothesize that early MD-supplementation might modulate neuronal processes that directly affect cell division. Another option is that early MD supplementation lastingly alters the way the animals behave and interact with their environment, and that this then indirectly alters levels of AHN. The notion that MD availability during early-life can lastingly alter behaviour is supported by some preclinical evidence: rat pups from dams that were fed a MD deficient diet during gestation and lactation, showed lasting behavioral deficits [48,49]. In line with this, our MD supplementation was able to partly rescue the learning and memory deficits induced by ES (and the ES-induced reduction in methionine) in male mice [54].
**ES Seems to Reduce the Running Induced Increase in Neurogenesis**

Physical activity is a very interesting environmental manipulation; it not only increases AHN in rodents, but also exerts cognitive benefits in humans throughout the life-span and is even associated with a reduced risk of dementia and improved academic performance and decision making [79].

Regarding AHN [23,24], the pro-neurogenic effects of exercise, e.g. in C57Bl/6 female mice, are generally much stronger than those of other classic AHN stimuli like antidepressants [80] and are usually maintained throughout life [25,79,81]. Although previous reports had shown that learning-induced AHN is repressed in rat offspring exposed to prenatal stress [82], little was known as to how ES affects the later neurogenic response to running. Interestingly, male mice exposed to ES exhibit a reduced survival of adult born cells, but we found AHN in female mice to be unaffected by ES [13]. Our current preliminary evidence suggests that female mice, while seemingly more resilient to ES, show an altered response when tested under conditions where neurogenic response is required/recruited, like running. This points towards a lasting effect of ES on adult neurogenic capacity and responsiveness.

A reduced response to running might have functional implications e.g. for cognitive functioning; intact exercise-induced AHN is required for improvement of spatial memory performance [83], and associated with spatial memory recovery after stroke [84] (but see [70]). Furthermore, running restores the deleterious effects of ageing [81] and adult corticosterone exposure [85] on AHN and spatial learning and memory. Thus, if ES indeed reduces the adult responsiveness to running, this is likely to be maladaptive. ES exposed female mice exhibited milder cognitive impairments when compared to males [13], but whether their cognitive functions under more demanding (e.g. stressful) conditions are affected has not yet been investigated. One could hypothesize that ES-exposed females might be less responsive to environmental stimuli in general; not only to positive stimuli as running, but also to negative stimuli, such as stress at an adult age. In line with the match-mismatch hypothesis [86], ES-induced unresponsiveness to any environmental stimulus could then be the result.

To understand how exercise fails to stimulate AHN after ES, it would be important to first elucidate the molecular mechanisms responsible for the effects of running on AHN itself. Various biological factors like angiogenesis, growth factor production, cytokines and various neurotransmitters and hormones may be involved [87]. When ES-exposed rats are subjected to exercise, they display a lower corticosterone response to stress than sedentary ES-animals [88]. Hence it would be interesting to study in future experiments if ES differentially affects running-induced CORT responses in mice as well.
To ensure that the difference between Ctl and ES animals is not due to differences in running activity between the groups, a better assessment of the duration and frequency of wheel running would be informative, especially since bodyweight gain was not equally reduced by running in all experimental groups. However, this requires individual housing of the animals and was not done in the present experiments as social isolation in female rats impedes the stimulatory effects of running on AHN [64,89].

So far there is no evidence for an additive effect of running combined with early MD supplementation. Possibly, an up-regulation of AHN beyond a certain optimum becomes no longer beneficial (such as in epilepsy [90]) and is therefore prevented. This hypothesis has also been postulated by Glenn et al. who found that both prenatal choline supplementation, as well as adult exploration behaviour increased proliferation, while the combined exposure to both manipulations had no additive effects [91]. Another reason could be a depletion of the neurogenic pool: if the MD-induced increase in AHN has an early onset, that is maintained throughout adulthood, the pool of progenitor cells might have become depleted by the time the animals are 8-months old and therefore a further increase of AHN (by exercise) might be impossible.

Based on our proliferation data, the early nutritional intervention seems more beneficial than adult exercise (as it is increases AHN in both ES and Ctl animals), but if this is due to i) the type of intervention, or ii) the timing of the intervention, remains unknown. How permanent endogenous and environmental factors alter levels of AHN is often dependent on their timing; while the effects of adult experiences (e.g. adult stress exposure) are often transient [92], factors early in life can have a long-lasting impact on the adult neurogenic capacity long after the event has taken place (see [17] for an overview of ES effects). Similarly, effects of early nutrition can exert enduring effects on AHN [93,94]. This offers an opportunity for early nutritional intervention; for instance, we have previously shown that early dietary supplementation with methyl donors could effectively ameliorate the lasting consequences of ES on cognitive function in male mice (without altering levels of AHN) [54], but the effects of early MD supplementation on the functional level in female mice remain yet unknown.

Our volume data indicates that adult exercise, but not MD supplementation, can increase volume of the dentate granular zone in both ES and Ctl females. However, AHN was only increased in Ctl, but not ES animals. Although increased DG volume might be causally related to running-induced AHN increments [95], it is not proven to be exclusively responsible for the volume effects of running, as new-born cells only account for 1-2% of the total granule cell population [96] and other factors (e.g. dendritic arborisation and vessel diameter) likely
contribute to volume changes. Thus, the trend towards the running-induced volume increase in ES-exposed females indicates that running, compared to sedentary housing, might exert certain beneficial effects on (some of) these factors in the ES-exposed brain.

**Sex-specific vulnerability or early-life experiences**

Sex of the offspring appears to be a crucial determinant for the effects of early-life experiences on brain structure: while ES effects on AHN where not detected in females under baseline conditions [13], these effects become apparent upon exposure to an additional neurogenic stimulus (e.g. wheel running). This points to a limited capacity to respond to stimuli requiring neurogenesis in female ES-exposed mice. It remains to be investigated how male ES-exposed mice respond to wheel running. We here showed that early MD-supplementation increases AHN in females while such effects have not been found in male mice [54]. While ES females clearly are differently affected as male mice, it remains an important question whether they are indeed protected and/or maybe more receptive to (nutritional) intervention.

Various studies provide evidence for a female-specific resilience to the effects of ES on AHN; e.g. stress or glucocorticoid exposure during the prenatal period reduces neurogenesis in adolescent males but not females [97-99], or the effects of prenatal stress becomes apparent at a much later age in female compared to male rats [100]. However, opposite findings have also been reported (see [16,17]). Possibly, this inconsistency can be explained by the fact that ES effects on neurogenesis largely depend on the type and duration of ES, the species examined and on the moment at which neurogenesis is determined. It would be interesting to investigate what underlies the sex-specific effects of ES on the brain. Gonadal steroids are obvious candidates and particularly estrogens are thought to have neuro-protective and growth promoting functions. They can have various effects on newly generated neurons, which do express estrogen receptors [101]. However, the effects of estrogens on AHN are complex and both increases as well as decreases have been reported [102].

Increased insight in the biological basis underlying these sex differences might not only help the development of sex-specific treatment strategies but might also provide insight in the mechanisms that underlie the sex differences in the prevalence of (stress-related) psychopathologies such as schizophrenia and depression [103]. Further research in this direction will increase our understanding of hippocampal plasticity over the life span and the effects of early-life experiences. Together, this might help to develop (sex-specific) intervention strategies to prevent or reverse the lasting consequences of ES exposure.
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