

## Effects of environmental enrichment on the behavioural consequences of early-life stress in rats

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- Early life stress
- Enriched environment
- Anxiety
- Memory
- Species typical behaviour
- oxidative stress

### Abstract

**Background:** Maternal separation (MS) is a major cause of chronic life stress and increased risk of psychiatric illness. Environmental enrichment (EE) enhances brain plasticity and neurogenesis. We investigated the consequences of maternal separation and early weaning and whether these could be prevented by environment enrichment. **Methods:** Wistar rat pups were divided into 3 groups; an MS group subjected to 3 h/day of maternal separation from postnatal day (PND) 4 till early weaning at PND 18; a maternal separation + environment enrichment (MSEE) group subjected to 3 h/day of maternal separation from PND 4 during which rats were transferred to an enriched cage, till early weaning at PND 18; and a control group. Rats were then subjected to a series of behavioural tests to assess exploratory behaviour, anxiety, memory, species-typical behaviour and depression. At PND 48, rats were sacrificed, and their brains excised for biochemical assessment. **Results:** Anxiety was increased in MS rats, evidenced by a marked reduction of time spent in the light side of the black-white alley. Anxiety was significantly decreased by EE. MS rats had suppression of their natural exploratory behaviour, with decreased number of squares crossed in the open field test ( $P = 0.031$ ), and a complete absence of rearing behaviour. Early life EE of MS rats restored exploratory behaviour to or towards control values in the open field test. Spatial memory showed a marked improvement by EE. **Conclusion:** Early life EE during and after maternal separation in rats ameliorates multiple adverse psychological consequences.

## **1. Introduction**

Exposure to stress in early life is linked to abnormalities in brain development and long-term behaviour(1,2). Epidemiologic studies show that children exposed to various forms of early life stress, including neglect, abuse and maternal deprivation, have an increased risk of developing psychiatric illness in adult life, including depression, anxiety and bipolar disorder(2–4).

Several animal models have been developed to mimic early life stress to enable studying of the neural mechanisms involved. Maternal separation together with early weaning (MSEW) is a recognized rodent model of early life stress(5,6). Several protocols of MSEW have been developed, with variations in the duration and timing of MS (7). MSEW in rodents has been shown to trigger perturbations in multiple organ systems. These include reduced insulin sensitivity(8), increased circulating corticosteroids (9), impairment of male reproductive functions (10), and increased susceptibility to inflammatory bowel disease(11). Additionally, MSEW has been shown to negatively impact neurological function and behaviour in rodents. MS has been shown in numerous studies in mice to induce depressive-like behaviour and memory deficits, but with inconsistent findings across mouse strains, with Balb/c mice showing the greatest sensitivity to the neurological effects of MS (7). On the other hand, a recent systematic review established that increased anxiety behaviour in response to MS is observed more consistently in rats than in mice (12). Underlying these behavioural abnormalities is an extensively studied spectrum of neuropathological changes in affected rodents, including neuroinflammation(13), aberrant

neuronal gene expression(14,15), accumulation of abnormal proteins in neurons(16), and neurodegenerative changes(17). The effects of early life stress on species-typical behaviour in rodents is, however, less well researched.

Besides early life stress (ELS), the broader early-life environment exerts a substantial influence on neurodevelopment(18,19). In rodent models of physical and psychiatric illness, early life EE was found to exert positive effects on brain structural development, memory and pain sensitivity (20–22). Furthermore, EE ameliorates depression- and anxiety-related phenotypes induced by early life physical or psychosocial insults (20–22). However, despite the established role of early life EE in promoting healthy development of neurological function and behaviour, and in ameliorating the impact of various neurological insults, the effect of EE on the behavioural perturbations caused by early life stress is less studied. The aim of this study was to further investigate the effects of early life stress caused by maternal separation and early weaning on rat behaviour, and how far these can be prevented by simultaneous exposure to early life EE.

## **METHODS**

### **Experimental design**

Wistar rats were procured from the Animal House Breeding Facility at the Faculty of Medicine–Alexandria University and housed in plastic cages on a standard 12-hr light-dark cycle with free access to food and water. All animal procedures were approved by the Ethics Committee of Alexandria University, approval no. 0304952. After mating, pregnant dams were housed separately. The day of birth was considered as postnatal day (PND) 0. On PND 4 pups were

randomly assigned to 3 groups: a control group (N = 9), an MS group (N = 8) and a maternal separation + environmental enrichment (MSEE) group (N = 7). Control group pups were only handled to change the cage bedding once weekly, they stayed with their dam till weaning at PND 22. In the MS and MSEE groups, pups were daily separated from their dam starting at PND 4 for 3 hrs /day (9:00 AM till 12:00 PM) till PND 18. To boost the stress effect, they were weaned early at PND 18 (as opposed to PND 22 in control rats) and were otherwise left undisturbed from days 18-48. For the MSEE group, the pups were separated in enriched cages for periods equivalent to the MS group till PND 18. Enriched cages (70 x 30 x 70 cm) contained running wheels, burrows (cardboard tubes), plastic cups, cardboard, metal foam and nesting material (compressed cotton) which were regularly replaced. The home cages of the MSEE group also contained nesting material and burrows. The use of material with different colours and textures like wheels, plastic cups and metal foam is important to potentiate the sensory experience in rats. To potentiate the species typical behaviour like nesting and burrowing; the enriched cage contains nesting material, running wheel and burrows. These tools are used also to simulate the natural environment of rats. (23) At PND 18, rats were weaned and housed either in a standard cage or in an enriched cage till PND 48. Rats were fed a standard chow diet and were housed in groups of 4 rats/group.

### **Behavioural tests**

Behavioural tests were conducted in a separate quiet room between 9:00 AM and 3:00 PM and at the same ages for all 3 groups (PND 18 to PND

42). All tests were conducted during the light phase under standard illumination by researchers blinded to the group identity.

#### *Exploratory behaviour*

**1- Open field test:** The apparatus was a dark grey 50 × 30 cm arena divided into 10 × 10 cm squares. Each rat was placed in a corner square facing the wall and observed for 3 minutes. The recorded parameters were: latency to leave the first square, total number of square crossings, time spent in central squares, latency to first rear, and number of rears. (24)

#### *Emotionality and anxiety*

**2- Black–white alley:** Two open-ended wooden boxes, one black, one white, each 60 × 9 × 30 cm, were placed together to form an alley. The rat was placed in the black end and the latency to cross to, and the time spent, in the white half were recorded for a duration of 1 minute. (24).

#### *Short-term working memory*

**3- Spontaneous T-maze alternation:** A T-shaped maze (start arm and lateral goal arms are 35 cm long and 7 cm wide and the central choice zone is a square 7 cm × 7 cm area) was used (25). The rat was placed in the start arm, facing away from the choice point. It was allowed to freely enter one of the arms and a sliding door was carefully closed, trapping it in the chosen arm for 30 s. It was then removed, the sliding door opened, and the rat replaced in the start arm. Each rat was given 10 trials (2h-24h interval) and the % of alternations was calculated.

#### *Spatial memory*

**4- Transparent Y-maze test:** The apparatus was a symmetrical Y-shaped maze (Acrylic 35 × 7 × 13) (26). Initially, one arm was closed off while the other two arms were accessible. The rat was placed

at the end of the start arm and was given a 4-minute exploration period after which it was removed for 1 minute. The closed arm was then opened, and the rat was replaced in the start arm and allowed to explore the maze freely for 2 minutes. The number of arm entries and the time spent in each were recorded. The latency to enter the novel (previously closed) arm was also recorded.

#### *Depression-related behaviour*

**5- Tail-suspension test:** Rats were suspended 50 cms above the bench by their tails using tape for 4 minutes(27). A video camera was used to record the total time of immobility.

**6- Glucose preference:** This test was used to assess preference of a sweetened drink over plain drinking water(28).Rats were placed in individual cages for 24 hours and provided with two sipper glass bottles, one with water and the other with 10% glucose solution 150 ml each. Volumes of remaining water and glucose solutions were measured after 24 hours, and the

$$\% \text{ glucose preference} = \text{calculated as } \frac{\text{Glucose intake} \times 100}{\text{total intake}}$$

#### *Species-typical behaviour*

**7- Burrowing test:** Many rodents dig out food pellets from a tube, this resembles burrowing behaviour in the wild. Rats were placed in individual cages, each containing one “burrow” consisting of a black plastic tube 20 cm in length and 6 cm in diameter, sealed at one end, filled with 200 g of food pellets. After 2 hours, the burrows were weighed to determine the weight of pellets displaced by each rat (29).

**8-Nesting scores:** Rats were placed overnight in individual cages, each containing one “Nestlet” which was , a square piece of pressed cotton (5 cm<sup>2</sup>, 0.7 mm in height) (29). Nests were scored on a 1-5 scale as follows:

1. Cotton largely untouched (>90% intact).
2. Cotton partially torn (50-90% remaining intact).
3. Cotton is mostly shredded but with no identifiable nest site: (10–50% intact)
4. Identifiable, but flat nest: > 90% of the cotton is torn up, the material is gathered into a nest, but the nest is flat, with walls no higher than rat body height
5. A (near) perfect nest: > 90% of the cotton is torn up, and arranged as a crater or mound with walls higher than the rat body

**9- Marble burying test:** Rats were placed in individual cages each containing twelve glass marbles of an equal size and shape, arranged at equal intervals as three rows of four on a 5-cm layer of sawdust bedding (30). Each rat was left for 30 mins, after which the number of marbles buried (to at least 2/3 depth) in sawdust were counted.

#### **Biochemical tests**

On PND 48, all rats were fasted for 12 hours, weighed and euthanized by terminal intraperitoneal anaesthesia (ketamine 50 mg/kg + xylazine 5 mg/kg). Blood and tissue samples were immediately collected and frozen (–80°C) for biochemical analysis.

Serum concentrations of total cholesterol, HDL-C, triglycerides, albumin, and glucose, and serum total antioxidant capacity, were measured by calorimetric assays on a Stat Fax 1904 Plus spectrometer (Awareness Technology, Inc., Palm City, Florida, USA), with absorbance at 474–505 nm.

#### **Statistical analysis**

Due to the skewed distribution of several behavioural test scores, non-parametric analysis was used for all parameters. Data are presented as median (25<sup>th</sup>, 75<sup>th</sup> percentiles). Kruskal Wallis

ANOVA was used to test for significant differences across all 3 study groups, followed, where applicable, by Mann-Whitney U tests for pairwise comparisons. PASW Statistics for Mac (20.0; SPSS Inc., Chicago, IL, USA) and GraphPad Prism (version 9.0 for Mac) were used for analysis and presentation of data. All tests were two-tailed and  $P < 0.05$  was considered significant.

## RESULTS

### Behavioural tests

#### Exploratory behaviour and anxiety

Exploratory behaviour, which, in rodents, is dependent on overcoming initial anxiety in a new arena, was tested using the open field and black-white alley tests. The MS group showed a marked decrease in exploratory behaviour in the open field compared to controls ( $p < 0.05$ ; Figure 1-B), manifested by a higher latency to leave the first square (Figure 1-A) and a 75% decrease in the number of squares crossed. This decreased exploration was completely rescued by EE, where MSEE rats showed values similar to controls. The

open field tests also suggested MS pups were more anxious as they exhibited no rears (Figure 1-D), compared to a median of 4 rears in the control group. EE significantly increased the number of rears (median of 2 rears,  $P = 0.021$  compared to MS).

Further assessment of anxiety was performed by the black-white alley test. The MS group demonstrated a significant ( $p = 0.023$ ) increase in the mean latency to cross to the white compartment (median [IQR]: 180 [63, 180] seconds; Figure 1-E) compared to the control group (13 [5, 19] seconds). In addition, the time spent in white compartment was significantly reduced to 0 [0, 2.5] seconds in the MS group versus the control group (15 [12, 22] seconds;  $p = 0.002$ ; Figure 1-F). EE reduced the latency to cross to the light compartment (14 [11, 55] to values comparable to controls ( $p = 0.68$  versus controls), and significantly increased the time spent in the white compartment to values exceeding the control group ( $p = 0.000$ ).

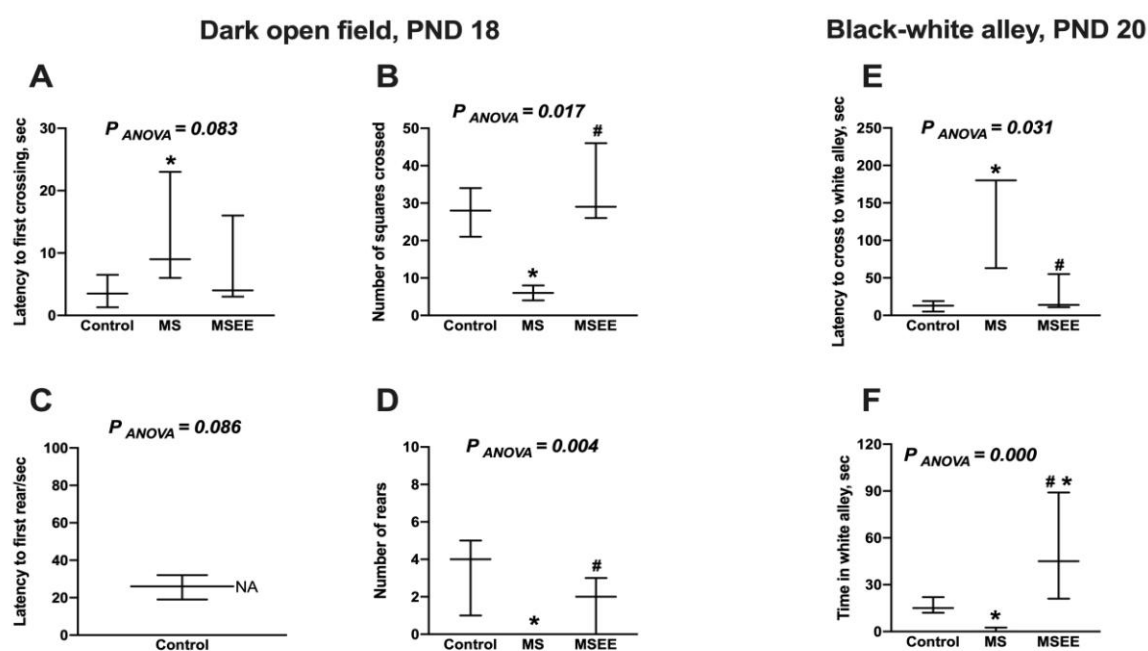


Figure 1

**Figure 1.** Rat performance in the open field (A-D) and black-white alley (E-F) tests. \* $p < 0.05$  vs controls; # $p < 0.05$  for MS vs MSEE groups. MS, maternally separated; MSEE, maternally separated rats with environmental enrichment; PND, postnatal day.

### Memory

Short-term working memory was not impaired by MS relative to controls; however, memory was significantly enhanced in the MSEE group, with a median of 100% correct alternation over 10 trials, compared with 75% in controls ( $p = 0.004$ ; Figure 2-A). Spatial memory was also not affected by MS or EE, as evidenced by lack of a difference in the number of entries to the novel arm (Figure 2-B). However, there was a tendency for decreased time spent in the novel arm by the MS group, that was partly rescued by EE ( $p$  ANOVA = 0.074; Figure 2-C).

### Depression

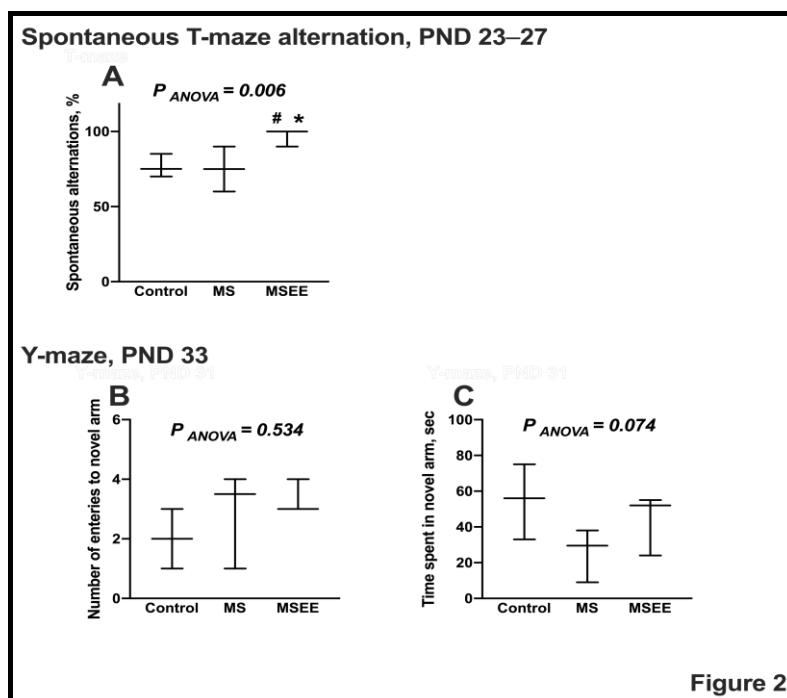
No evidence of depressive behaviour was detected in MS rats by either the glucose preference test or the tail suspension test ( $p$  ANOVA  $\geq 0.22$  for both tests; Figure 3).

### Species-typical behaviour

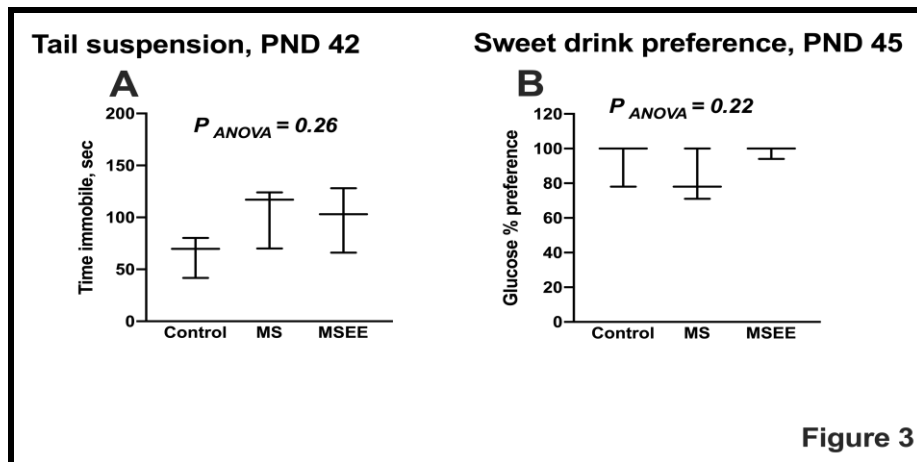
Nest building was impaired in the MS group, with none of the rats qualifying as good nesters, compared with 60% of the control group. EE tended to improve nesting behaviour, with 42% of rats in the MSEE being good nesters ( $p = 0.059$ ; Figure 4-b). Neither burrowing nor marble burying were significantly affected by MS or EE ( $p \geq 0.47$ , Figure 4-A and 4-C).

### Serum biochemistry

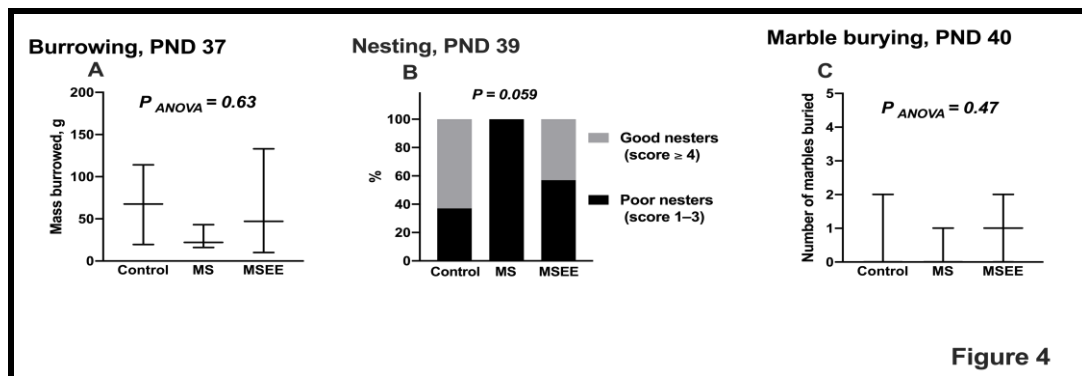
Fasting blood glucose level was not significantly affected by MS or EE. HDL cholesterol showed significant increase in MSEE group compared to control or MS groups ( $P=0.023$ ). Total antioxidant capacity was higher in the MSEE group.



**Figure 2.** Rat performance on spontaneous T maze alternation (A) and the Y maze (B,C) tests. \* $p < 0.05$  vs controls; # $p < 0.05$  for MS vs MSEE groups. MS, maternally separated; MSEE, maternally separated rats with environmental enrichment; PND, postnatal day.



**Figure 3.** Tail suspension test (A) and sweet drink preference (B) tests. \* $p < 0.05$  vs controls; # $p < 0.05$  for MS vs MSEE groups. MS, maternally separated; MSEE, maternally separated rats with environmental enrichment; PND, postnatal day.



**Figure 4.** Burrowing, nesting, marble burying (A,B,C) tests. \* $p < 0.05$  vs controls; # $p < 0.05$  for MS vs MSEE groups. MS, maternally separated; MSEE, maternally separated rats with environmental enrichment; PND, postnatal day.

**Table 1. Fasting plasma clinical biochemistry in the rats at PND 48.<sup>a</sup>**

	Control	MS	MSEE	<i>P</i> -ANOVA
Glucose, mg/dL	119 (104, 130)	123 (120, 131)	136 (120, 149)	0.45
Protein, mg/dL	4.80 (4.40, 4.90)	4.10 (4.00, 4.20)	4.40 (3.50, 5.10)	0.14
Albumin, mg/dL	2.60 (2.60, 2.80)	2.60 (2.50, 2.80)	2.60 (2.40, 2.80)	0.79
Total cholesterol, mg/dL	106 (95.0, 116)	113 (109, 119)	116 (113, 118)	0.27
HDL-cholesterol, mg/dL	38.3 (36.7, 42.1)	38.8 (38.6, 40.7)	48.8(46.4, 51.6)*	0.023
Total antioxidant capacity, mmol/L	1.00 (0.90, 1.20)	1.20 (1.10, 1.50)	1.50 (1.20, 1.70)*#	0.030

<sup>a</sup>Data are median (25<sup>th</sup>, 75<sup>th</sup> percentiles) from 7-8 rats per group.

<sup>b</sup>*P* values are from Kruskal-Wallis ANOVA, followed, where significant ( $P < 0.05$ ), by pairwise comparisons using Mann-Whitney U test. \* $P < 0.05$  MS or MSEE vs control; # $P < 0.05$  MS vs MSEE  
MS, maternal separation; MSEE, maternal separation with environmental enrichment

## DISCUSSION

Among the major events associated with high incidence of stress is loss. Maternal separation has serious adverse effects on mental health. Early life EE is recognized to exert favourable influences on

neurodevelopment and behaviour, but it's possible role in reversing the deleterious effects of early life stress is less well studied. In the present study in Wistar rats, early life stress caused by MS and early weaning impaired exploratory behaviour in

the open field, black-white alley, and Y-maze, with clear evidence of increased anxiety; these effects were reversed by EE. A trend towards defective nest-building behaviour was also observed in rats exposed to ELS, and nesting performance was improved by EE in these rats. Neither memory nor depressive behaviour, however, were impaired by early life stress in this model, suggesting that behavioural impairments in response to early life stress in rodents vary by strain and model. Overall, our results suggest that psychosocial consequences of ELS in rats can be effectively ameliorated by simultaneous exposure to an enriched environment during development.

Depressive-like behaviour, as tested by tail suspension or glucose preference tests, was not detected in rats exposed to early life stress in the present study. The tail suspension test is commonly used to investigate the depressive behaviour induced by stress, or to demonstrate relief of stress by antidepressants(27). Different mouse strains respond in different ways to the tail suspension test, suggesting that this test is sensitive to genetic influence (31). Depression is notoriously difficult to measure in rodents, and earlier studies have suggested that the tail suspension test does not in fact emulate the pathophysiology of depression, but that it is more sensitive in assessing the therapeutic effects of anti-depressants(32).

EE in our study elicited a phenotype characterized by nearly normal behaviour of rats subjected to early life stress in the open field and dark light box, with a greater exploratory drive and less anxiogenic attitude compared with rats exposed to early life stress without EE. An enriched cage was shown to induce better coping with restraint-

induced stress as evidenced by upregulation of hippocampal mineralocorticoid and glucocorticoid receptors with rapid recovery after acute stress(33). Early life stress was shown in previous studies to induce anxiety and depressive behaviour; this has been attributed to upregulation of the hypothalamic-pituitary-adrenal axis (4,34). Rats housed in enriched cages also exhibited an increase in cortical thickness and hippocampal neurogenesis with satisfactory performance in memory tasks and fewer anxiety features (33) compared to controls. The present study demonstrates similar positive effects of EE in rats subjected to early life stress, where EE improved memory performance and exploratory behaviour. The increased plasma HDL-cholesterol and improved plasma antioxidant capacity in MSEE rats relative to controls may be partly related to the increased physical activity in these rats as demonstrated in the open field test.

Species-typical behaviour reflects the general well-being of an animal, which is sensitive to disruption with any psychological or physical illness. It has been proposed that species-typical behaviour tests in rodents mimic the activities of daily living, such as making one's bed (35). Impairment in species-typical behaviour tests has been demonstrated to occur early in dementia, before the development of other signs (35). In the present study we hypothesized that early life stress may be associated with impaired species-typical behaviour. Indeed, rats subjected to early life stress had worse nest-building performance than controls; this was partly rescued by EE. Nest-building behaviour, an instinctive behaviour that ensures protection and warmth, has been consistently shown to be impaired in pups that



have undergone maternal separation (36). Early environmental enrichment was reported to improve physical activity and social interaction in animals species (37), and this may have contributed to the rescue effect of environmental enrichment on nest-building in our model. However, other species-typical activities such as burrowing, and marble-burying were not impaired by early life stress in our model, suggesting a lower dependency of these behaviours on maternal influences.

### Summary

In conclusion, early life stress due to MS and early weaning in Wistar rats induced a clear phenotype of increased anxiety, associated with inhibition of their natural exploratory behaviour and impairment of nest-building performance. These effects were partly or completely reversed by exposure of the rats to an enriched environment during the MS hours. On the other hand, early life stress did not affect short-term working memory, spatial memory, or depressive behaviour. MS or EE showed no significant effect on fasting blood glucose level. However, total antioxidant capacity was higher in the MSEE group. These results suggest that early life stress in rats influences certain behavioural domains more than others, and highlight the value of EE in limiting the psychological damage caused by early life stress due to MS.

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