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The effects of stress and environmental enrichment on cognitive functions and stress-related gene expressions in the brain of aged rats

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Abstract. We aimed to investigate whether environmental enrichment (EE) would alter possible adverse effects of chronic unpredictable mild stress (CUMS) in elderly rats regarding corticosterone levels, stress-related gene expressions in some brain regions, and learning and memory. Wistar male rats (over 20 months) weighing 450–550 g were housed in enriched or standard cages for the duration of the study (10 weeks). After 8 weeks of CUMS application, body weight gain, adrenal weight, and corticosterone levels were measured. Morris water maze (MWM), and novel object recognition test were performed. Glucocorticoid receptor (GR), corticotropin-releasing hormone (CRH), and corticotropin-releasing hormone receptor 1 (CRHR1) expression levels were determined in the hypothalamus and hippocampus. In the stress group, body weights decreased over time. Regarding the distance swum by rats to find the platform in the MWM, while there was no significant difference between the 3rd and 4th days in the EE+CUMS group, the decrease continued until the 4th day in the standard control (SC)+CUMS group. Stress application reduced the GR and CRHR1 gene expressions in the hypothalamus. We conclude that chronic stress and EE caused brain region-specific changes, thus affecting the neurobiological and cognitive functions in the elderly. In this respect, our study will contribute to neurobiological and neurodegenerative studies on aging.

Key words: CUMS — Environmental enrichment — Hypothalamus — Hippocampus — Gene expressions — Learning and memory

Highlights

- Chronic stress affect learning in MWM in aged rats
- EE has a positive effect on learning in the stressed group in MWM in aged rats
- Stress caused specific changes in CRH, CRHR1, and GR mRNA levels

Introduction

Stress affects cognitive processes such as learning and memory (Klier and Buratto 2020) and plays a negative role in the quality of life of living organisms (Kim and Diamond 2002). Chronic stress exposure causes functional and morphological impairments in various brain regions such as the hippocampus (HC) and hypothalamus (HT) in animals (Lupien et al. 2009; Leite et al. 2023) and these changes have adverse effects on learning, memory recall, and retention as well as decision-making and behaviors (Herman et al. 2005; McEwen 2006; McCallum et al. 2024). Stress also increases the severity of degeneration in neuronal structures, the impairments in cognitive functions, and peripheral circulation,

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related to aging. Long-term exposure to stress hormones increases the effects of aging (Yau et al. 1995; Aguilera 2011; Borges et al. 2023). Furthermore, stress was reported to be associated with accelerated epigenetic aging (Harvanek et al. 2021).

Many studies reported the neuroprotective effects of environmental enrichment (EE) in neurodegenerative diseases such as Parkinson's disease. Although the molecular mechanisms underlying such effects are not yet completely understood, modulation of dopaminergic, cholinergic, glutamatergic, and GABAergic systems and increased expression of neurotrophic factors i.e. BDNF and GDNF are considered to play a role in these effects (Alarcón et al. 2023). EE has also been reported to have many positive effects on rodent models of dementia, with improved cognitive function i.e. learning and memory, and alleviated anxiety levels (Mohd Sahini et al. 2024). EE improves learning and memory and positively affects cognition in aging (Harati et al. 2011; Speisman et al. 2013; Cortese et al. 2018). EE is the most frequently used experimental environment in rodents to show increased brain plasticity and neurogenesis (Speisman et al. 2013; Cortese et al. 2018). Moreover, EE increases glucocorticoid receptor (GR) expression in the hippocampus, regulates hypothalamic synthesis of corticotropin-releasing hormone (CRH), alters the hypothalamic-pituitary-adrenocortical (HPA) axis function (Issa et al. 1990; van Praag et al. 2000; Fox et al. 2006). It also increases brain weight, dendritic branching, and synaptogenesis (Leggio et al. 2005; Rossi et al. 2006).

In adult rats, EE is known to attenuate the detrimental effects of chronic stress (Leggio et al. 2005; Hutchinson et al. 2012). The timing and duration of the onset of EE may alter its impact on old age. Elderly rats exposed to lifelong EE show better performance in the water maze than elderly rats exposed to late EE. Although late-onset EE is not as beneficial as adult-onset EE, it does mitigate the memory loss associated with aging (Kumar et al. 2012). This result shows that late-onset EE applications also yield favorable results (Issa et al. 1990; Kobayashi et al. 2002; Kumar et al. 2012; Speisman et al. 2013).

Although there are many studies examining the effects of EE on stress-related changes, the results vary widely and the mechanisms underlying such effects are not fully understood (Joushi et al. 2021; Dandi et al. 2023, 2024; Vaquero-Rodríguez et al. 2023). Furthermore, the effects of EE on stress-related changes in elderly rats are not well known, and studies in this area are limited. Therefore we aimed to investigate the possible impact of EE on chronic stress-related behavioral, physiological, and molecular changes in elderly rats. We hypothesize that EE will mitigate the adverse effects of chronic unpredictable stress on the physiological, behavioral, and molecular aspects of aged rats. By testing these hypotheses, we aim to provide insights into the potential therapeutic effects of EE in counteracting the negative consequences of chronic stress on both physiological parameters and molecular processes, ultimately influencing cognitive function in aged rats. For this purpose, data such as body weights, corticosterone levels, and adrenal weights were measured after a mild stress protocol applied to aged rats housed in standard cages and in EE conditions. Molecular mechanisms related to stress response were examined in brain regions such as the hypothalamus and hippocampus. CRH, CRHR1, and GR gene expression levels were determined in the same regions. Furthermore, the learning and memory abilities of rats were evaluated using MWM and new object recognition (NOR) tests.

Materials and Methods

Experimental animals and housing conditions

The experimental study was approved by the Local Ethics Committee for Animal Experiments of Bagcilar Training and Research Hospital (Project 95. board/2019-48 dated 29.12.2019). Experiments were conducted following the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

The study involved 32 male Wistar Hannover rats aged 21 months with an average weight of 450–550 g. The average laboratory rat lives approximately three years (Suter et al. 1979; Ghasemi et al. 2021) and 20–22 months of rats are considered aged and used in the experimental studies (Stanley and Shetty 2004; Kumar et al. 2012). All animals were born and maintained in the same laboratory under the same housing conditions until the study.

The rats were housed under standard laboratory conditions with 50–60% humidity, 22 ± 2°C temperature, 15 cycles of ventilation *per* hour, and 12 hours of light and dark cycle (lights on, 06:00 to 18:00). Animals were fed *ad libitum*. Food pellets and 750 ml drinker cups were placed on a stainless-steel wire grid (PLEXX, Netherlands). Body weights were measured (Kern FCB 12K1, Germany) every ten days. Thirty-two rats were randomly divided into 4 groups with 8 animals in each group (Table 1). The standard cage (SC) and SC+chronic unpredictable mild stress (CUMS) groups were housed in 425×265×180 mm polycarbonate conventional Type 3H cages (PLEXX, The Netherlands) in pairs. The EE and EE+CUMS groups were housed in a plastic living area measuring 110×75×70 cm with 8 rats. Animals had 2 weeks adaptation period to standard and enriched housing conditions. After the 15 day adaptation period, the EE+CUMS and SC+CUMS groups were taken to another room until the end of the experiment to prevent other groups from being affected by stressors and exposed to CUMS for 8 weeks starting at the same time. Afterwards behavioral experiment was carried out for 2 weeks. Blood samples were taken before

Table 1. Animal groups and the procedures

Group	Protocols applied
SC	Housed in standard cages.
SC+CUMS	Housed in standard cages + stressors in the chronic unpredictable mild stress protocol were applied.
EE	Environmental enrichment protocol was applied. They were not exposed to any stressors during the experiment.
EE+CUMS	Environmental enrichment protocol was applied + stressors in the chronic unpredictable mild stress protocol were applied.

SC, standard cage; CUMS, chronic unpredictable mild stress; EE, environmental enrichment.

the behavioral experiments and after the behavioral experiments finished, animals were killed. The exact dates of each procedure in the experiment are shown in the Table S1 in Supplementary material.

Chronic unpredictable mild stress protocol

The chronic unpredictable mild stress protocol described by Willner et al. (1987) was modified and applied (Willner et al. 1987, 1992). The following stressors (Table 2) were alternated to prevent adaptation. Care was taken to ensure that the same stressor was not applied on two consecutive days and that the order of stressors was different.

The stressors were applied randomly to the animals in the SC+CUMS group and the EE+CUMS group, for 8 weeks (Jeong 2006; Castelhano-Carlos et al. 2014). The CUMS protocol was performed in a separate room to avoid affecting the SC and EE groups with stressors.

Environmental enrichment protocol

A 110×75×70 cm living area was created for the EE groups (Bakos et al. 2009; Castelhano-Carlos et al. 2014), which included materials that increased physical activity and social interaction (Fig. S1). A standard cage (425×265×180 mm) was placed there to provide them with food and water. For the adaptation period, the EE protocol was started 2 weeks before the 8-week stress protocol in the EE and EE+CUMS groups. The EE materials were cleaned once a week. The location of the materials was changed after each cleaning.

Behavioral experiments

At the end of the experiment, the Morris Water Maze (MWM) test (Morris 1984) and the Novel Object Recognition (NOR) test (Bevins and Besheer 2006) were performed to evaluate hippocampus-dependent learning and memory processes. Three days before the start of the tests, rats were kept in the experimental room for 15 minutes a day and moved to the room 1 hour before the tests. The animals in different groups were tested in random order. Recordings were analyzed using the NOLDUS video tracking system and appropriate software (Ethovision XT, Noldus Information Technology, Netherlands). To check the accuracy of the results obtained from video tracking software, some recordings, which were chosen randomly, were scored by an observer blind to experimental conditions.

MWM test

MWM test was performed in a standard pool with a diameter of 150 cm and a depth of 60 cm (Morris 1984). The pool was

Table 2. Stressors applied in the chronic unpredictable mild stress protocol

Applied stressors	Duration of applications (h)
Crowded grouping in a limited area	4
Holding in a tilted cage (30°)	4
Exposure to cat noise	3
Stay on wet bedding (100 g corn cobs + 200 ml water)	24
Housing in a 15 cm high cage with hot water (40°C) without the bedding material	0.5
Housing with a different group of animals by swapping partners	14
Cage housing without a water bottle	15
Food deprivation followed by 1 h exposure to inaccessible food	14
Water deprivation followed by exposure to an empty bottle for 1 h	14
Light/dark cycle reversal	24
Light/dark application at 30-min intervals	10

hypothetically divided into four equal quadrants which were numbered. The pool was filled with water to rise 1.5 cm above a 15 cm wide platform placed equidistant from the center and walls into the center of one of the quadrants (number 4). The water temperature was kept at 24°C and the platform was made invisible by adding a non-toxic paint to the water. The MWM consisted of learning exercises and memory testing phases. During the learning phase, each rat was tested four times a day at 10-minute intervals. In each exercise, the rats were released into the water from a different quadrant, facing the wall of the pool. The rats were supposed to find the platform by swimming. Once the rats found the platform they were allowed to stay on the platform for 30 s. Each exercise lasted a maximum of 60 s. At the end of this time, the rat that could not find the platform was directed to the platform and was expected to stay on the platform for 15 s. In the memory test, the platform was removed from the pool on the day following the learning exercises (day 5). Rats started to swim from the quadrant (number 2) furthest from the platform in the learning exercises. Rats were allowed to swim for the duration of the test (60 s).

Novel object recognition test

The novel object recognition test was conducted in a 50×50×50 cm Plexiglas open-top setup in a semi-dark environment. The test was conducted over a three-day period including acclimatisation, exercise (E) and test (T) days. During the familiarisation period, the rats were allowed to acclimatise to the apparatus for 10 min without any objects in the environment. Training and testing consisted of a three-minute period each and were repeated at 24-h intervals. In the training phase, the same two objects were placed in the apparatus and the animal was allowed to recognize these objects by moving freely (E). After 24 h, one of the objects presented in E was changed and the rat was again placed in the same apparatus and allowed to spend free time with the two objects (T). At T, the duration and the frequency of the interest in both objects were measured. The NOR discrimination index was calculated by using the following formula; time of novel object exploration minus time of familiar object exploration divided by time of novel plus familiar object exploration, multiplied by 100 (Brivio et al. 2020).

Collection of blood and tissue samples

Before the behavioral experiments, blood samples (0.5–1 ml) were taken from the jugular vein, at the onset of darkness (18:00–19:00 h) to determine the highest corticosterone level (zenith) and at the onset of light (06:00–07:00 h) to determine the lowest corticosterone level (nadir). Serum was obtained by centrifugation at 14,000 rpm for 10 min.

After the behavioral experiments finished the animals were killed (by decapitation without anesthesia). Afterwards, the brain was removed and placed on dry ice and then in the brain matrix (Electron Microscopy Sciences, Hatfield, PA, catalog No. 69026-C). From 2 mm thick brain slices whole hypothalamus and hippocampus sections were removed (Paxinos and Watson 2007), and stored at −80°C.

Determination of corticosterone levels

Serum corticosterone levels were measured in serum samples by the ELISA method according to the manufacturer's protocol (ENZO Corticosterone ELISA Kit Cat No. ADI-901-097, PA, USA).

Real-time polymerase chain reaction (RT-PCR)

Real-time PCR was carried out to determine CRH, GR, and CRHR1 mRNA levels in relevant brain regions. RNA isolation from tissues was performed using a commercial kit (Jena Bioscience Cat. No. PP-210L) according to the kit protocol. A260/A280 and A260/A230 ratios were used to determine the purity and quality of the nucleic acid samples (Lucena-Aguilar et al. 2016). Total RNA was measured using a nanodrop spectrophotometer (Implen NanoPhotometer NP80) prior to cDNA synthesis. After RNA quantification, cDNA synthesis was performed using 1 ng/µl RNA from each sample. Jena Bioscience brand SCRIPT cDNA Synthesis Kit (Cat. No. PCR-511S) was used to sythesise first-strand complementary DNA (cDNA) from total RNA. The real-time gene expression was performed on an RT-PCR instrument (ABI 7500 Real-Time PCR Systems, Applied Biosystems) using the qPCR ProbesMaster (Jena Bioscience, Germany) kit (Cat. No. PCR-360L). TaqMan Gene Expression assay kits (Thermofisher Scientific, Waltham, USA) (https://www.thermofisher.com/tr/en/home/life-science/pcr/real-time-pcr/real-time-pcr-assays/taqman-gene-expression.html) containing the primer-probe mix for each gene were as follows; GAPDH(Rn01775763-g1), GR (Rn00561369-m1), CRH (Rn01462137-m1) and CRHR1 (Rn00578611-m1). GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene was used as a housekeeping gene.

The samples were amplified in the RT-PCR device according to the conditions in the protocol. And threshold cycle (Ct) values were determined. The mRNA expression levels of tested genes were normalized to those of GAPDH (ΔCt). The data were analyzed using the ΔΔCt method (Livak and Schmittgen 2001). Fold changes of genes were calculated using the expression $2^{-\Delta\Delta Ct}$ with respect to the mean value of ΔCt in the control group.

Statistics

SPSS 22.0 program was used for statistical analysis. According to Shapiro-Wilk and histogram graphs, it was determined

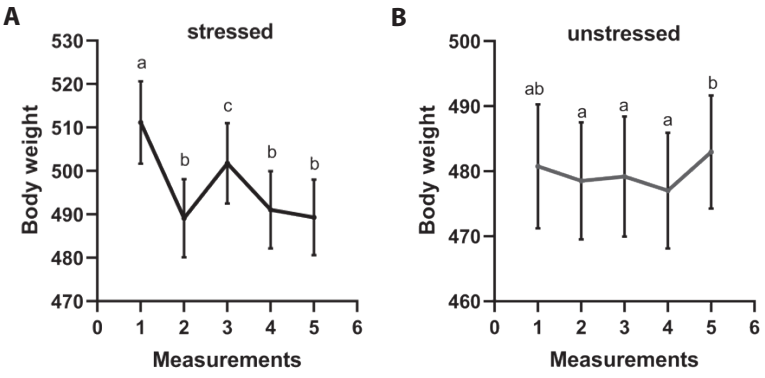

Figure 1. Change of body weight averages over time. The stress-time interaction is significant, $p = 0.000$. **A.** The body weight of animals decreased over time in the stressed groups, $p = 0.000$. **B.** The effect of time was significant on body weights of unstressed groups, $p = 0.003$. However, there was no periodic decrease in the body weight of unstressed animals. Differences between the letters show the significance of the body weight measurements. Data are presented as mean ± S.E.M.

whether the data were normally distributed. Where the normality assumption was not met, the Mann-Whitney U test (corticosterone levels at each point, hypothalamus gene expressions, hippocampus CRH expression) and Wilcoxon Test (corticosterone repeated measures) were used. If the normality assumption was met, a two-way analysis of variance (relative adrenal weight, NOR and MWM tests, hippocampus GR, and CRHR1 expressions) and a two-way analysis of variance for repeated measures (body weight, MWM learning parameters) were used. A factorial design was applied 2×2 (stress effect: CUMS (+) – CUMS (–)) × (housing effect: enriched cage – standard cage). The significance value was set at $p \leq 0.05$.

Results

Body weight gain

The main effect of time on body weight gain was significant ($F_{(4,112)} = 25.92$, $p = 0.000$). However, stress ($F_{(1,28)} = 1.74$, $p = 0.20$) and EE ($F_{(1,28)} = 0.27$, $p = 0.61$) had no significant effect on body weights. The interaction of stress and time was significant regarding body weights ($F_{(4,120)} = 20.92$, $p = 0.000$).

The change in body weight over time in the unstressed ($F_{(4,27)} = 5.38$, $p = 0.003$) and stressed ($F_{(4,27)} = 56.38$, $p = 0.000$) groups was statistically significant. The body weight in the stressed group decreased over time. However, there was no periodic decrease in the body weights of non-stressed rats and no significant difference between the initial and final weights (Fig. 1).

Relative adrenal weight

Stress and enrichment had no significant effect on the relative adrenal weights of animals ($F_{(1,28)} = 1.01$, $p = 0.32$), ($F_{(1,28)} = 0.18$, $p = 0.67$). Furthermore, the interaction between stress and EE was not significant, ($F_{(1,28)} = 3.68$, $p = 0.65$) (Fig. S2A).

Corticosterone levels

A statistically significant difference was observed between the nadir and zenith corticosterone levels ($Z = -3.4$, $p = 0.001$) (Fig. 2). Zenith's corticosterone levels were higher than those of nadir levels. Stress did not affect nadir ($U = 63$, $p = 0.18$) and zenith corticosterone levels ($U = 71.5$, $p = 0.51$). Similarly, EE did not affect nadir ($U = 81$, $p = 0.68$) and zenith corticosterone levels ($U = 75.5$, $p = 0.65$) (Fig. 2).

Behavioral tests

NOR test

The main effects of stress ($F_{(1,28)} = 1.23$, $p = 0.28$) and EE ($F_{(1,28)} = 1.53$, $p = 0.23$), and also stress X EE interaction ($F_{(1,28)} = 0.01$, $p = 0.94$) were not significant on NOR discrimination index and the time spent exploring novel and familiar objects (Fig. S2B, S2C). Data are presented as mean ± s.e.m.

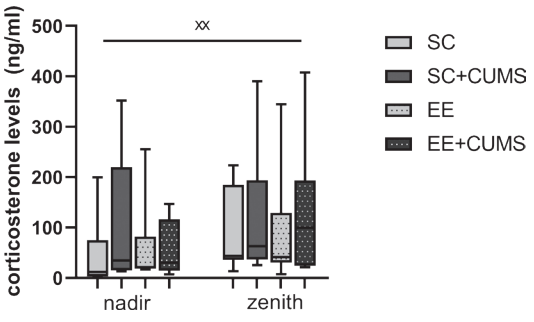

Figure 2. Serum corticosterone levels (ng/ml) at the end of the experimental period at nadir (6–7 a.m.) and zenith (6–7 p.m.). Corticosterone levels of animals were higher at the zenith than the nadir; $^{xx}p = 0.000$ indicating the general effect of time. Data is presented by box plots where the central lines represent the median, and the whiskers represent the minimum and maximum values.

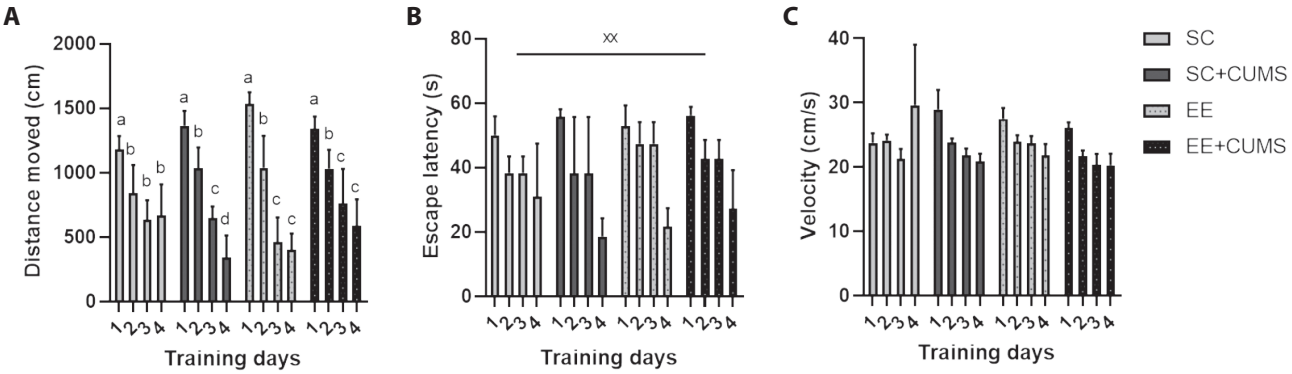

Figure 3. Morris water maze (MWM) training test. **A.** Distance traveled before finding the platform in MWM test. Stress×housing×time interaction is significant, $^{a,b,c,d}p = 0.032$. Different letters (a,b,c,d) show significant differences in distance traveled between the days for each group. The decrease in distance between days in each group was different. **B.** Time to find the platform in MWM training trials. The main effect of time was significant for all groups. Escape latency decreased over the days of training trials for all groups, $^{xx}p \leq 0.001$, indicating the main effect of the time **C.** MWM training trials' average velocity values. Data are presented as mean ± s.e.m.

MWM test

In the training part of the test, the interaction of stress, EE, and time was significant regarding the distance moved until the rats found the platform in the learning exercises ($F_{(3,81)} = 3{,}51$, $p = 0.03$). In terms of groups, the decrease in distance between days in each group was different. Regarding stressed groups, while there was no significant difference between the 3rd and 4th days in the EE group, the decrease continued until the 4th day in the SC group. Among the non-stress groups, there was no significant difference between the 3rd and 4th days in the EE group and between the 2nd, 3rd, and 4th days in the SC group (Fig. 3A). The main effect of time for rats to find the platform (escape latency) was significant ($F_{(3,84)} = 33.16$, $p = 0.000$). However, stress ($F_{(1,28)} = 0.94$, $p = 0.34$) and housing ($F_{(1,28)} = 0.22$, $p = 0.64$) had no significant effect on the time to find the platform (Fig. 3B). The effect of time on rats' average velocity was insignificant ($F_{(3,63)} = 1.71$, $p = 0.20$). Also, stress ($F_{(1,21)} = 3.93$, $p = 0.06$) and housing ($F_{(1,21)} = 1.37$, $p = 0.25$) had no significant effect on mean velocity (Fig. 3C).

Stress ($F_{(1,27)} = 0.02$, $p = 0.90$) and housing ($F_{(1,27)} = 1.2$, $p = 0.28$) had no statistically significant effect on the distance traveled in the probe test (Fig. 4A). Stress ($U = 112$, $p = 0.77$) and housing ($U = 118.5$, $p = 0.95$) did not affect the time spent in the target quadrant (Fig. 4B). Stress ($F_{(1,27)} = 0.01$, $p = 0.93$) and housing ($F_{(1,27)} = 1.28$, $p = 0.27$) had no statistically significant effect on the average speed of rats in the probe test (Fig. 4C).

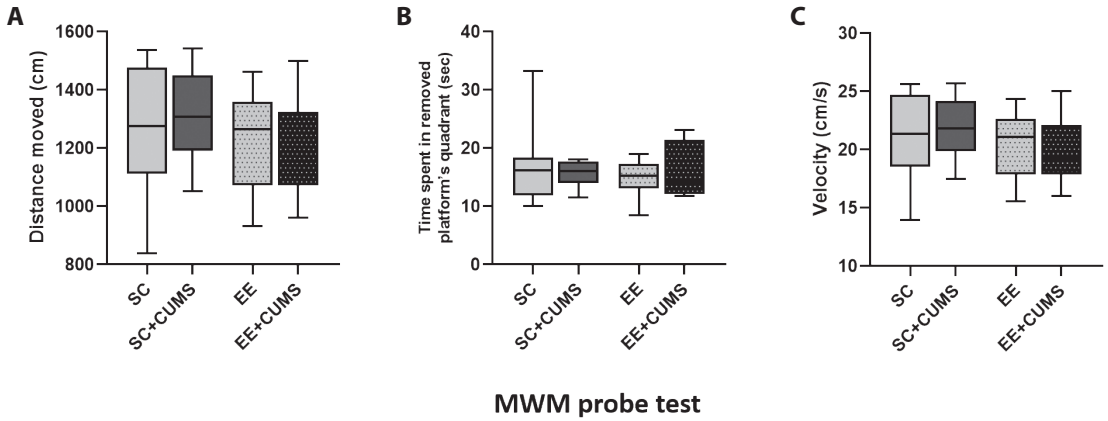

Figure 4. MWM probe test. Stress and EE had no significant effect on the parameters measured in the probe test. **A.** Distance traveled in the MWM probe test. **B.** Time spent in the target quadrant in the MWM memory test. **C.** MWM probe test average velocity parameter. Data is presented by box plots where the central lines represent the median, and the whiskers represent the minimum and maximum values.

Gene expressions

Hypothalamic GR, CRH, and CRHR1 gene expressions

Stress factor had a significant effect on HT-GR gene expression ($U = 55$, $p = 0.02$) (Fig. 5) and HT-CRHR1 gene expression ($U = 47$, $p = 0.01$); stress decreased the expression of these genes. However, EE did not have a significant effect on GR ($U = 102$, $p = 0.68$) and CRHR1 gene expressions ($U = 91$, $p = 0.56$) in the hypothalamus (Fig. 5A). Stress ($U = 89$, $p = 0.5$) and EE ($U = 82$, $p = 0.33$) had no significant effect on HT-CRH gene expression (Fig. S2D).

Hippocampus GR, CRH, and CRHR1 gene expressions

Stress had no significant effect on HC-CRH ($U = 117$, $p = 0.92$) expression. Although not statistically significant, HC-CRH gene expression tended to decrease with EE treatment ($U = 71$, $p = 0.08$) (Fig. 5B).

Stress had no significant effect on HC-GR ($F_{(1,28)} = 0.07$, $p = 0.79$), HC-CRHR1 ($F_{(1,28)} = 0.15$, $p = 0.70$) and EE had no significant effect on HC-GR ($F_{(1,28)} = 0.01$, $p = 0.96$) and HC-CRHR1 ($F_{(1,28)} = 0.14$, $p = 0.72$) gene expressions (Fig. S2E,F).

Discussion

We found that CUMS exposure affected BW, learning in MWM and stress-related gene expressions in a brain region specific manner in aged rats. EE application had a positive effect on learning in MWM in stressed animals but did not show any other impact on the adverse effects of CUMS exposure.

A decrease in body weight gain was observed over time compared to the initial weight in the animals subjected to CUMS group. A decrease in body weight indicates the impact of stress exposure (Westenbroek et al. 2005). In line with our findings, it has been reported in previous studies that body weight decreased in the animals subjected to CUMS (Forbes et al. 1996; Nielsen et al. 2000).

A statistically significant difference was observed between the nadir and zenith corticosterone levels. This shows that diurnal corticosterone secretion works in its normal rhythm (Lightman et al. 2020). However, in our study, no statistically significant effect of stress and housing on the nadir and zenith corticosterone levels was observed (Bourke and Neigh 2011). It is known that stress and EE may affect corticosterone levels in rats (Moncek et al. 2004; Castelhano-Carlos et al. 2014). However, we may fail to capture the dynamic nature of the HPA axis drive by only conducting end-point hormone sampling. Nevertheless, neurochemical and endocrine changes may not always reflect the impact of chronic stress (Harris 1997; Moncek et al. 2004; Westenbroek et al. 2005). Similar to our results, some studies have shown that stress did not affect adrenal weight and corticosterone levels in rats subjected to CUMS (Harris 1997; Bourke and Neigh 2011). Although there is no change in adrenal weight and corticosterone levels, decreased body weight is used as an indicator of stress exposure (Häidkind et al. 2003; Westenbroek et al. 2005; Eraslan et al. 2023). In our study, although corticosterone levels and adrenal weights did not increase after the stress treatment, weight loss over time in the CUMS exposed group indicates that the applied stress was effective.

It was found that the effect of time was statistically significant on the distance traveled until finding the platform, time to find the platform, and time spent on the platform quadrant during the learning phase of the MWM test.

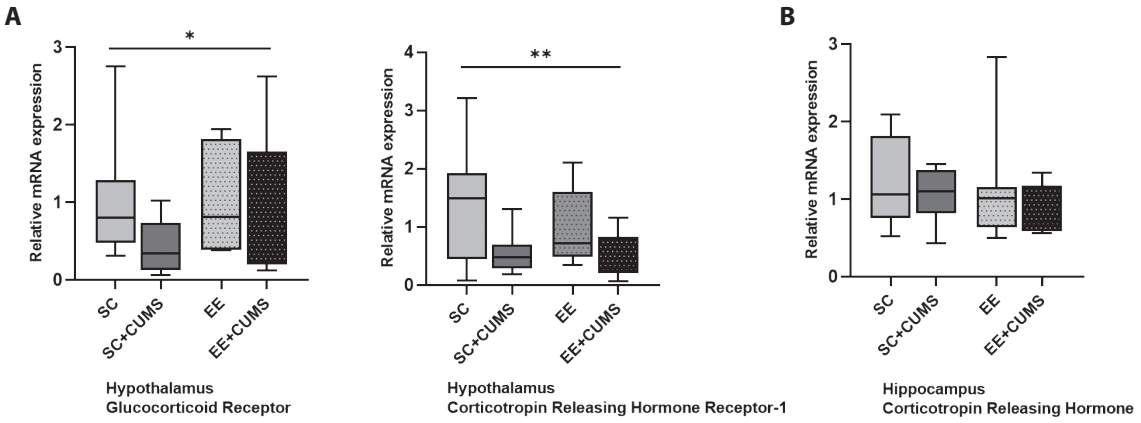

Figure 5. Effects of stress and EE on gene expressions in the brain. **A.** Hypothalamus glucocorticoid receptor (GR) and corticotropin-releasing hormone receptor 1 (CRHR1) expressions. Stress decreased the expression of GR, * $p = 0.02$, and CRHR1, ** $p = 0.01$, indicating the main effect of stress. **B.** EE tended to decrease hippocampus corticotropin-releasing hormone (CRH) gene expression, $p = 0.08$. Data is presented by box plots where the central lines represent the median, and the whiskers represent the minimum and maximum values.

The decrease in the values of these parameters over time shows that learning has taken place in all animals (Morris 1984). In the stressed animals, while a significant decrease continued until the last day in the SC+CUMS group, the average distance traveled decreased until the third day in the EE+CUMS group. This shows that learning was completed earlier in the stressed EE group, whereas, learning was prolonged until the last day in the SC group. These results suggest that EE applications may have a positive impact on learning in stressed animals. On the other hand, there was no significant difference in the mean distance traveled until finding the platform between the third and fourth days in the non-stressful EE group and between the second, third, and fourth days in the SC group. This finding may indicate that in the absence of stress, the application of EE does not affect learning processes. Previous studies have shown that late-term and early-term EE applications have different effects on learning processes in aged rats (Simpson and Kelly 2011; Fuchs et al. 2016).These differences can be explained by the fact that the other studies started the EE application at an earlier period or applied it for a longer period. In addition, the distance traveled in the non-stressed SC group did not change after the second day, but the decrease in the distance traveled in the stressed SC group continued until the last day can be evaluated in the direction that stress prolongs the learning process. Similarly, studies are reporting that stress prolongs the learning process in MWM (Hölscher 1999; Hu et al. 2017). In the NOR test, no statistically significant difference was found between the groups as a result of the CUMS and EE treatments. This may be related to the nature of the stressors and EE applications and the period of application (Burke et al. 2010).

Various results have been reported about the effect of stress and EE on gene expressions in different brain regions (Olsson et al. 1994; Kentner et al. 2018). While some of these results support our results (Francis et al. 2002; Fan et al. 2021), some of them are not in accordance with ours (Sampedro-Piquero et al. 2014; Wang et al. 2014). From the results of previous and our studies, we suggest that changes in gene expressions are specific to the type and duration of treatments, and brain region investigated. We found that chronic stress decreased GR gene expression in the hypothalamus in aged rats, whereas EE did not have an effect. Although some studies have reported that chronic stress does not change GR mRNA levels in the hypothalamus (Sapolsky et al. 1984; Mizoguchi et al. 2003), there are studies in which stress application decreased GR gene expression in the hypothalamus (Herman et al. 1995; Lu et al. 2015). Different results in mRNA GR levels in the hypothalamus after different stress treatments reveal that receptor expression levels are stressor-specific. Changes in GR and corticosterone levels may not be parallel to each other. Similar to our results it has also been shown that changes in GR levels in brain regions may not be related to the HPA axis, ACTH and corticosterone responses (Wei et al. 2004; Gądek-Michalska et al. 2013).

According to our study, chronic stress did not affect CRH gene expression but decreased CRHR1 gene expression in the hypothalamus in aged rats. In contrast to our findings, chronic stress has been reported to increase CRH and CRHR1 gene expression in the hypothalamus (Herman et al. 1995; Imaki et al. 1996; Eraslan et al. 2015). However, CRH mRNA level in the PVN of mice subjected to acute restraint stress increased after 2 h and decreased to basal level after 4 h (Greetfeld et al. 2009). In a stress comparison study between mice and rats, CRHR1 mRNA expression in the PVN increased in rats but did not change in mice (Imaki et al. 2003). These results are compatible with our data. In our study, the decrease in HT CRHR1 in the stress group and the prolongation of learning until the last day in the stressed SC group in the MWM test may be related. In support of this interpretation, a study in mice reported that the interaction of CRH with CRHR1 is not necessary to affect memory performance (Contarino et al. 1999). EE factor has no significant effect on CRH and CRHR1 gene expression in our study. Consistent with our results, studies have reported that EE does not affect CRH (Francis et al. 2002) and CRHR1 (Fan et al. 2021) gene expression in the hypothalamus.

In previous studies, it was reported that different stress treatments decreased GR gene expression in HC (Kitraki et al. 1999; Park et al. 2015; Shilpa et al. 2017). Consistent with our study, stress application did not alter GR gene expression in HC (Lam et al. 2019; Palumbo et al. 2020; Osacka et al. 2021).

In support of the lack of effect of EE on GR mRNA in the hippocampus in our study, another study reported that EE did not affect GR gene expression (Francis et al. 2002).

In our study, chronic stress had no significant effect on GR, CRH, and CRHR1 gene expression in the hippocampus. Previous studies had various results about the effect of stress on these gene expressions. The effects of stress on the hippocampus are variable and complex and are affected by the duration of stress, age, and gender (McEwen et al. 2011).

In our study, EE application tended to reduce CRH gene expression in the hippocampus of aged rats. Moreover, learning in the MWM was completed on the 3rd day in the EE groups. It was observed to continue until the last day in the non-EE groups. The increase in hippocampus-dependent cognitive function may be related to the decreasing trend in CRH gene expression in HC after EE application. Further studies are needed (Bakshi and Kalin 2000) to elucidate the reasons for this situation.

This study has potential limitations. Corticosterone levels could have been measured at various sampling points during the stress application period. Comparing male rats with females and aged rats with younger groups would make this work more comprehensive. Furthermore, we detected

receptor mRNA levels which are not necessarily predictive of protein levels. Differences in mRNA do not always translate to differences in proteins. Therefore, further studies are required to determine whether or not the alterations detected in gene expressions are linked with the functional receptors.

In our study, the decrease in body weights over time in the stress-treated groups indicates that the CUMS was effective. In MWM, the EE treatment was found to have a positive effect on learning in the stressed group. It was observed that the effects of stress and EE on GR, CRH, and CRHR1 mRNA levels occurred in different ways specific to brain region, type, and duration of stress, nature of EE, and application period. In conclusion, we can say that chronic stress and EE affect neurobiological and cognitive functions in the elderly. More studies are needed to explain exactly how these effects occur in terms of the underlying mechanisms. We believe that this study may make a contribution to neurobiological and neurodegenerative research on aging.

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Data availability statement. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contribution. The authors declare that all data were generated in-house and that no paper mill was used. Conceptualization, EE; methodology, EE, DSO; formal analysis and investigation, EE, DSO; writing – original draft preparation, DSO; writing – review and editing, EE; funding acquisition, EE; supervision, EE.

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Supplementary Material

The effects of stress and environmental enrichment on cognitive functions and stress-related gene expressions in the brain of aged rats

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Supplementary Figures

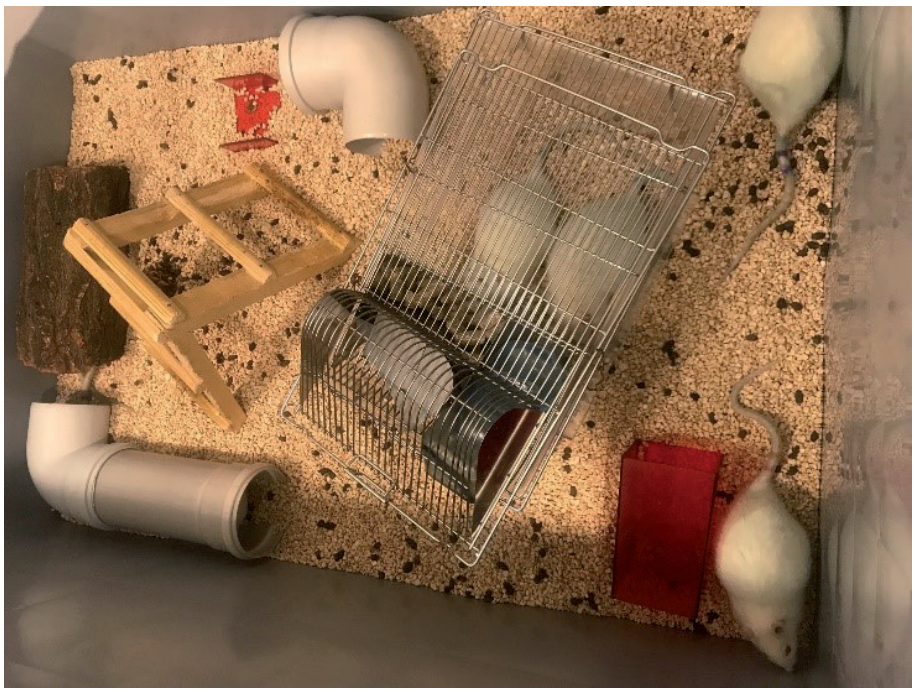

Figure S1. The area created for EE (environmental enrichment) groups

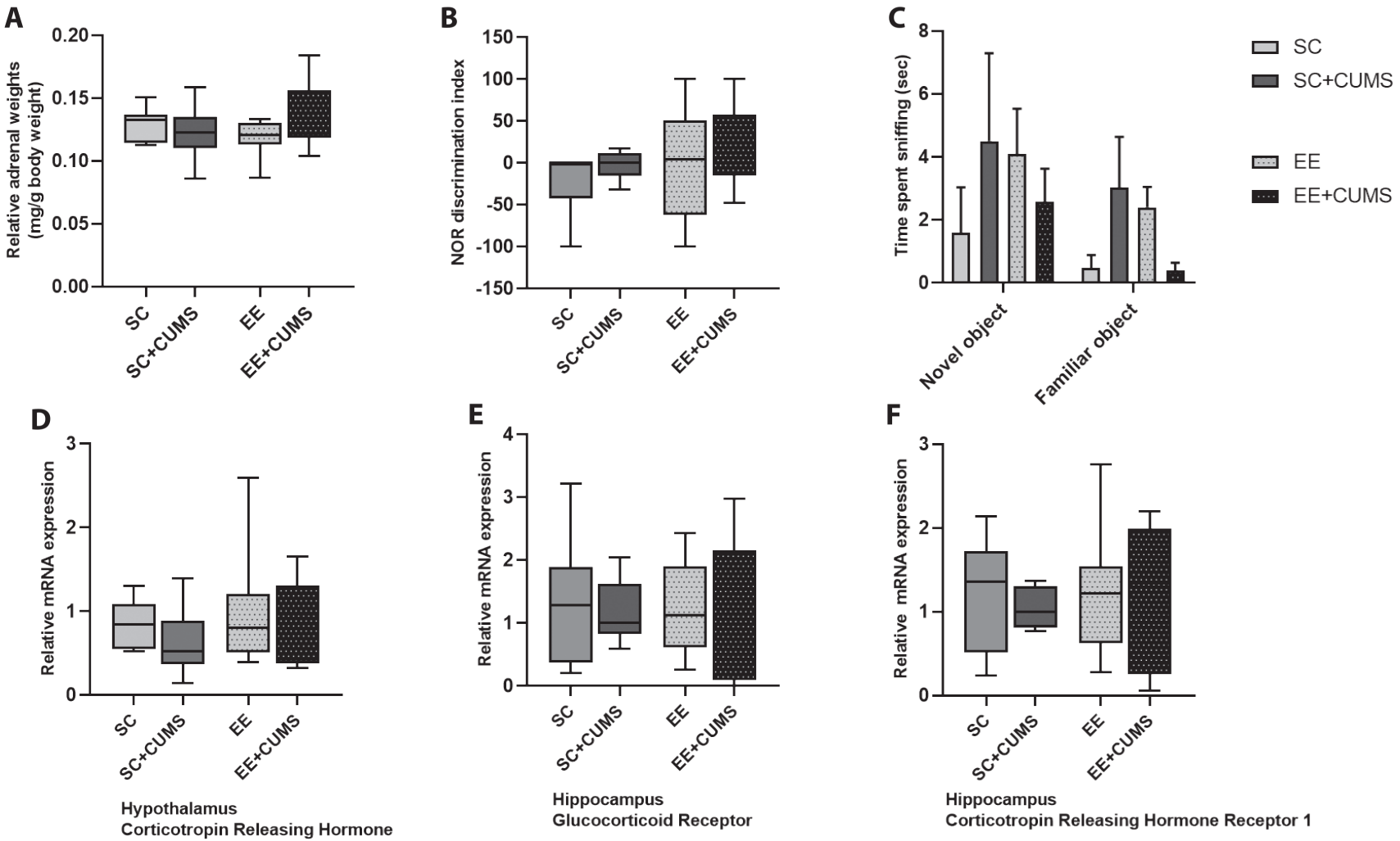

Figure S2. A. The relative adrenal weight of animals (mg/g body weight). **B.** NOR discrimination index. **C.** Time spent in the exploration of novel and familiar objects in the NOR test. **D.** Hypothalamus CRH mRNA expression. **E.** Hippocampus GR mRNA expression. **F.** Hippocampus CRHR1 mRNA expression. Normally distributed data is presented as means ± s.e.m. Non-normally distributed data is presented by box plots; the central lines represent the median, and the whiskers represent the minimum and maximum values. Effects of stress and EE were not significant on the presented parameters.

Table S1. Application schedule of experimental procedures

	Monday	**Tuesday**	**Wednesday**	**Thursday**	**Friday**	**Saturday**	**Sunday**
October 19-25	adaptation Day 15 EE adaptation start	handling + weighing	handling	handling	handling + cage cleaning + enrichment cage toy relocation	handling	handling
October 26-November 1	handling	handling + weighing	handling + cage cleaning + enrichment cage toy relocation	handling	handling	handling	handling
November 2-8	handling	handling + weighing	handling + cage cleaning + enrichment cage toy relocation	handling	handling	**STRESS – EE + STRESS** **Light/dark cycle reversal**	**STRESS – EE + STRESS** **Stay on wet bedding (100 g corn cobs + 200 ml water) (24 h)**
November 9-15	**STRESS – EE + STRESS** **8.00-11.00 Exposure to cat noise (3 h)** **12.00-16.00 Crowded grouping in a limited area**	handling + weighing **STRESS – EE + STRESS** **07:30-15:30 Light/dark application at 30-min intervals** **17.30 Cage housing without a water bottle (15 h)**	handling + **stress** + cage cleaning + enrichment cage toy relocation **STRESS – EE + STRESS** **7.30-8.30 Water deprivation followed by exposure to an empty bottle for 1 h** **8.30-12.30 holding in a tilted cage (30°)(4 h)** **17:00 Housing with a different group of animals by swapping partners (14 h)**	**STRESS – EE + STRESS** **Light/dark cycle reversal (24 h)**	handling+weighing **STRESS – EE + STRESS** **12:30- 16:30 Crowded grouping in a limited area** **17.30 Food deprivation**	**STRESS – EE + STRESS** **07.30-8.30 Food deprivation followed by 1 h exposure to inaccessible food** **08.30-11.30 Exposure to cat noise (3 h)**	**STRESS –EE + STRESS** **Stay on wet bedding (100 g corn cobs + 200 ml water) (24 h)**

(continued)

Table S1. (continued)

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
November 16-22	**STRESS – EE + STRESS** **10.00-14.00 holding in a tilted cage (30°)(4 h)** **17.30 Cage housing without a water bottle (15 h)**	handling + weighing **STRESS – EE + STRESS** **7.30 Water deprivation followed by exposure to an empty bottle for 1 h** **10.00-13.00 Exposure to cat noise (3 h)** **17:00 Housing with a different group of animals by swapping partners (14 h)**	handling + stress + cage cleaning + enrichment cage toy relocation **STRESS – EE + STRESS** **7.30-16.30 Light/dark application at 30-min intervals** **17.30 Food deprivation (14 h)**	**STRESS – EE + STRESS** **07.30-8.30 Food deprivation followed by 1 h exposure to inaccessible food** **8.30-12.30 holding in a tilted cage (30°)(4 h)** **17.30 Light/dark cycle reversal (24 h)**	**STRESS – EE + STRESS** **7.00- 17.30 Light/ dark cycle reversal (24 h)**	**STRESS – EE + STRESS** **Housing with a different group of animals by swapping partners (14 h)**	
November 23-29	**STRESS – EE + STRESS** **7.30-17.30 Light/dark application at 30-min intervals (10 h)**	**STRESS – EE + STRESS** **08.30-12.30 Crowded grouping in a limited area (4 h)** **17.30 Cage housing without a water bottle (15 h)**	handling + stress + cage cleaning + enrichment cage toy relocation **STRESS – EE + STRESS** **7.30 Water deprivation followed by exposure to an empty bottle for 1 h** **08.30-12.30 holding in a tilted cage (30°) (4 h)**	**STRESS – EE + STRESS** **Light/dark cycle reversal (24 h)**	handling + weighing **STRESS – EE + STRESS** **12.00-15.00 Exposure to cat noise (3 h)** **17.00 Housing with a different group of animals by swapping partners (14 h)**	**STRESS – EE + STRESS** **08.30-12.30 holding in a tilted cage (30°) (4 h)**	**STRESS – EE + STRESS** **Stay on wet bedding (100 g corn cobs + 200 ml water)(24 h)**
November 30-December 6	**STRESS – EE + STRESS** **18.30 Food deprivation (14 h)**	**STRESS – EE + STRESS** **Food deprivation followed by 1 h exposure to inaccessible food**	handling + stress + cage cleaning + enrichment cage toy relocation **STRESS - EE+STRESS** **08.30-12.30 holding in a tilted cage (30°)(4 h)** **Light/dark cycle reversal (24 h)**	**STRESS – EE + STRESS** **Light/dark cycle reversal (24 h)**	**STRESS – EE + STRESS** **08.30-12.30 Crowded grouping in a limited area (4 h)** **13.00-16.00 Exposure to cat noise (3 h)**	handling+weighing **STRESS – EE + STRESS** **Housing with a different group of animals by swapping partners (14 h)**	**STRESS – EE + STRESS** **08.30-12.30 holding in a tilted cage (30°)(4 h)**

(continued)

Table S1. (continued)

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
December 7-13	**STRESS - EE + STRESS** **Light/dark cycle reversal (24 h)**	handling + weighing **STRESS - EE + STRESS** **08.30-12.30 Crowded grouping in a limited area (4 h)** **17.30 Cage housing without a water bottle (15 h)**	handling + stress + cage cleaning + enrichment cage toy relocation **STRESS- EE+STRESS** **7.30 Water deprivation followed by exposure to an empty bottle for 1 h** **08.30-12.30 holding in a tilted cage (30°) (4 h)**	**STRESS – EE + STRESS** **7.30-17.30 Light/dark application at 30-min intervals (10 h)**	**STRESS – EE + STRESS** **08.00-11.00 Exposure to cat noise (3 h) (3 h)** **17.00 Housing with a different group of animals by swapping partners (14 h)**	**STRESS – EE + STRESS** **08.30-12.30 holding in a tilted cage (30°) (4 h)**	**STRESS-EE+STRESS** **Stay on wet bedding (100 g corn cobs + 200 ml water)(24 h)**
December 14-20	**STRESS - EE + STRESS** **12.00-16.00 holding in a tilted cage (30°) (4 h)** **Light/dark cycle reversal (24 h)**	**STRESS – EE + STRESS** **Light/dark cycle reversal (24 h)**	handling + stress + cage cleaning + enrichment cage toy relocation+ weighing **STRESS-EE+STRESS** **08.30-12.30 Crowded grouping in a limited area (4 h)**	blood samples were taken from the jugular vein at the onset of light (06:00-07:00 h) **STRESS- EE+STRESS** **12.00-15.00 Exposure to cat noise (3 h)**	blood samples were taken from the jugular vein at the onset of darkness (18:00-19:00 h) **STRESS- EE+STRESS** **09.00-13.00 holding in a tilted cage (30°) (4 h)** **Food deprivation (14 h)**		**Behavioral experiments**Novel Object Recognition test **STRESS – EE + STRESS** **Housing with a different group of animals by swapping partners (14 h)**
December 21-27	**Behavioral experiments** Novel Object Recognition test **STRESS-EE+STRESS** **13.00-17.00 holding in a tilted cage (30°) (4 h)**	**Behavioral experiments** Novel Object Recognition test **STRESS – EE + STRESS** **17.30 Cage housing without a water bottle (14 h)**	handling + stress + cage cleaning + enrichment cage toy relocation **STRESS – EE + STRESS** **Water deprivation followed by exposure to an empty bottle for 1 h**	**STRESS- EE+STRESS** **08.30-12.30 Crowded grouping in a limited area (4 h)** **13.00-16.00 Exposure to cat noise (3 h)**	**Behavioral experiments** Morris water maze test **STRESS – EE + STRESS** **Housing with a different group of animals by swapping partners (14 h)**	**Behavioral experiments** Morris water maze test **STRESS – EE + STRESS**	**Behavioral experiments** Morris water maze test **STRESS – EE + STRESS** **12.00-16.00 holding in a tilted cage (30°) (4 h)**

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Table S1. (continued)

	Monday	**Tuesday**	**Wednesday**	**Thursday**	**Friday**	**Saturday**	**Sunday**
December 28 – January 3	**Behavioral experiments** Morris water maze test **STRESS – EE + STRESS** **Light/dark cycle reversal (24 h)**	**Behavioral experiments** Morris water maze test **STRESS - EE + STRESS** **Housing with a different group of animals by swapping partners (14 h)**	**STRESS – EE + STRESS** **12.00-16.00** **holding in a tilted cage (30°) (4 h)**	Sacrification			