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Behavior, BDNF and epigenetic mechanisms in response to social isolation and social support in middle aged rats exposed to chronic stress

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ABSTRACT

Social deprivation can be stressful for group-living mammals. On the other hand, an amazing response of these animals to stress is seeking social contact to give and receive joint protection in threatening situations. We explored the effects of social isolation and social support on epigenetic and behavioral responses to chronic stress. More specifically, we investigated the behavioral responses, corticosterone levels, BDNF gene expression, and markers of hippocampal epigenetic alterations (levels of H3K9 acetylation and methylation, H3K27 methylation, HDAC5, DNMT1, and DNMT3a gene expressions) in middle-aged adult rats maintained in different housing conditions (isolation or accompanied housing) and exposed to the chronic unpredictable stress protocol (CUS). Isolation was associated with decreased basal levels of corticosterone, impaired long-term memory, and decreased expression of the BDNF gene, besides altering the balance of H3K9 from acetylation to methylation and increasing the DNMT1 gene expression. The CUS protocol decreased H3K9 acetylation, besides increasing H3K27 methylation and DNMT1 gene expression, but had no significant effects on memory and BDNF gene expression. Interestingly, the effects of CUS on corticosterone and HDAC5 gene expression were seen only in isolated animals, whereas the effects of CUS on DNMT1 gene expression were more pronounced in isolated than accompanied animals. In conclusion, social isolation in middle age showed broader effects than chronic unpredictable stress on behavioral and epigenetic alterations potentially associated with decreased BDNF expression. Moreover, social support prevented the adverse effects of CUS on HPA axis functioning, HDAC5, and DNMT1 gene expressions.

1. Introduction

The adaptative responses to acute stress are normally explained by the fight or flight response, which involves the activation of the sympathetic division of the autonomic nervous system and stimulation of the hypothalamus-pituitary-adrenal axis (HPA) [1]. The resulting increase in catecholamines and glucocorticoids orchestrates an integrated response of organs and systems, adapting the body to deal with stressful situations. However, as the stress becomes chronic, these physiological mechanisms can become maladaptive, resulting in dysfunctions of different organs and systems, including the brain [2].

Chronic psychological stress, such as social distancing, plays a major role in neurocognitive health [3,4]. A growing body of evidence indicates that social isolation and loneliness, important consequences of social deprivation, are major environmental factors associated with adverse effects on cognition, mood disorders, and the development of

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comorbid psychiatric illnesses and dementia [5]. Animal models suggest that the behavioral changes induced by social isolation, such as anxiety-like and depression-like behaviors, are associated with altered functioning of the HPA axis and modifications of brain neurochemistry, structure, and function [2,6–8]. Nevertheless, most of these studies comprise the effects of early life stress (such as maternal separation or social isolation at a young age) on endocrine responses to stress at adulthood [9,10]. Thus, the effects of social isolation during adulthood, especially in middle-aged and older animals, remain to be elucidated. This is especially important because the investigation of causal pathways that associate social isolation and loneliness with neurocognitive aging and neuropathological changes in humans is not readily amenable to be studied using randomized controlled trials [11].

Human beings are intensely social. Therefore, most of us find social deprivation stressful [12]. On the other hand, an amazing response of humans to stress is to come together, forming groups to give and receive joint protection in threatening situations [13,14]. Animal models show that behavioral reactions and alterations in the HPA axis due to stressful situations can be ameliorated by social support, i.e., by the presence of members of the same species [15–20]. The investigations of physiological mechanisms underlying the behavioral effects of social buffering are restricted to the role of the HPA axis, oxytocin, and vasopressin [15]. However, it is becoming increasingly apparent that epigenetic mechanisms could be the basis for the lasting effects that a history of stress exposure can have on future stress reactivity and maladaptation [2]. By now, only one study has explored the association between social support and epigenetic mechanisms. Borges and colleagues [16] found that social support prevented the adverse effects of chronic unpredictable stress (CUS) on HDAC5, a histone deacetylase. Thus, more studies on the epigenetic effects of social support are warranted.

The hippocampus is a component of the limbic system and one of the most sensible brain regions to stress [17]. It has a central role in functions like emotion, motivation, and memory processing [2]. The association of chronic stress with cognitive dysfunction and psychiatric conditions is due, at least partially, to the negative effects of stress on hippocampal neurogenesis, plasticity, and neuronal survival [17]. Among the processes underlying these effects of stress seems to be the control of brain-derived neurotrophic factor (BDNF) availability by epigenetic mechanisms [16].

Most epigenetic modifications involve histone alterations and DNA methylation. Histone acetylation (H3 and H4) is associated with active transcription and is modulated by the activity of acetyltransferases (HATs), responsible for the increase in acetylation, and deacetylases (HDACs), responsible for the decrease in acetylation [18,19]. Histone methylation is associated with both transcriptional activation (H3K4 and H3K36) and repression (H3K9, H3K27, and H3K20), depending on the residue and valence state of the methylation [20,21]. DNA methylation, catalyzed by DNA methyltransferases (DNMTs), regulates gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA [22,23].

Various stress protocols, such as prenatal stress, maternal separation, restrain stress and chronic unpredictable stress can affect histone acetylation and methylation, as well as DNA methylation, in the hippocampus. Of special interest are the epigenetic modifications associated with the downregulation of BDNF, such as the decrease in H3K9 acetylation, the increase in HDAC5 expression, H3K27 methylation and DNA methylation promoted by upregulated DNMT1 and DNMT3a activity [24–29]. Chronic stress was already shown to decrease BDNF levels in humans [30,31] and animals [2]. As BDNF has a major effect on neurogenesis, neuronal survival and plasticity, there are suggestions that the maladaptive effects of chronic stress on mental health are, at least partially, associated with the epigenetic modulation of BDNF levels [8, 32]. This study was designed to explore the effects of social isolation and social support on epigenetic and behavioral responses to chronic stress. More specifically, we investigated the behavioral responses, corticosterone levels, and markers of hippocampal epigenetic alterations (levels of H3K9 acetylation and methylation, H3K27 methylation, HDAC5, DNMT1 and DNMT3a, and BDNF gene expressions) in middle-aged adult rats maintained in different housing conditions (isolation or accompanied housing) and exposed to chronic stress (CUS protocol). Our main hypotheses were that: (1) isolation and chronic unpredictable stress would lead to negative outcomes on the investigated variables (such as memory impairment, a chronic increase of corticosterone levels, a decrease of BDNF expression, and epigenetic alterations potentially involved in the decline of BDNF gene expression); (2) isolation and chronic unpredictable stress would interact, inducing increased negative outcomes; (3) paired housing would be protective against some of the epigenetic alterations induced by chronic unpredictable stress.

2. Methods

2.1. Animals

Adult male Wistar rats (CrlCembe: WI, 17 months old, n = 46) were obtained from the university breeding facility (Centro de Modelos Biolo&#xfffd;gicos Experimentais da Pontifi&#xfffd;cia Universidade Cato&#xfffd;lica do Rio Grande do Sul, CeMBE/PUCRS). Animals were maintained in standard cages with sawdust bedding, room temperature of 21 ± 1ºC, a 12 h light/dark schedule and ad libitum access to standardized pellet food and water. The experiments were carried out in conformity with the Guide for the Care and Use of Laboratory Animals and performed according to the recommendations of the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI). Experimental protocols were approved by the Ethics Committee for the Use of Animals of the Pontifical Catholic University of Rio Grande do Sul (CEUA, registration No. 7142). All efforts were made to reduce sample size and minimize animal suffering.

2.2. Experimental design

At the start of the experiment all animals were weighed and randomly divided into four experimental groups: Accompanied (two animals/home cage); Accompanied + CUS (two animals/home cage and one of them daily submitted to the CUS protocol); Isolated (one animal/home cage); Isolated + CUS (one animal/home cage daily submitted to the CUS protocol). The CUS protocol was initiated two days after the beginning of the new housing (paired or isolated) conditions.

2.3. Chronic unpredictable stress protocol (CUS)

The CUS protocol was designed according to the literature to induce stress of mild to moderate intensity [54–56]. The stress protocol lasted 4 weeks and the stressors were presented in a random and unpredictable fashion (Table 1). In the last week, the stress protocol was interrupted for three days for the behavioral tasks and resumed for another two days. This interruption aimed to avoid confounding repercussions of the acute effects of the stressors of the CUS protocol on behavior. Animals were weighed and euthanized by decapitation two days after the end of the CUS protocol.

2.4. Behavioral tasks

All behavioral tasks were conducted during the light phase, between 9:00 am and 1:00 pm, under red lighting. Before the first task (open field), animals were handled for 90 s for habituation to the experimenter and the testing room. The animals were also transferred to the testing room 1 h before the beginning of all behavioral tasks, ensuring adaptation to the room.

2.4.1. Open field

Open-field testing was performed as previously described [16]. In short, animals were placed in a 50 × 55 × 50 cm high open-field cage

Table 1
Weekly protocol of chronic unpredictable stress (CUS) repeated four times during the stress period.

Stressor	Start and ending time of the stressors						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Water deprivation*	10 am						
Wet bedding		13–17 pm					
Light*			8 am				
Immobilization				16–16:45 pm			
Food deprivation*					11 am		
Strobe light						14–16 pm	
Cage tilt							7–11 am

* 24-hour long treatments: start and ending time of the stressors are the same with a 24-hour interval in between.

divided into 16 equal-sized sections for 5 min under red lighting. The light lamp was positioned to ensure the same lighting conditions in the central (the four inner squares of the apparatus) and peripheral zones of the arena. Between each session, feces and urine were removed from the apparatus. Animals were videotaped and locomotor, exploratory, and anxiety responses were scored offline by researchers blind to the experimental condition with high inter-rate reliability (Pearson's > 0.9). The number of squares the animal crossed (number of crossings), the number of rearings, and the proportion of time spent in the inner zone were determined as measures of locomotion, exploratory behavior, and anxiety, respectively.

2.4.2. Inhibitory avoidance

The Inhibitory Avoidance task was performed to evaluate long-term aversive memory and followed the procedures previously described [16]. The apparatus was an acrylic box (50 × 25 × 25 cm) whose floor consisted of parallel-caliber stainless-steel bars (1 mm diameter) spaced 1 cm apart, and a platform that was 7 cm wide and 2.5 cm high. During the training session, animals were placed on the platform and their latency to step down on the grid with all four paws was measured. Animals received a 0.4-mA, 3.0-s foot shock after stepping down on the grid and were immediately removed from the apparatus. The test session was carried out 24 h after training, no foot shock was given, and the step-down latency (maximum of 180 s) was used as a measure of memory retention.

2.5. Biochemical analysis

2.5.1. Corticosterone levels in hair samples

2.5.1.1. Hair samples. The hair of an area bounded cranially by the eighth lumbar vertebra, caudally by the ischial tuberosity and laterally by the hind limb was totally shaved immediately before the beginning of the different housing conditions and the CUS protocol. The hair grown in the bald area during the experimental procedures was then collected immediately after the euthanasia. The samples were stored in 2 mL centrifuge tubes, in the dark, at room temperature, until the analysis of corticosterone levels. As hair is capable of accumulating corticosterone for a period of weeks to months [33], results from this analysis express the effects of chronic stress, associated with housing conditions and the CUS protocol, on the body of animals.

2.5.1.2. Analysis of hair corticosterone levels by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The corticosterone extraction method was adapted from the work by Xiang and colleagues (2016) [34]. Briefly, each sample was transferred into a 15 mL Falcon tube previously weighed. The samples were cleaned three times by the addition of 3 mL of isopropanol and orbital homogenization at 20 RPM, at room temperature, for 5 min. At each cleaning step, the total volume of isopropanol was discarded, and the residual alcohol volume of the last step was evaporated by dry air insufflation for 10 min. Then, the dried samples were weighed (50 mg). The extraction was performed by the addition of 5 mL of methanol and trituration by an Ultra-turrax system (IKA-Werke GmbH, Germany). The mixture was homogenized whit an orbital mixer at 20 RPM, for 72 h at room temperature, in the dark. After this, the tubes were centrifuged at 4000 RPM for 20 min, at 4 oC. The liquid phase was transferred to a new 15 mL Falcon tube and evaporated by dry air insufflation. The extracts were resuspended with 0.2 mL methanol and 0.1 mL of water, homogenized in a vortex (approx. 30 s), transferred to a 0.6 mL centrifuge tube, centrifuged at 14 RMP, for 10 min at 4 °C, and finally injected into LC-MS/MS system.

The corticosterone quantification by LC-MS/MS was based on a modification of the method reported by Ranganathan and colleagues (2020) [35]. The LC-MS/MS system consisted of a Xevo TQ-S micro mass spectrometer coupled to an Acquity Class I Plus UPLC chromatograph (Waters; Milford, MA, USA), equipped with a ZORBAX RRHT Extend-C18 (2.1 ×50 mm, 1.8 μm, Agilent, Paolo Alto, USA) column and a mobile phase of 0.1% formic acid (A) and 0.1% formic acid in methanol (B). The chromatographic separation was carried out in gradient mode at a flow rate of 0.5 mL/min., at 40 °C, starting with 40% B and programmed to reach 80% B after 1.5 min. After 1 min the gradient was programmed to 95% B. This composition was maintained for 1 min before returning to the start condition. The sample volume injected into the system was 5 μL. The mass spectrometer was equipped with an ESI source operated in positive mode and the ions were monitored by multiple reaction monitoring. The transitions of m/z 347.0 > 293.2, and m/z 347.0 > 121.1 were used for both quantitation and confirmation purposes, respectively. The calibration curve was constructed with a corticosterone standard (>98,5%) in the mobile phase at concentrations of 1.0; 5.0; 10.0; 15.0 e 20.0 ng/mL. The corticosterone standard and the solvents with LC-MS grade, including isopropanol, methanol, and formic acid were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). Type 1 water was prepared with a Milli-Q system (Millipore Corp., Bedford, MA, USA).

2.5.2. Histone acetylation, methylation, and gene expression in the hippocampus

2.5.2.1. Hippocampus samples. Animals were euthanized by decapitation during the light cycle (between 9:00 am and 3:00 pm), 48 h after the last session of the CUS protocol. Brains from randomly selected animals from each group were immediately removed, and the hippocampi were quickly dissected. The left hippocampi were placed in a cooled RNA-later solution (Sigma-Aldrich, São Paulo, Brazil) for RT-qPCR assays, and the right hippocampi in a cooled protease inhibitor solution (Complete Mini, Roche Applied Science, Mannheim, Germany) for the Western blot assays. Samples were snap-frozen in nitrogen and were stored at − 80 °C for further analysis, as explained below.

2.5.2.2. Analysis of histone acetylation and methylation by Western blotting. The dissected and nitrogen-frozen hippocampus samples were homogenized, placed in EDTA-free (Sigma-Aldrich, St. Louis, MO, USA) solution 1x containing a protease inhibitor cocktail tablet, and stored at − 80 °C for subsequent analysis. For histone extraction, PBS buffer

(Phosphate-Buffered-Saline) containing 250 μL Triton and 10 mg NaN3 was added to the homogenate samples to a 50 mL final volume. After 10 min on ice, samples were centrifuged at 6500 g for 10 min at 4 °C. The supernatant was collected and acid extraction (0.2-N HCl) of histones was carried out overnight at 4 °C. Samples were centrifuged once again (6500 g for 10 min at 4 °C), supernatants were saved, and the protein content was determined using the Coomassie Blue method, with bovine serum albumin as a standard [60].

Western blot analysis of acetylated H3K9 (H3K9ac), methylated H3K9 (H3K9me3), and methylated H3K27 (H3K27me3) was done as follows. Twenty-five μg total protein was separated on a 10% SDS polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in TBS containing 0.05% Tween 20 and were incubated overnight with the following antibodies: anti-H3 (ab1791, Abcam) at 1:3000, anti-H3K9ac (ab10812, Abcam) at 1:500, anti-H3K9me3 (ab8898, Abcam) at 1:500, anti-H3K27m2/me3, (ab6147, Abcam) at 1:500. Goat anti-rabbit radish-conjugated secondary antibodies (ab97051, HRP) were used and detected using ECL Western Blotting Substrate Kit (Abcam, Cambridge, UK). Pre-stained molecular weight protein markers (SuperSignal Molecular Weight Protein Ladder, Thermo Scientific, Rockford, USA) were used to determine the detected band's molecular weight and confirm the target specificity of antibodies. Analysis of band intensities was performed in a Carestream Gel Logic 2200 PRO Imaging System and the associated Image Analysis Software. Data for acetylated histones were corrected for total histone protein.

2.5.2.3. Analysis of BDNF, HDAC5, DNMT1 and DNMT3a gene expression by real-time PCR (RT-qPCR). RT-qPCR was used to analyze the mRNA levels of BDNF, HDAC5, DNMT1, and DNMT3a. PCR primer sequences can be seen in Table 2. The total cellular RNA of the hippocampus was extracted with SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's protocol. RNA was re-suspended in nuclease-free water and was quantitated by spectrophotometry. The total RNA was used for reverse transcription (RT) reactions. RT reactions were performed using Invitrogen Superscript IV One-Step Rt-PCR System (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and this was followed by real-time PCR of the target gene. TaqMan probes and the One-Step RT-PCR System (Applied Biosystems, Foster City, CA) were used in our experiments. PCR reactions were performed using 20 × Assays-On-Demand Gene Expression Assay Mix (containing unlabeled PCR primers and Taq-Man probe) and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. PCR conditions were 95 °C for 10 min, followed by 95 °C for 15 s and 60 °C for 1 min repeated for 40 cycles. Experiments were performed in duplicate for each data point. Beta-actin was evaluated as an internal RNA control. Quantitative values were obtained from the cycle number (CT value) at which the increment in fluorescent signal associated with the exponential growth of PCR products started to be detected. The amount of target gene mRNA expression was normalized to the endogenous level of Beta-actin. Analysis was performed by obtaining the relative threshold cycle (ΔCT), in relation to the CT of the control gene to measure the relative expression level (2–ΔΔCT) of the target gene [45].

2.6. Statistical analysis

The assumptions of normality and homogeneity of variances were assessed with the Shapiro-Wilk test and with Levene's test for equality or error of variances, respectively. Parametric data are expressed as mean ± standard deviation and were analyzed by two-way analysis of variance (2-way ANOVA), with housing condition (accompanied or isolated) and stress (exposed or not exposed to the CUS protocol) as between-group variables. Whenever significant interactions occurred, simple effect analyses were performed. Non-parametric data are expressed as median (interquartile ranges) and were analyzed with Kruskal-Wallis tests, followed by the Mann-Whitney test. Effect size [eta squared (η^2) or partial eta squared (η^2_ρ)] was reported for all behavioral and biochemical data analysis. In all comparisons, p values less than 0.05 were considered to indicate statistical significance, except for the simple effect analysis, where p values of less than 0.025 were considered to indicate statistical significance.

3. Results

3.1. Weight alterations during the experimental period

The two-way ANOVA revealed a significant main effect of stress [$F(1,39) = 122.828$, $\eta^2_\rho = 0.759$, $p < 0.001$] on weight alteration during the experimental period. No significant main effect of housing condition [$F(1,39) = 0.532$, $\eta^2_\rho = 0.013$, $p = 0.47$], or interaction between housing condition and stress [$F(1,39) = 1.983$, $\eta^2_\rho = 0.048$, $p = 0.16$], was identified by the statistical analysis. As can be seen in Fig. 1, experimental groups submitted to the CUS protocol showed a much greater weight loss in comparison to the accompanied-only and isolated-only subgroups.

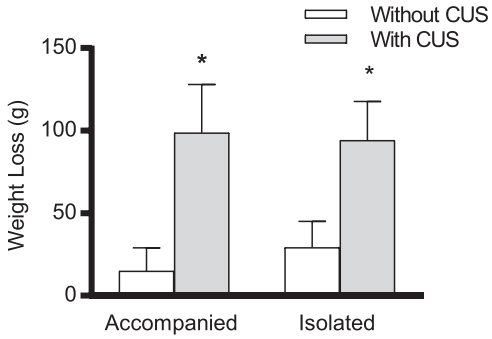

Fig. 1. Weight alteration of animals during the experimental procedures, calculated as the difference between weight at the end (immediately before animals were euthanized) and at the start (when animals were assigned to different housing conditions) of the experiment. Statistical analysis was performed using 2-way ANOVA, with housing conditions [accompanied or isolated] and stress [submitted or not submitted to the chronic unpredictable stress (CUS) protocol] as fixed factors. Data are expressed as mean ± standard deviation. Sample size *per* group: accompanied without CUS, n = 10; accompanied with CUS, n = 11; isolated without CUS, n = 10; isolated with CUS, n = 12 *p < 0.001 in comparison to subgroups without CUS, indicating a significant effect of stress.

Table 2
Primer sequences used in the RT-qPCR analysis.

Primers	Forward Sequence (5′−3′)	Reverse Sequence (5′−3′)
BDNF	AAGCTCAACCGAAGAGCTAAAT	CTGAGGGAACCCGGTCTCA
HDAC5	CAGCCAGAAGATGTACGCCA	GCTGTGATGGCTACGGAGTT
DNMT1	CCTAGTTCCGTGGCTACGAGGAGAA	TCTCTCTCCTCTGCAGCCGACTCA
DNMT3a	GCCGAATTGTGTCTTGGTGGATGACA	CCTGGTGGAATGCACTGCAGAAGGA
B-ACTN	ACCGAGCATGGCTACAGCGTCACC	GTGGCCATCTCTTGCTCGGAGTCT

3.2. Open field

The two-way ANOVA showed a significant effect of housing condition [$F(1,35) = 18.287$, $\eta_p^2 = 0.339$, $p < 0.001$] on the latency to start locomotion and on the time spent in the center of the apparatus [$F(1,35) = 5.301$, $\eta_p^2 = 0.131$, $p = 0.046$]. As can be seen in Table 3, isolated animals took longer to start locomotion and spent less time in the center zone, suggesting that they are more anxious than the accompanied animals. The statistical analysis of the time spent in the inner zone also indicated a significant main effect of stress [$F(1,35) = 9.461$, $\eta_p^2 = 0.212$ $p = 0.004$], which could also be seen on the number of crossings [$F(1,35) = 5.735$, $\eta_p^2 = 0.140$, $p = 0.022$]. These results indicate that animals submitted to the CUS protocol spent less time in the center zone and made more crossings, implying greater anxiety and hyperactivity in comparison to animals not submitted to this type of stress. No interaction was found between housing condition and stress on the variables described above. On the other hand, a significant interaction between housing condition and stress [$F(1,35) = 4.686$, $\eta_p^2 = 0.102$, $p = 0.037$] was found for rearing, despite the lack of main effects of housing condition [$F(1,35) = 0.933$, $\eta_p^2 = 0.025$, $p = 0.341$] and stress [$F(1,35) = 0.010$, $\eta_p^2 < 0.001$, $p = 0.921$] on this variable. However, this interaction was not confirmed by the simple effect analysis [$F(1,35) = 4.9$, $\eta_p^2 = 0.10$, $p = 0.03$].

3.3. Inhibitory avoidance

As indicated by the Kruskal-Wallis test, latency to step down the platform in the training session was not significantly different ($\eta^2 = 0.023$, $p = 0.837$) between the accompanied [5.19 (3.27/6.12)], accompanied + CUS [4.78 (3.5/7.75)], isolated [4.67 (3.66/5.13)] and isolated + CUS [5.4 (3.16/6.87)] groups. The latency to step down increased significantly from the training to the test session in all experimental groups, as indicated by the Wilcoxon test ($\eta^2 = 0.888$, $p = 0.008$ for the accompanied group; $\eta^2 = 0.860$, $p = 0003$ for the accompanied + CUS group; $\eta^2 = 0.907$, $p = 0.012$ for the isolated group; and $\eta^2 = 0.600$, $p = 0.028$ for the isolated + CUS group). Further analysis with the Mann-Whitney post hoc test indicated that the isolated ($\eta^2 = 0.783$) and isolated + CUS ($\eta^2 = 0.780$) groups had worse performance on the memory retention test than the accompanied group (all $p < 0.001$). Additionally, the isolated ($\eta^2 = 0.673$) and isolated + CUS ($\eta^2 = 0.750$) groups also had worse long-term memory than the accompanied + CUS group (all $p < 0.001$) (Fig. 2). On the other hand, no significant differences were identified between the accompanied and accompanied + CUS group ($\eta^2 = 0.016$, $p = 0.085$) and between the isolated and isolated + CUS groups ($\eta^2 = 0.014$, $p = 0.124$).

Table 3
Open field behavior.

	Experimental Groups			
	Accompanied	Accompanied + CUS	Isolated	Isolated + CUS
Latency to Start (s)	0,89 ± 0,38	1,11 ± 0,74	2,81 ± 1,48*	3,23 ± 2,02*
Number of crossings	36,4 ± 8,31	43,2 ± 10,61[!]	39,11 ± 7,96	45,7 ± 7,52[!]
Time in center (s)	29,16 ± 9,24	18,55 ± 10,17[!]	21,65 ± 11,29*	13,26 ± 6,44*[!]
Number of rearings	19,7 ± 5,39	16,6 + 2,22	17,9 ± 5,58	21,3 ± 4,98

Statistical analysis was performed using a two-way analysis of variance, with housing conditions (accompanied or isolated) and stress (submitted or not submitted to the CUS protocol) as fixed factors. Data are expressed as mean ± standard deviation. n = 10 per group, except for the accompanied + CUS group (n = 9). *$p < 0.05$ in comparison to the accompanied groups, indicating a significant housing effect; [!]$p < 0.05$ in comparison to animals not submitted to CUS, indicating a significant stress effect.

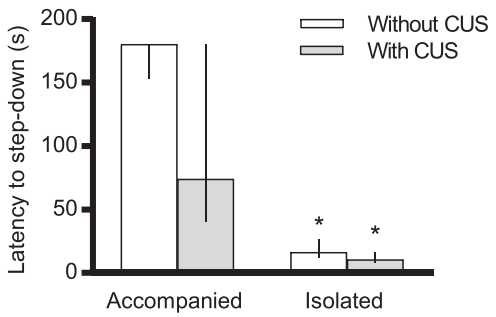

Fig. 2. Long-term retention of Inhibitory Avoidance memory in animals submitted to different housing [accompanied or isolated] and stress [submitted or not submitted to chronic unpredictable stress (CUS)] conditions. The retention test was run 24 h after the training session. Statistical analysis was performed using the Kruskal-Wallis test and Mann-Whitney's post hoc test. Data are expressed as median and interquartile range. Sample size *per* group: accompanied without CUS, n = 9; accompanied with CUS, n = 11; isolated without CUS, n = 8; isolated with CUS, n = 9. *$p < 0.001$ in comparison to the accompanied subgroups, indicating a significant housing effect.

3.4. Hair corticosterone

The two-way ANOVA indicated a significant main effect of housing condition [$F(1,27) = 8199$, $\eta_p^2 = 0.233$, $p = 0.008$] on hair corticosterone levels. As can be seen in Fig. 3, isolated animals had lower levels of corticosterone than accompanied animals. Additionally, a significant interaction between housing conditions and the CUS protocol [$F(1,27) = 5.703$, $\eta_p^2 = 0.174$, $p = 0.024$] was found. Simple effect analyses indicated that isolated rats submitted to the CUS protocol had lower corticosterone levels than isolated animals not submitted to the CUS protocol [$F(1,27) = 13.43$, $\eta_p^2 = 0.332$, $p = 0.001$].

3.5. BDNF expression

A significant main effect of housing condition [$F(1,14) = 29.879$, η_p^2

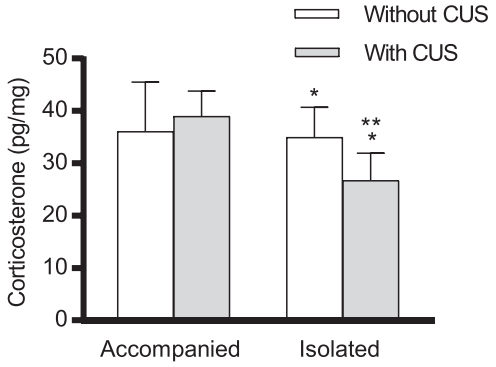

Fig. 3. Hair corticosterone levels of rats submitted to different housing [accompanied or isolated] and stress [submitted or not submitted to chronic unpredictable stress (CUS)] conditions. The hair of an area bounded cranially by the eighth lumbar vertebra, caudally by the ischial tuberosity, and laterally by the hind limb was totally shaved immediately before the beginning of the different housing conditions and the CUS protocol. The hair grown in the bald area during the experimental procedures was collected immediately after the euthanasia for the analysis of corticosterone levels by LC-MS/MS. Statistical analysis was performed using 2-Way ANOVA, with housing and stress conditions as fixed factors. Interactions were confirmed with simple effect analyses. Sample size *per* group: accompanied without CUS, n = 7; accompanied with CUS, n = 8; isolated without CUS, n = 9; isolated with CUS, n = 7. Data are expressed as mean ± standard deviation. *$p = 0.008$ in comparison to the accompanied subgroups, indicating a significant housing effect; **$p = 0.001$ in comparison to all other groups, indicating an interaction of housing condition and stress.

= 0.681, p < 0.001] was indicated by the two-way ANOVA on the BDNF gene expression. As can be seen in Fig. 4, isolated animals had lower levels of BDNF expression than accompanied animals. Neither main effects of stress [F(1,14)= 0.067, η_p^2 = 0.005, p = 0.80] nor interactions between housing condition and stress [F(1,14)= 0.097, η_p^2 = 0.007, p = 0.76] were seen on BDNF gene expression.

3.6. H3K9 acetylation levels and HDAC5 gene expression

Significant main effects of housing condition [F(1,19) = 38.01, η_p^2 = 0.667, p < 0.001] and stress [F(1,19)= 14.32, η_p^2 = 0.430, p = 0.001] were found for H3K9ac. As can be seen in Fig. 5 A, isolated animals had lower levels of H3K9ac than accompanied animals. Additionally, H3K9ac decreased in animals submitted to the CUS protocol. However, no significant interactions between housing conditions and stress were seen on H3K9ac levels [F(1,19)= 0.191, η_p^2 = 0.010, p = 0.667).

The statistical analysis of HDAC5 indicated a significant main effect of the housing condition [F(1,11) = 18.045, η_p^2 = 0.621, p < 0.001]. As can be seen in Fig. 5B, the HDAC5 expression was significantly higher in isolated animals. Although no significant main effect of stress was observed [F(1,11) = 2.910, η_p^2 = 0.020, p = 0.116], statistical analysis revealed a significant interaction between housing condition and stress on HDAC5 expression [F(1,11) = 10.178, η_p^2 = 0.481, p = 0.009]. Simple effect analyses indicated that isolated rats submitted to the CUS protocol had higher HDAC5 levels than isolated animals not submitted to the CUS protocol [F (1,11) = 25.687, η_p^2 = 0.700, p < 0.001]. Overall, this pattern of results suggests that accompaniment can mitigate the effects of CUS on HDAC5 expression.

3.7. H3K9 and H3K27 methylation levels

Only housing condition had a significant main effect on H3K9me [F (1,15) = 7.36, η_p^2 = 0.329, p = 0.016], whereas only stress has a significant main effect on H3K27me [F(1, 18) = 5.63, η_p^2 = 0.238, p = 0.029]. As can be seen in Fig. 6, isolated animals had higher levels of H3K9me than accompanied animals, and CUS increased H3K27me levels in comparison to the accompanied-only and isolated-only experimental groups. No interactions were found between housing conditions and stress for H3K9me [F(1,15)= 0.438, η_p^2 = 0.028, p = 0.51] and H3K27me [F(1,18)= 0.07, η_p^2 < 0.001, p = 0.93] levels.

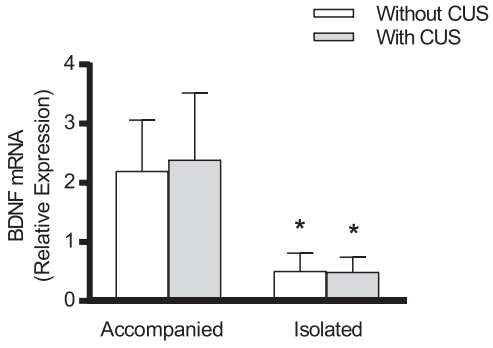

Fig. 4. Alterations of brain-derived neurotrophic factor (BNDF) expression in the hippocampus of rats submitted to different housing [accompanied or isolated] and stress [submitted or not submitted to chronic unpredictable stress (CUS)] conditions. Samples were normalized to Beta-actin gene expression and run in duplicate. Statistical analysis was performed using 2-way ANOVA, with housing and stress conditions as fixed factors. Sample size *per* group: accompanied without CUS, n = 4; accompanied with CUS, n = 4; isolated without CUS, n = 5; isolated with CUS, n = 5. Data are expressed as mean ± standard deviation. *p < 0.001 in comparison to the accompanied subgroups, indicating a significant effect of housing conditions.

3.8. DNMT1 and DMNT3A gene expression

Main effects of housing condition [F(1,12) = 22.017, η_p^2 = 0.647, p = 0.001] and stress [F(1,16) = 15.133, η_p^2 = 0.558, p = 0.002] on the expression of DNMT1 were identified by the two-way ANOVA. As can be seen in Fig. 7 A, animals submitted to social isolation had higher levels of DNMT1 expression than accompanied animals, and experimental groups submitted to the CUS protocol had higher levels of DNMT1 expression than groups that were not submitted to stress. Moreover, a significant interaction between housing condition and stress was found [F(1,12) = 14.294, η_p^2 = 0.544, p = 0.003]. Simple effect analyses indicated that isolated rats submitted to the CUS protocol had higher DNMT1 expression than isolated animals not submitted to the CUS protocol [F (1,12) = 31.79, η_p^2 = 0.726, p < 0.001]. Importantly, stress effects were more pronounced on isolated than accompanied animals, suggesting that social support can prevent the effects of CUS on DNMT1 expression. On the other hand, no main effects of housing condition [F (1,13) = 0.157, η_p^2 = 0.012, p = 0.699] and stress [F(1,13) = 2.536, η_p^2 = 0.165, p = 0.135] on DNMT3a expression were identified with the two-way ANOVA. Interactions between housing conditions and stress were also absent [F(1,13) = 0.085, η_p^2 = 0.007, p = 0.775]. Thus, no significant group differences were found for the DNMT3a expression (Fig. 7B).

4. Discussion

The present study has investigated the effects of social isolation and chronic unpredictable stress (CUS) on epigenetic mechanisms potentially involved in the modulation of BDNF gene expression and behavior. The main findings indicate that the social isolation protocol is associated with decreased basal levels of corticosterone, impaired long-term memory, and decreased expression of the BDNF gene, besides altering the balance of H3K9 from acetylation to methylation and increasing the DNMT1 gene expression. The CUS protocol was also able to decrease H3K9 acetylation, besides increasing H3K27me and DNMT1 gene expression, but had no significant effects on memory and BDNF gene expression. Interestingly, the effects of CUS on corticosterone and HDAC5 gene expression were seen only in isolated animals, whereas the effects of CUS on DNMT1 gene expression were more pronounced in isolated than accompanied animals, suggesting that social support prevented some of the adverse effects of CUS.

Stress induced by social isolation and CUS resulted in anxiety-like behaviors, as suggested by the increased time to start locomotion and reduced time spent in the inner zone of the arena. Anxiety-like behaviors are common among rats submitted to these types of stress, but some controversies do exist, mainly due to confounding factors such as methodological issues and strain-, sex- and age-related differences among animals [16,36]. Additionally, animals submitted to the CUS protocol had an important weight loss, a usual finding of most studies that address this type of stress [37–39].

Long-term memory impairment and decreased BDNF gene expression were seen only in animals submitted to social isolation. These findings agree with a former study with younger adult rats, which also showed that social isolation, but not the CUS protocol, caused impairment of inhibitory avoidance memory and decreased BDNF levels [16]. The extracellular release of BDNF and its interaction with tropomyosin-related kinase B (TrkB) receptors are known to be important for hippocampal memory consolidation of inhibitory avoidance [40–42]. Thus, a decrease in BDNF gene expression could induce memory impairment, as suggested by Bambah-Mukku and colleagues [43]. However, the role of the anxiety-like behavior of isolated animals on memory impairment cannot be ruled out.

Different epigenetic mechanisms could be involved in the decrease of the BDNF gene transcription. In this study, we found that social isolation was capable to increase HDAC5 gene expression and reduce H3K9 acetylation. These results reproduce previous findings of our research

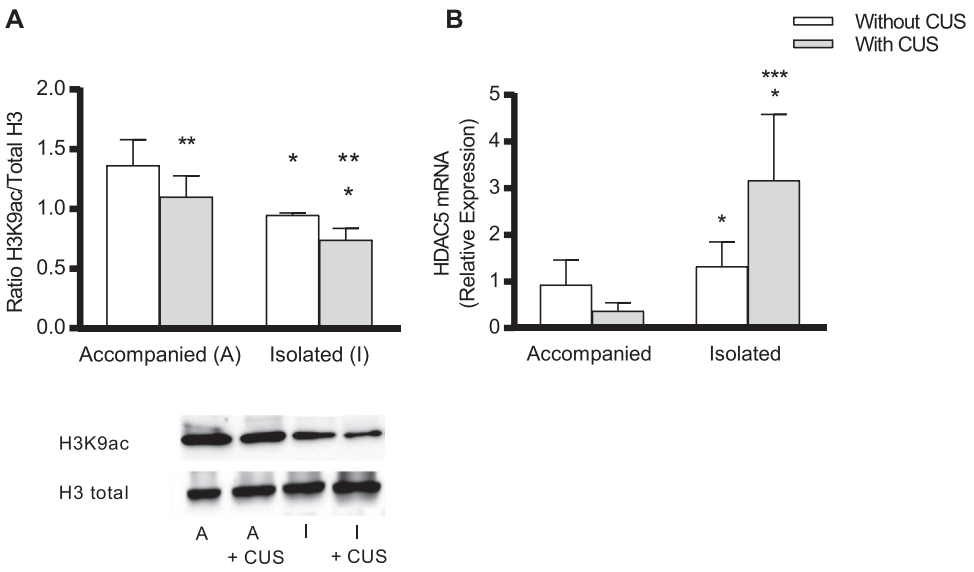

Fig. 5. Quantification and representative western blot of acetylated histone 3 lysine 9 [H3K9ac] (A) and gene expression of Histone Deacetylase 5 [HDAC5] (B) in the hippocampus of rats exposed to different housing [accompanied or isolated] and stress [submitted or not submitted to chronic unpredictable stress (CUS)] conditions. Statistical analysis was performed using 2-way ANOVA with housing conditions and stress as fixed factors. Interactions were confirmed with simple effect analyses. Sample size *per* group for Western Blotting: accompanied without CUS, n = 6; accompanied with CUS, n = 6; isolated without CUS, n = 6; isolated with CUS, n = 5. Sample size per group for real-time PCR: accompanied without CUS, n = 4; accompanied with CUS, n = 4; isolated without CUS, n = 4; isolated with CUS, n = 3. Data are expressed as mean ± standard deviation. *p < 0.001 in comparison to the accompanied subgroups, indicating a significant housing effect; **p = 0.001 in comparison to accompanied-only and isolated-only subgroups, indicating a significant effect of stress; ***p < 0.001 in comparison to all other groups, indicating an interaction of housing condition and stress.

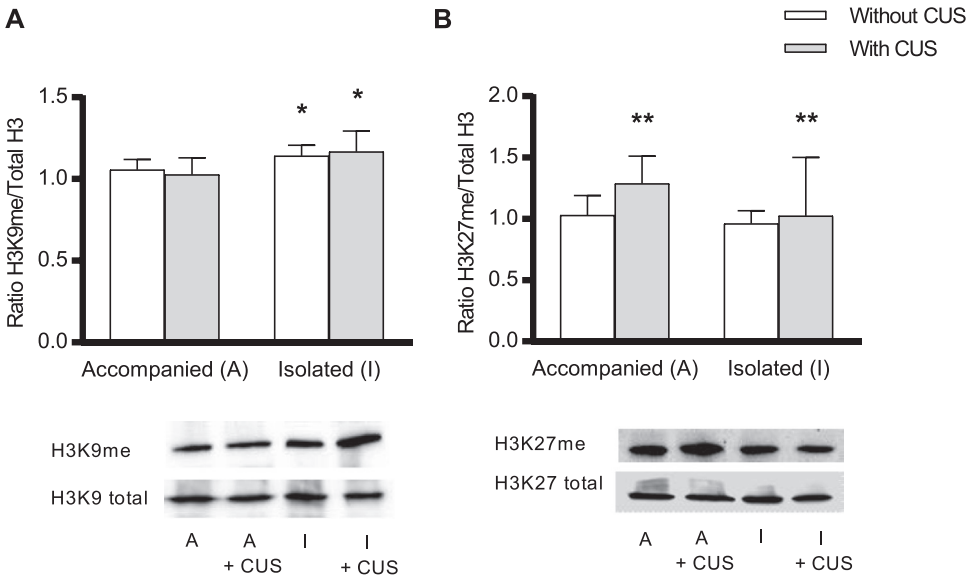

Fig. 6. Quantification and representative western blots of histone 3 methylation at lysine 9 [H3K9me] (A) and at lysine 27 [H3K27me] (B) in the hippocampus of rats exposed to different housing [accompanied or isolated] and stress [submitted or not submitted to chronic unpredictable stress (CUS)] conditions. Statistical analysis was performed using 2-way ANOVA with housing conditions and stress as fixed factors. Sample size *per* group for H3K9me analysis: accompanied without CUS, n = 6; accompanied with CUS, n = 5; isolated without CUS, n = 6; isolated with CUS, n = 5. Sample size *per* group for H3K27me analysis: accompanied without CUS, n = 5; accompanied with CUS, n = 5; isolated without CUS, n = 5; isolated with CUS, n = 4. Data are expressed as mean ± standard deviation. *p = 0.016 in comparison to the accompanied subgroups, indicating a significant housing effect; **p = 0.029 in comparison to accompanied-only and isolated-only subgroups, indicating a significant effect of stress.

group in younger rats, in which changes in HDAC5 expression and H3K9 acetylation levels were associated with decreased BDNF protein levels [16]. This pattern of results was formerly shown to exist from early development, as seen in studies of maternal separation [27,44], to social isolation in adolescence [45] and young adult animals [16]. We now extend the findings to middle-aged rats, reinforcing the hypothesis that the decrease in H3K9 acetylation, possibly mediated by an increased HDAC5 expression and activity, is a conserved response to social isolation along different life stages.

Besides the decrease in H3K9 acetylation, we also found an increase in H3K9 methylation in isolated animals. H3K9 methylation is a well-known indicator of silenced transcription and heterochromatin structure [46]. To the best of our knowledge, this is the first study to explore the effects of late-life isolation on H3K9 methylation. Our results are in line with studies on early life isolation, such as maternal separation or isolation rearing from the first day of weaning, which induce increased H3K9 methylation in middle-aged animals associated with lowered BDNF mRNA and protein levels, and memory impairment [47,48]. In This study, the increase in H3K9 methylation induced by social isolation was accompanied by an increased expression of DNMT1, a DNA methyltransferase. Traditionally considered for its prominent role during the early developmental stages [49], DNMT1 is also one of the main DNMTs expressed in the adult brain [50]. Growing evidence suggests a role for DNMT1 in the modulation of BDNF expression. [51–53], neuronal survival [50], neuronal excitability, and synaptic function [54,55] in the adult brain. Although there are no other studies in the literature that evaluate the effect of social isolation on DNMT1 expression in adulthood, our results are in line with studies that showed that maternal separation (an early life model of social isolation) can induce a long-lasting increase in DNMT1 gene expression in different brain structures [56,57], including the hippocampus, where it was associated to decreased BDNF protein and mRNA levels [52]. Curiously, contrary to

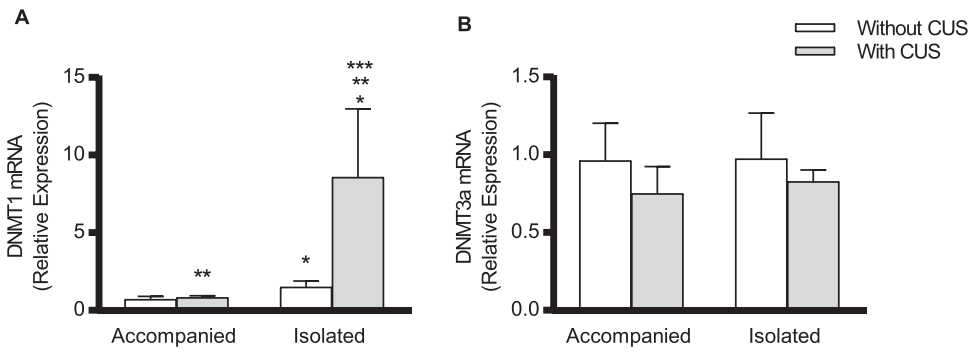

Fig. 7. Levels of DNA methyltransferase 1 [DNMT1] (A) and DNA Methyltransferase 3 A [DNMT3a] (B) expression in the hippocampus of rats submitted to different housing [accompanied or isolated] and stress [submitted or not submitted to chronic unpredictable stress (CUS)] conditions. Samples were normalized to Beta-actin gene expression and ran in duplicate. Statistical analysis was performed using 2-way ANOVA with housing conditions and stress as fixed factors. Interactions were confirmed with simple effect analyses. Sample size *per* group for DNMT1 analysis: accompanied without CUS, n = 4; accompanied with CUS, n = 4; isolated without CUS, n = 5; isolated with CUS, n = 3. Sample size *per* group for DNMT3a analysis: accompanied without CUS, n = 3; accompanied with CUS, n = 6; isolated without CUS, n = 5; isolated with CUS, n = 3. Data are expressed as mean ± standard deviation. *p = 0.001 in comparison to the accompanied subgroups, indicating a significant housing effect; **p = 0.002 in comparison to accompanied-only and isolated-only subgroups, indicating a significant effect of stress; *** p < 0.001 in comparison to all other groups, indicating an interaction of housing condition and stress.

these studies, which also showed increased DNMT3a expression, no significant alterations in DNMT3a mRNA levels were seen in middle-aged rats submitted to social isolation.

The middle-aged animals of this study were also sensible to the CUS protocol, showing decreased H3K9 acetylation and increased H3K27methylation in isolated as well as accompanied animals. Moreover, the CUS protocol exacerbated the effects of social isolation on DNMT1 and HDAC5 expression. Although these epigenetic effects of CUS were accompanied by weight loss and anxiety-like behavior, as discussed before, no significant alterations were seen on BDNF gene expression and performance on the memory task, suggesting that the CUS protocol was less severe than the social isolation protocol. These findings agree with a former study from our research group, in which the CUS protocol also induced decreased H3K9 acetylation and increased HDAC5 gene expression in young adult rats in the absence of BDNF protein and memory alterations[16]. Until now no other studies have evaluated the effects of CUS on global H3K27me levels. However, increased H3K27me3 at the p11 promoter was observed in mice submitted to CUS in adulthood [58]. Expression of p11, also called S100A10, plays a critical role in depression-like behaviors and responses to antidepressant drugs [59], besides mediating the antidepressant-like effects of BDNF [60].

As mentioned before, the effects of CUS on HDAC5 gene expression were seen only in isolated animals, whereas the effects of CUS on DNMT1 gene expression were more pronounced in isolated than accompanied animals, suggesting that social support prevented some of the adverse effects of CUS. A former study of our research group had already unraveled the effects of social support on protecting from the negative outcomes of the CUS protocol on HDAC5 mRNA levels in young adult animals [16]. In this study, we extend the findings on epigenetic mechanisms sensible to social buffering to the expression of the DNMT1 gene expression. There are surprisingly few studies on the mechanisms through which social support operates. Affiliative behavior, group cohesion and liking are natural responses seen in humans exposed to stress or anticipating stressful events [51–63], and the effects of social buffering are far-reaching, improving health outcomes in various diseases, and even supporting increasing longevity [15]. Experimental and therapeutic drugs capable to inhibit HDAC5 and DNMT1 effects can revert disturbances associated with different psychiatric disorders [64–68]. Our results suggest that social buffering could act on some of the mechanisms targeted by these pharmacological interventions, warranting future studies on the effects of social support on epigenetic mechanisms.

Corticosterone levels in hair were used in this study as a biomarker of the physiological impact of social isolation and CUS on the animals. Differently from corticosterone levels in blood samples, which reflect transient changes in the HPA axis, hair corticosterone reflects the total retrospective activity of the HPA axis over the preceding weeks, being more adequate to evaluate the effects of chronic stress. Literature shows that the direction and amount of corticosterone alterations in response to stress can be very variable, depending on the type, chronicity, and timing of the stress protocol, as well as the age and strain of animals [69, 70]. In this study we observed that corticosterone levels decreased in animals submitted to isolation, reaching the lowest levels in isolated+CUS rats. Other studies evaluating plasma corticosterone in adult rats and mice also observed decreased corticosterone levels in isolated animals [71,72]. Moreover, the additional decrease of the corticosterone levels in isolated+CUS animals is also in agreement with former studies that associated these two types of stress [73,74]. Although the mechanisms that cause this decrease in glucocorticoids are not fully understood, they probably involve an enhanced negative feedback sensitivity to glucocorticoids in the hypothalamus, as well as a decrease in the responsiveness of adrenal glands to the adrenocorticotropic hormone (ACTH) [75–77]. Even though glucocorticoids can affect epigenetic mechanisms through their glucocorticoid receptors, e.g. increasing the expression of histone deacetylases e DNA methyltransferases [57,78], it is important to consider that the alterations in BDNF levels, memory and epigenetic mechanisms seen in this study could be the result of regulatory mechanisms independent of corticosterone, since the HPA axis showed a blunted activity in the isolated and isolated+CUS animals.

It is important to have in mind that mRNA expression is useful, but certainly far from perfect, in predicting protein levels, since it takes several steps to get from gene to protein. Thus, the analysis of protein levels of BDNF, HDAC5, DNMT1, and DNMT3a would be more accommodating for drawing stronger conclusions about the effects of social isolation and the CUS protocol on these variables. Future studies should address this issue. Moreover, the results of this study cannot be generalized to females, especially considering the literature on sex differences in the effects of social isolation, including the response to stress and subsequent HPA axis adaptations [79].

In conclusion, social isolation in middle age showed broader effects than chronic unpredictable stress on behavioral and epigenetic alterations potentially associated with decreased BDNF expression. Moreover, some of the negative outcomes induced by social isolation were exacerbated by stress, namely HDAC5 and DNMT1 expression, which showed the highest mRNA levels in the isolated+CUS group. Accompanied animals, on the other hand, promoted social buffering of the stress effects on HPA axis functioning, HDAC5 and DNMT1 gene expressions. The present results expand the knowledge of the effects of social isolation and social support on epigenetic mechanisms in middle age, a developmental time window in which epigenetics of neuroplasticity and memory mechanisms have been scarcely explored. In

humans, middle age is a time when key risks for social isolation and loneliness accumulate (such as retirement, children leaving the family home, divorce and bereavement), predisposing to negative health outcomes believed to have significant impacts on the aging organism [11, 80]. On the other hand, it is known that as tighter someone is embedded in a network of friends, the less likely they will become ill and the longer they will live [12]. Thus, future studies should focus on the effects of social isolation and social support on the epigenetic control of specific genes involved in psychiatric and neurodegenerative disorders in middle-aged and older animals.

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CRediT authorship contribution statement

Juliano Viana Borges: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Vivian Naziaseno Pires**: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Betânia Souza de Freitas**: Investigation, Formal analysis. **Gabriel Rübensam**: Investigation, Formal analysis. **Vitória Corrêa Vieira**: Investigation, Visualization. **Cristophod de Souza dos Santos**: Investigation. **Nadja Schröder**: Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Elke Bromberg**: Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. All authors read and approved the final manuscript.

Ethics approval

All experimental procedures were approved by the Institutional Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS/SIPESQ # 7142).

Declaration of Competing Interest

All the other authors have no conflict of interest to disclose.

Data availability

Data will be made available on request.

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