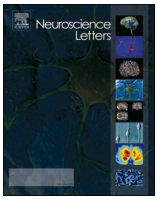
Neuroscience Letters 820 (2024) 137589

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet



Acute restraint stress regulates brain DNMT3a and promotes defensive behaviors in male rats

Richard L. Spinieli [a,*], Rafael Cazuza [a], Amanda J. Sales [b], Ruither Carolino [c], Janete A. Franci [c], Maral Tajerian [d,e], Christie R.A. Leite-Panissi [a,*]

[a] Department of Psychology, School of Philosophy, Science and Literature of Ribeirão Preto of the University of São Paulo, Brazil
[b] Department of Pharmacology, Medical School of Ribeirão Preto, University of São Paulo, São Paulo, Brazil
[c] Department of Basic and Oral Biology, Dental School of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil
[d] Department of Biology, Queens College, City University of New York, Flushing, NY, United States
[e] The Graduate Center, City University of New York, New York, NY, United States

ARTICLE INFO

Keywords:
Acute restraint stress
DNA methylation
Prefrontal cortex
Anxiety-like behaviors

ABSTRACT

Depending on its duration and severity, stress may contribute to neuropsychiatric diseases such as depression and anxiety. Studies have shown that stress impacts the hypothalamic–pituitary–adrenal (HPA) axis, but its downstream molecular, behavioral, and nociceptive effects remain unclear. We hypothesized that a 2-hour single exposure to acute restraint stress (ARS) activates the HPA axis and changes DNA methylation, a molecular mechanism involved in the machinery of stress regulation. We further hypothesized that ARS induces anxiety-like and risk assessment behavior and alters nociceptive responses in the rat. We employed biochemical (radioimmunoassay for corticosterone; global DNA methylation by enzyme immunoassay and western blot for DNMT3a expression in the amygdala, ventral hippocampus, and prefrontal cortex) and behavioral (elevated plus maze and dark-light box for anxiety and hot plate test for nociception) tests in adult male Wistar rats exposed to ARS or handling (control). All analyses were performed 24 h after ARS or handling. We found that ARS increased corticosterone levels in the blood, increased the expression of DNMT3a in the prefrontal cortex, promoted anxiety-like and risk assessment behaviors in the elevated plus maze, and increased the nociceptive threshold observed in the hot plate test. Our findings suggest that ARS might be a helpful rat model for studying acute stress and its effects on physiology, epigenetic machinery, and behavior.

1. Introduction

Exposure to stress is an unavoidable life event. When an organism is confronted with a stressful situation, the hypothalamic–pituitary–adrenal (HPA) axis is activated and, subsequently, glucocorticoids are released into the bloodstream by the adrenal glands where they circulate, cross the blood-brain barrier, and ultimately influence the brain by binding to mineralocorticoid and glucocorticoid receptors located in the amygdala, hippocampus, and prefrontal cortex [1–3].

A single exposure to traumatic stressors may exaggerate the response to a future stressful event. For example, an acute, single exposure to acute restraint stress (ARS) in the rat induced anxiety-like behaviors manifested by reduced percentage of the number of entries and/or time spent in the open arms of the elevated plus maze [4–6]. Depending on the intensity and frequency of stress, the regulatory function of the HPA axis can be imbalanced and consequently trigger molecular and behavioral changes [7–9], potentially leading to the development of neuropsychopathological conditions such as depression and anxiety [10], and even post-traumatic stress disorders (PTSD) [11].

DNA methylation, a covalent reaction often at the 5′ position in cytosine-guanine dinucleotides, has been proposed as a molecular mechanism of how stress changes gene expression in the brain and how it can be associated with behavioral abnormalities [12]. DNA methylation is mainly regulated by three enzymes: DNMT1, DNMT3a and DNMT3b, where DNMT3a is reported to regulate neural function. Rats that experienced ARS for 60 min had elevated corticosterone levels in the blood, reduced global DNA methylation in the prefrontal cortex and

* Corresponding authors at: Department of Neurology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215, USA (R.L. Spinieli); Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto Universidade de São Paulo, Ribeirão Preto, São Paulo 14040-901, Brazil (C.R.A. Leite-Panissi).
E-mail addresses: rspiniel@bidmc.harvard.edu (R.L. Spinieli), christie@usp.br (C.R.A. Leite-Panissi).

https://doi.org/10.1016/j.neulet.2023.137589
Received 25 September 2023; Received in revised form 16 November 2023; Accepted 6 December 2023
Available online 14 December 2023

hippocampus, and concomitant reduced levels of the DNMT1 in the periaqueductal grey matter [13]. Additionally, mice submitted to social defeat showed diminished DNA methylation and DNMT3a gene expression in the prefrontal cortex [14]. To our knowledge, the effects of ARS on DNMT3a regulation are not well described.

Last, our group and others have shown that anti-nociception might accompany fear and anxiety in guinea pigs and mice, and the neural mechanisms of fear/anxiety and pain might overlap [12,15–17]. Further, Wang and colleagues (2017) reported that mice submitted to neuropathic pain and that were more susceptible to display increased anxiety-like behaviors presented with decreased expression of DNMT3a in the central nucleus of amygdala [18]. Little attention has been paid to the effects of ARS in the nociceptive response.

Considering the above, we hypothesized that the ARS activates the HPA axis, alters brain DNA methylation via DNMT3a, and augments anxiety-like behaviors with concomitant risk assessment behavior in the EPM. Additionally, we hypothesized that ARS changes nociceptive behavior. To address these hypotheses, we used a radioimmunoassay (RIA) to detect corticosterone in the blood; an enzyme immunoassay for global DNA methylation and western blot to measure the expression of DNMT3a in the amygdala, ventral hippocampus, and prefrontal cortex to screen the DNA methylation in brain areas involved in the emotional regulation; EPM and dark-light box (DLB) to assess anxiety-like behaviors; and hot plate test to measure the nociceptive behavior in rats exposed to ARS or control.

Our findings suggest that a single exposure to ARS is accompanied by HPA axis activation, DNMT3a upregulation in the prefrontal cortex, increased anxiety-like behaviors in the EPM, and decreased sensitivity to painful heat. We propose the rat ARS model is a suitable model for the study of stress and its molecular and behavioral correlates.

2. Materials and methods

Ethical approval

All animal experiments were approved by our local Animal Care and Use Committee of the University of São Paulo-Brazil, Campus of Ribeirão Preto (Protocol number 2014.1.508.58.1) and followed the Conselho Nacional de Controle de Experimentação Animal – Ministério da Ciência e Tecnologia (Brazil). The number of rats used in each protocol was chosen empirically and all efforts were made to minimize animal suffering during the experiments. We followed the ARRIVE (Animal Research: Reporting of *in Vivo* Experiments) guidelines in this study.

2.1. Animals

Adult male Wistar rats were provided by our local animal vivarium (*Biotério Central do Campus Administrativo da USP de Ribeirão Preto*, The original breeding pairs were purchased from Charles River Laboratories (Massachusetts, USA). Rats were used for the experiments when they reached 8–12 weeks of age following postnatal day 0. A total of 3–4 rats were kept in Plexiglas boxes (35×19×25 cm) with shavings in a room with controlled temperature (24C ± 1C) and standard light cycle (lights on at 7:00 AM, lights off at 7:00 PM). The rats had water and chow food *ad libitum*. All experiments were performed between 11 am and 1 pm to avoid major circadian fluctuations. Experiments performed in the EPM and DLB were vi. deotaped for subsequent analysis. A total of 57 animals were used for experiments reported in this manuscript.

2.2. Acute restraint stress protocol

Animals were exposed either to handling in the vivarium or 2-hr acute stress in a separate room. Rats were individually placed and held in a metallic tube (6.3×19.3 cm) for 2 h in a single physical restraint episode. The metallic apparatus restrained body movements but kept respiratory movements intact. Twenty-four hours following the completion of the 2 h restraint episode or handling control, animals were exposed to behavioral testing, or were euthanized through decapitation for harvesting of the brain and collection of the blood. The animals did not have access to water or food during the stress period. This stress model has been previously described [4].

2.3. Experimental groups

The following experiments were conducted in independent cohorts of animals, i.e. rats were not re-used in multiple experiments.

Cohort 1: rats were submitted to the ARS protocol (n = 6) or ~5 min handling control (n = 5) and were decapitated for brain harvest and blood collection 24 h later to be used for measurement of blood corticosterone, and analysis of global DNA methylation and expression of the DNMT3a in the amygdala, ventral hippocampus, and prefrontal cortex.

Cohort 2: rats were submitted to the ARS protocol (n = 8) or handling control (n = 7) and tested in the EPM 24 h later.

Cohort 3: rats were submitted to the ARS protocol (n = 7) or handling control (n = 8) and were exposed to the DLB 24 h later.

Cohort 4: all 16 rats had their baseline nociceptive threshold measured in the hot plate test (50±1 °C). Twenty-four hours later were submitted to either the ARS protocol (n = 8) or handling control (n = 8) and had their withdrawal latency measured again in the hot plate test 24 h later.

A priori exclusion criteria included animals with signs of illness or absence of jumps, paw lickings, or withdrawal in the hot plate test in 20 s after been placed in the apparatus. No animals were excluded from the study. For didactic reasons, the groups and protocols are depicted in Fig. 1.

2.4. Corticosterone

To determine whether our ARS model activated the HPA axis, we used an RIA to quantify corticosterone in the blood. Animals were exposed either to handling in the vivarium or 2-hr acute stress in a separate room the day prior to blood draw. On the day of the blood draw, animals were moved from the vivarium to the laboratory, where they were left to acclimate 2 h. The order of decapitation for individual animals was randomized to avoid any inter-group biases. Decapitation and blood collection was carried out in a separate room to minimize stress due to odorant cues. Trunk blood (~5 mL) of the animals of from experiment 1 was collected in heparinized tubes and immediately centrifuged (4000 rpm, 20 min, 4 °C). After centrifugation, the plasma in the liquid phase in the heparinized tubes (~2 mL) was collected, transferred to another labeled tube, and kept at −20 °C until the RIA. The RIA followed (with modifications) the manufacturer's instructions for the Corticosterone Antiserum Developed in rabbit (C8784-100TST, Sigma). The corticosterone present in the plasma was extracted with ethanol. We added 500 µL of 100 % ethanol for each 25 µL of plasma. The mixture of ethanol and plasma was vigorously agitated for 15 s, centrifuged (2.500g, 15 min, 4 °C), and the supernatant was collected and left to dry completely in room temperature. All samples were resuspended in 0.1 % gelatin phosphate buffer (pH 7.4) and were maintained at −20° until the analysis. We incubated the extracted samples or the unlabeled corticosterone with the antiserum and tritiated hormone {[1,2,6,7 – 3H (N)]-corticosterone, Perkin Elmer} at 4 °C for 24 h. Each tube was added with dextran-coated charcoal (0.5 % activated charcoal, 0.5 % dextran in phosphate buffer, pH 7.4) to separate free/bound tritiated corticosterone. The tubes were then vortexed for 1 min, incubated at 4 °C for 15 min, and centrifuged (2,000g for 15 min at 4 °C). The antiserum-bounded 3H-corticosterone in each supernatant was mixed with the scintillation liquid, and the radioactivity of each tube was measured using the beta Trilux counter (Perkin Elmer). We set up a standard curve with known concentrations of unlabeled corticosterone and it was used to estimate the corticosterone levels in the unknown samples. The data generated were analyzed by Multicalc

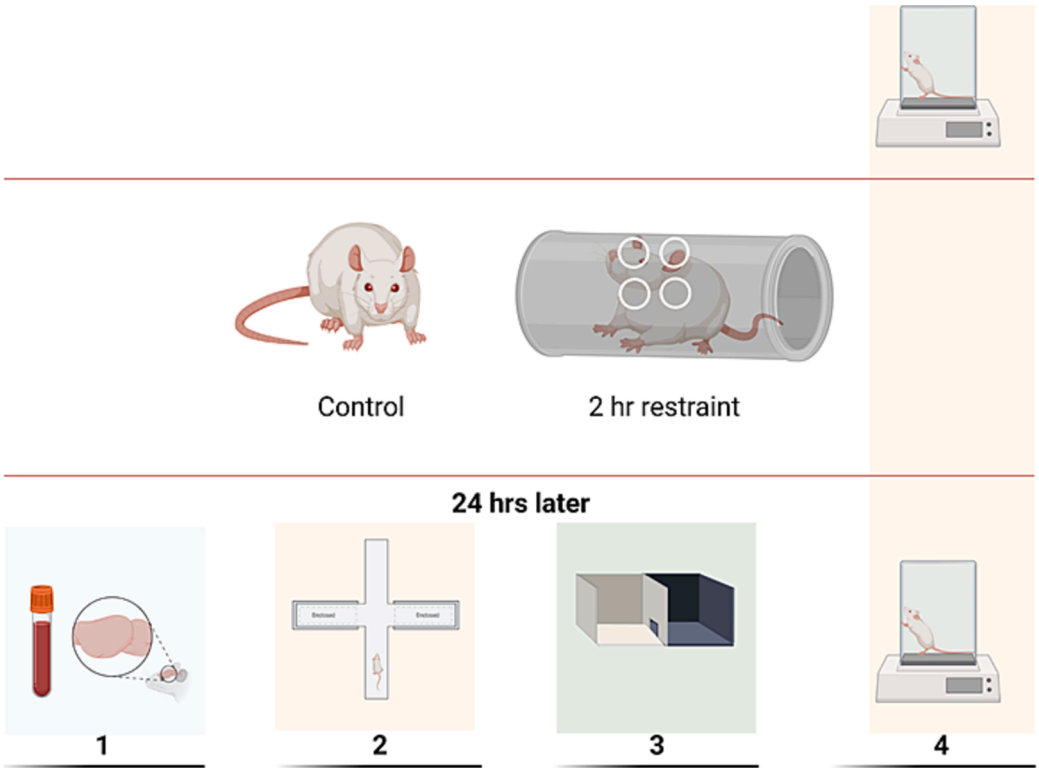


Fig. 1. Experimental protocol. Image illustrated using Biorender© software.

software. The lowest limit for detection for corticosterone was 0.05 ng/mL. The intra-assay coefficient of variation was 9 %. All samples were measured in duplicate in the same assay to avoid inter-assay error. This RIA protocol has been previously published [19].

2.5. DNA methylation

The following areas were dissected: amygdala (Bregma −1.92 mm to −3.12), ventral hippocampus (Bregma −1.92 mm to −1.44 mm), and prefrontal cortex (Bregma −4.68 mm to −2.76 mm) using mice from cohort 1 (Fig. 1). The dissected structures were split in two approximately equal amounts of tissue and put in two labeled tubes, where the tissue in one tube was assigned for global DNA methylation analysis and the other tube for western blot of the DNMT3a protein expression.

2.5.1. Global DNA methylation

Genomic DNA of the above-mentioned brain areas was extracted using the SDS/proteinase K method. The tissue was put in 550 µL lysis buffer (NaCl 0.1 M, Tris 50 mM, EDTA 50 mM, final pH 8), with the addition of 27.5 µL of 20 % SDS, and 20 µL of proteinase K (#P2308, Sigma). Tissue was then incubated for 2 h at 60° C for total tissue digestion. After total digestion of the tissue, 600 µL of 4 M sodium chloride was added to the lysis buffer followed by 15 s of vortexing and centrifuging (14,000 RPM, room temperature, 30 min). We then added an aliquot (1 mL) of the mixture lysis buffer + NaCl containing the digested tissue of the samples and placed in another labeled tube and two equal volumes of 100 % ethanol. The samples were kept in −20 °C for 16–18 h and centrifuged (14,000 RPM, 4 °C, 30 min) the following day. After discarding the supernatant, the DNA was washed (by centrifuging 14,000 RPM 4 °C, 5 min) three times with 700 µL of 70 % ethanol and left at room temperature to allow complete drying. We resuspended the DNA with 50 µL of ultrapure molecular grade water. We quantified the DNA concentration by spectrophotometry (Nanodrop 2000c, Thermo Fisher Scientific). Following genomic DNA extraction, we digested the DNA with Nuclease P1 (#P2640, Sigma, 2 U/mg of DNA, 4 h, 65 °C in acetate buffer 20 mM pH 5.3) and alkaline phosphatase enzymes (#N8630, Sigma, 1 U/mg of DNA, 2 h, 65 °C in Tris-HCl buffer 20 mM pH 7.5). The digested DNA was precipitated with 100 % ethanol, and NaCl 5 M at −20 °C for at least 18 h. The samples were then centrifuged at 15,000g for 15 min. The pellet that remained in the bottom of the tubes was re-suspended in ultrapure water and the global levels of DNA methylation were analyzed using a DNA Methylation EIA kit (#589324, Cayman Chemicals). A SpectraMax 190 plate reader (Version 6.2.1, Molecular Devices) measured the absorbance of the samples. We generated the standard concentration curve from different concentrations of the purified 5-methyl-2′-deoxycytidine (provided by the kit). The concentrations (ng/mL) were calculated based on the standard curve equation. The results were expressed as methylated cytidine/total DNA (pg/ng). All samples were measured in the same assay.

2.5.2. Western blot

The tubes with part of the tissue assigned for western blot analysis of the protein DNMT3a were homogenized in RIPA buffer (Sigma-Aldrich # R0278) containing protease inhibitors (10 %, dilution 1:10, Sigma-Aldrich) and 0.5 % phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich). After agitation in a cold room (~4° C) for 2 h, the samples were centrifuged (3500 rpm, 20 min, 4 °C) to collect the supernatant. After protein quantification, 60 µg of protein of each sample was diluted in a 2x Laemmli buffer (1:1; Sigma-Aldrich, S3401). The separation of proteins was done by electrophoresis (125 V, 90 min) in 12 % polyacrylamide gels. After the electrophoresis, the proteins were transferred to a nitrocellulose membrane (0,45 µm pore size; Amersham) in a tank blotting system (125 V, 90 min) semi-immersed in a transfer buffer containing 20 % methanol. One well of each gel was a marker of molecular weight (10 kDa to 250 kDa; Prism Ultra Protein Ladder, ABCAM ab116028) for assessment of the protein transfer. The membranes were subsequently blocked with 5 % BSA for 60 min in slow agitation in room temperature to block non-specific binding, incubated overnight (~14 h) in 4° C in a PBS-T BSA 1 % buffer (phosphate buffered saline + 1 %

tween 20 + 1 % BSA) containing anti- DNMT3a (rabbit, 1:1000, H-295 sc-20703, Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cristalli et al 2022) and β-actin (mouse, 1:1000, C4 sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. The membranes were subsequently incubated with PBS-T containing BSA 1 % and secondary HRP (horseradish peroxidase)-conjugated antibodies (anti-rabbit, 1:10000; Abcam, ab6721; and anti-mouse 1:10000; Merck, 12–349) for 2 h in room temperature. We used a chemiluminescent kit (SuperSignal Chemiluminescent Substrates, Thermo Fisher Scientific) to detect the immunolabeled bands in the membranes, and the ImageLab 5.2.1 (BioRad) software for densitometric analysis. The ratio of band optical density of DNMT3 to β-actin (housekeeping protein) was calculated.

2.6. Behavioral and nociceptive tests

2.6.1. Elevated plus maze

The EPM used in our study has been previously described by our group [20]. The apparatus consists of two equal (50×10 cm) open and enclosed arms, 50 cm above the ground. The enclosed arms have 40 cm walls. The light was positioned in a manner to ensure that there were no shadows present on the open arms. The brightness in the maze was measured at 50 lx. We considered the number of times the animals entered (with four paws) the open and enclosed arms, and the percentage of time spent in the open arms (raw time spent in the open arms/ total time of the test X 100). We also counted the number of end-arm explorations (when the animal walks all the way to the end of the open arm) and instances of head dipping (exploratory movement of the head of the animal in the lateral or end of the open arms; the head projects below the level of the maze floor). Finally, we analyzed behaviors such as stretched-attend postures (the animal displays a vigorous stretch of the forepaws while the hindpaws are stationary, and then return to its previous position), peeping out (a stretch of the head and shoulder towards the center of the maze when the animal is still in the enclosed arm), and flat-back approach (the animal walks fully stretched but moves forward cautiously). The number of stretched-attend postures, peeping out, and flat-back approaches were summed and reported as “risk assessment behaviors” [21,22] throughout the manuscript.

2.6.2. Dark-light box

The DLB consists of a chamber with two equal compartments (50 cm length × 80 cm width × 60 cm height) with a small opening (15 cm height × 10 cm width) to allow the animal to cross between the compartments. Half of the apparatus is dark (with a dark lid to prevent illumination) and the other half is white (with an acrylic lid that allowed light inside the box). There was no additional light used to illuminate the light side. Instead, the luminosity of the room (50 lx) illuminated the light side (the walls of the light side are translucent). The dark side was covered and completely dark. The animals were placed in the DLB in the white compartment with their faces towards the small opening described above. They were then allowed to explore the apparatus for 5 min. The number of crossings between compartments, time spent in the white compartment, and first latency to cross from the white to the dark compartments were considered [23,24]. Upon the completion of the 5 min of free exploration, the chamber was cleaned with alcohol 70 % and another animal was placed in the box after 5 min.

2.6.3. Hot plate test

Rats, previously submitted to the ARS or control, were placed in the center of a hot aluminum plate (50±1 °C; Bonther, Brazil) for no more than 40 s (cut-off time) to prevent tissue damage [25,26]. We used a removable acrylic enclosure (28 cm height × 26 cm length × 18 cm width) to prevent the animals from escaping the experimental apparatus before the cut-off time. The nociceptive threshold was determined by the latency for the animals to exhibit behaviors such as paw licking (forepaw and hindpaw), withdrawal, or jumps. The animals were placed in the hot plate during 60 min in non-fixed intervals (at 0 min, 5 min, 15 min, 23 min, 35 min, 50 min, 60 min). The basal latencies were taken 3 times with no fixed intervals (BL 1, BL 2, BL 3). Twenty four hours after the completion of the experiment, we 1) inspected the paws of the animals and looked for redness and swelling; 2) we also inspected for motor impairments such as paw withdrawal to ambulation and excessive licking. These were the additional exclusion criteria for this experiment. No animals were excluded from the analysis. The apparatus was cleaned with 70 % alcohol between animals, and we allowed 5 min for alcohol evaporation.

2.7. Statistical analysis

Two-tailed, unpaired Student t-tests were used to analyze the corticosterone levels, brain DNA methylation (i.e., global DNA methylation and DNMT3a expression in the amygdala, ventral hippocampus, and prefrontal cortex), and behavior in the EPM and DLB. Welch’s correction was applied when the assumption of equal variances was not met. ROUT outlier test was performed with outlier values circled in red in the relevant figures. The exclusion of outlier values did not change the interpretation of result significance.

The nociceptive threshold in the hot plate test was analyzed using two-way ANOVA for repeated measures followed by Sidak’s post hoc test when appropriate. Time (time point where the animal was tested in the hot plate), and treatment (control or ARS) were considered as factors. Results were considered significant is $p < 0.05$. Our data are reported as mean ± standard error of the mean (SEM) and were analyzed by GraphPad Prism® for Windows, version 7.0 (GraphPad Software, USA).

3. Results

3.1. ARS activates the HPA axis and increases the expression of DNMT3a in the prefrontal cortex

The first step of our study was to investigate whether ARS changes corticosterone concentrations in the plasma, an indicator of stress in rodents. We observed that ARS increased corticosterone levels in the plasma ($t(5.4) = 2.716$, $p = 0.04$, Welch’s correction, Fig. 2a) when compared to the control group. We and others have reported that the activation of the HPA axis and global DNA methylation may be paralleled [12]. With this in mind, we also assessed the global DNA methylation levels in the amygdala, ventral hippocampus, and prefrontal cortex, but we did not observe significant changes in these brain areas (amygdala $t(4.5) = 1.205$, $p = 0.26$, Fig. 2b; ventral hippocampus $t(5.4) = 0.808$, $p = 0.44$, Fig. 2c; prefrontal cortex $t(5.4) = 1.646$, $p = 0.13$, Fig. 2d). As a last step of our molecular analysis, we quantified the expression of the DNMT3a, a *de novo* methyltransferase protein. We found that ARS increased the expression of DNMT3a in the prefrontal cortex ($t(5.3) = 3.125$, $p = 0.01$, Fig. 2g) compared to the control group, but no significant differences were observed between ARS and control groups in the amygdala ($t(2.2) = 0.3673$, $p = 0.73$, Fig. 2e*)* and ventral hippocampus ($t(4.4) = 0.4129$, $p = 0.69$, Fig. 2f).

3.2. ARS induces anxiety-like and risk assessment behaviors in the elevated plus maze

It has been reported that ARS induced anxiety-like behaviors in rats [4]. We confirmed these findings by observing that ARS led to decreased percentage of the time spent in the open arms of the EPM ($t(7.6) = 3.109$, $p = 0.0083$, Fig. 3a) and reduced number of entries in the open arms ($t(6.7) = 5.741$, $p < 0.0001$, Fig. 3b) when compared to the control group. ARS did not induce a significant effect on the number of entries in the enclosed arms of the EPM when compared to the control group ($t(6.7) = 1.368$, $p = 0.19$, Fig. 3c).

Considering that ARS induced signs of stress confirmed by the

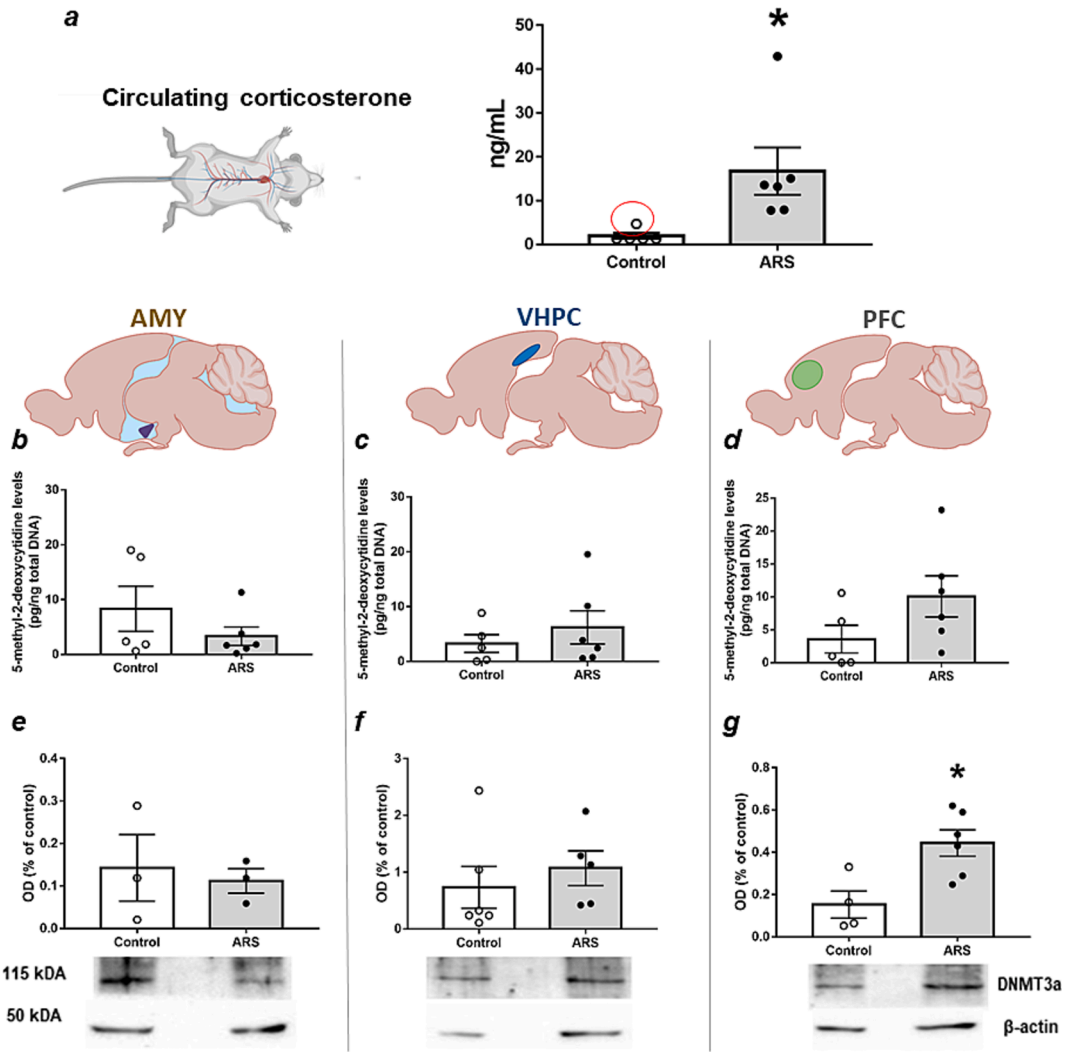


Fig. 2. A single Acute Restraint Stress exposure activates the HPA axis, does not change global DNA methylation but increases the expression of DNMT3a in the prefrontal cortex. Plasma levels of corticosterone measured by radioimmunoassay 24 h after a single episode (2 h) of physical restraint in the rat (a). The empty dots (n = 5) represent the control group and the black dots (n = 6) represent the group submitted to the ARS. Global DNA methylation levels in the amygdala (b), ventral hippocampus (c) and prefrontal cortex (*d*) after exposure to ARS (black dots, n = 6) or not (empty dots, control, n = 5) are presented. Each sample result is expressed as 5-methyl-2-deoxycidine/total DNA (pg/ng). The quantification of DNMT3a levels (relative to β-actin) in the amygdala (e, empty dots, n = 3; black dots, n = 3), ventral hippocampus (f, empty dots, control = 5; black dots, ARS n = 5) and prefrontal cortex (g, empty dots, control n = 4; black dots, ARS n = 6) are presented in the lower side along with their respective representative bands. The upper bands of the representative blots represent the protein DNMT3a, and the lower bands represent the housekeeping control (β-actin), and their respective molecular weight is indicated. Results are expressed as mean ± the standard error of the mean (SEM) and were analyzed by Student *t* test. * $p < 0.05$. OD: optical density. Red circle indicates an outlier value determined by ROUT test. The inclusion or exclusion of the outlier data point did not change the significance of the results in panel a.

observed elevated corticosterone in the blood and augmented anxiety-like behaviors in the EPM, we found it was reasonable to also investigate the "complementary ethological categories" in the EPM described by Albrechet-Souza and colleagues [21]. Through factorial analyses of behaviors of rats in the EPM, behaviors such as head dipping, end-arm exploration, and risk assessment were classified as additional measures that might represent signs of anxiety-like behaviors (i.e., head dipping and end-arm exploration) and elaboration of anti-predatory strategies (i.e., risk assessment behaviors). Our findings showed that ARS promoted reduction in the number of end-arm explorations (t (7.6) = 3.427, p = 0.0045, Fig. 3d) and head dipping (t (6.7) = 6.066, p < 0.0001, Fig. 3e), and increased risk assessment behaviors (t (7.6) = 2.816, p = 0.01, Fig. 3f).

No significant changes between the ARS and control groups were observed in the DLB regarding total crossings (t (7.6) = 0.8588, p = 0.41, Fig. 3g), time in the white compartment (t (7.6) = 1.014, p = 0.33, Fig. 3h), and first latency (t (7.6) = 1.858, p = 0.11, Fig. 3i).

3.3. ARS promotes antinociception in the hot plate test

Previous reports have shown that fear induces anti-nociception in rodents [15–17]. Taking into account that ARS activates the HPA axis and increases defensive behaviors in the EPM, we were interested to also assess nociception in independent groups of rats submitted (or not) to ARS. Two-way ANOVA for repeated measures evidenced significant effects of time [F (9.126) = 5.465, p < 0.0001; Fig. 4)] and treatment × time interaction [F (9. 126) = 2.309, p = 0.02; Fig. 4), and did not show a significant effect of treatment [F (1.14) = 3.76, p = 0.07, Fig. 4). Neither time nor treatment were significant factors for baseline measurements (Time: p = 0.19, Treatment: p = 0.46). A post hoc analysis (Sidak's post hoc test) revealed a higher nociceptive threshold in the ARS group at the 15 min time point only (p = 0.0067, Fig. 4). Fig. 5.

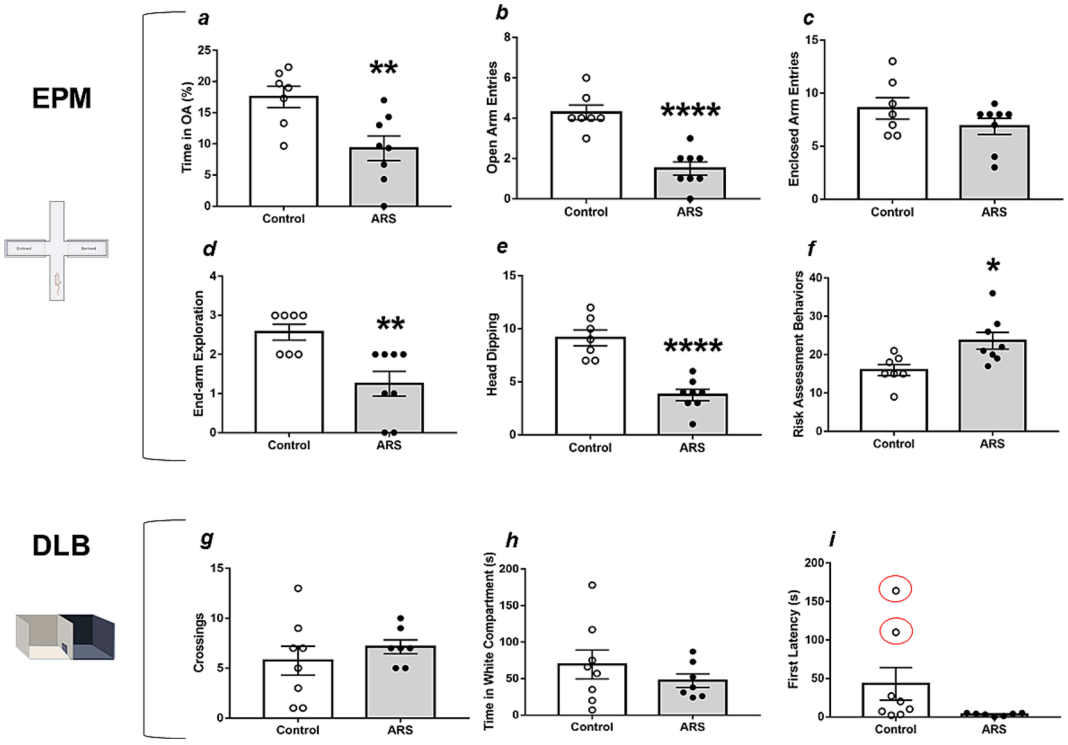


Fig. 3. Acute Restraint Stress induces anxiety-like and risk assessment behaviors in the elevated plus maze. In the EPM, we measured the percentage of time spent in the open arms (*a*), frequency of entries in the open arms (*b*), number of entries in the enclosed arms (*c*), end-arm exploration (*d*), head dipping (*e*), and risk assessment behaviors (*f*) in animals exposed to ARS (black dots, n = 8) or control (handling) (empty dots, n = 7). For the DLB, we measured in ARS (black dots, n = 7) and control (empty dots, n = 8) animals, the number of crossings between the compartments (*g*), time in the white compartment (in seconds) (*h*), and first latency to enter the black compartment of the apparatus (in seconds) (*i*). Results are expressed as mean ± the standard error of the mean (SEM) and were analyzed by Student *t* test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. OA: open arms. Red circles indicate outlier values determined by ROUT test. The inclusion or exclusion of outliers did not change the significance of the results in panel i.

4. Discussion

The salient findings of the study are: 1) a single 2-hour exposure to ARS impacts the HPA axis by increasing corticosterone release in the blood; 2) ARS leads to increased protein levels of DNMT3a, a *de novo* methyltransferase, in the prefrontal cortex; 3) ARS is linked with augmented anxiety-like behaviors in the EPM; 4) ARS increases the nociceptive threshold in the hot plate test. Our investigation showed that ARS increases corticosterone release in the blood 24 h after the stress challenge, thereby corroborating previous findings where 2 h of immobilization increased basal levels of corticosterone and adrenocorticotropic hormone in the blood as well as anxiety-like behaviors at 1 and 7 days after stress exposure [27].

The mechanistic link between stress, corticosterone release, and changes in DNA methylation in the adult rat brain and behavior remains unclear [7]. There is evidence that stress, anxiety, and fearfulness that are often co-morbid with chronic pain are associated with changes in global DNA methylation [28], but the data lacks specificity in terms of cell type. For example, increased DNA methylation has been observed in the olfactory bulb of mice and is associated with an increased salience of unpleasant memories [29]. On the other hand, decreased global methylation levels were observed in the prefrontal cortex and amygdala of the mouse [30] and amygdala of the rat [12], changes which were linked to signs of pain-related anxiety. We did not observe alterations in the global DNA methylation levels in the amygdala, ventral hippocampus, or prefrontal cortex in rats submitted to ARS at the studied timepoint. Our DNA methylation results are in contrast with previous reports of reduced global DNA methylation in prefrontal cortex, hippocampus, and periaqueductal grey matter in rats that experienced ARS [13]. One explanation could be the different experimental timelines. While Rodrigues et al. [13] euthanized the animals and proceeded with tissue collection right after the stress exposure, we performed all our analyses (biochemical and behavioral) 24 h after the stressful exposure.

DNMT3a regulation in the prefrontal cortex has been involved in the behavioral manifestations of stress and helplessness in rodent models, albeit with inconsistent results. For example, learned helplessness in adult rats was shown to parallel increased DNMT3a levels, a phenomenon that was reversed/prevented with various antidepressant treatments [31,32]. Similarly, adolescent rats undergoing chronic unpredictable stress demonstrated DNMT3a downregulation and associated decrease in glutamatergic neurotransmission in the prefrontal cortex and DNMT inhibition was able to capitulate anxiety signs in unstressed animals [33]. On the other hand, our molecular results are in contrast to the findings in Elliot et al. [14], where they found reduced DNMT3a transcript abundance in socially defeated mice along with anxiety-like behaviors. One methodological difference between our studies is that we measured the protein expression, while they analyzed transcript abundance. Besides the fact that a social defeat model is a much more prolonged stimulus than a single 2-hour restraint, the correspondence of protein expression and transcript abundance is about 50 % [34–36]. Finally, while there is accumulating evidence for the involvement of DNMT3a in stress, the exact relationship between the two remains elusive. For example, in a model of early life stress, DNMT3a alterations were dependent on the region of brain studied as well as the age of the animal [37]. While these observations of DNMT3a abundance do not provide a thorough understanding of the precise mechanistic underpinnings of DNA methylation of behavior, they strongly support the concept of epigenetic plasticity that is present when organisms undergo significant environmental alterations.

Methyltransferases are responsible for catalyzing the addition of methylation across the genome. However, their abundance is not always reflected in changes of genomic methylation [38]. For example,

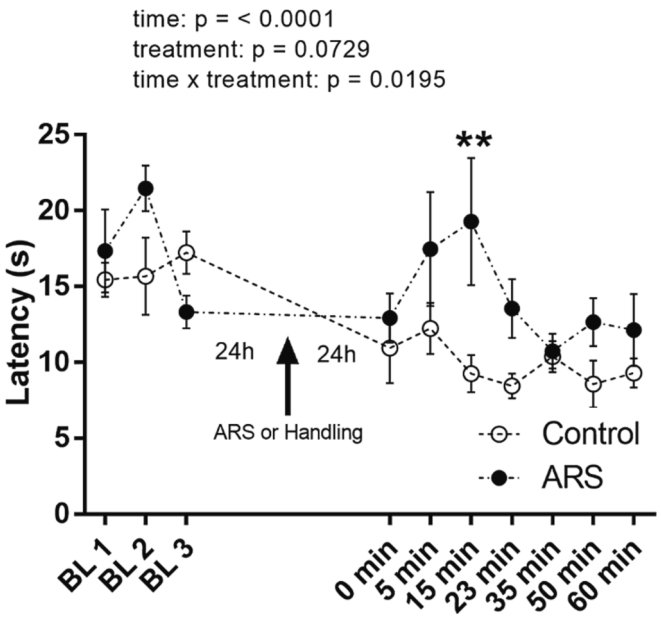


Fig. 4. Acute Restraint Stress promotes antinociception in the hot plate test. Twenty-four hours after baseline measurements of the nociceptive thresholds (BL 1, BL2, and BL3), animals were submitted to the ARS (black dots, n = 8) or to handling (empty dots, n = 8). Then, the same rats were tested again in the hot plate test 24 h for 7 times in non-fixed intervals (0 min, 5 min, 15 min, 23 min, 35 min, 50 min, 60 min) after the exposure to ARS or handling. The nociceptive threshold was determined by the latency (in seconds) the animals took to display jumps, paw lickings, or withdrawals. Effects of time, treatment, and time × treatment are indicated in the figure. Note that the animals of the ARS group had an increased nociceptive threshold at one time point (15 min) (two-way repeated measures ANOVA followed by Sidak's post-test for multiple comparisons, ** $p < 0.01$). Results are expressed as mean ± the standard error of the mean (SEM).

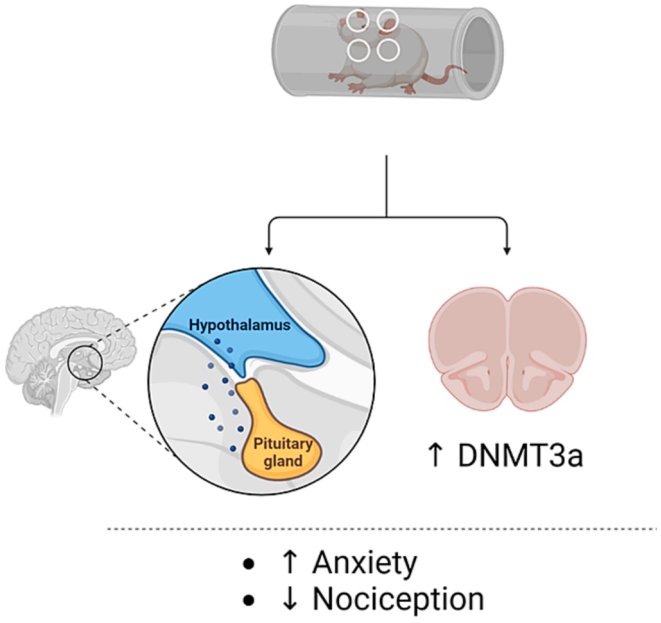


Fig. 5. Summary of findings. Image illustrated using Biorender© software.

methylation patterns can be set from differentiated cell lineages with genomic hypomethylation occurring passively through rounds of cell division. This can also be modulated by the presence or absence of DNA demethylating processes. In this case, the action methyltransferases (DNMTs) and DNA demethylation (TET + MBD4) maintain an equilibrium that can affect global patterns [39,40]. Even the function of DNA methyltransferases are subject to various post translational modifications that can modulate their activity. For example, phosphorylation of ser127/143 can affect DNMT1 stability reducing its ability to methylate DNA. In contrast, sumoylation of DNMT1 can also increase its ability to methylate DNA [41]. Therefore, the absence of DNMT3a changes in the amygdala and the ventral hippocampus in our model does not imply a diminished role for epigenetic regulation of the stress phenotype.

We replicated previous findings [4,5] by confirming that a single episode of ARS induced decreased time spent and entries in the open arms of the EPM 24 h after the exposure. The rationale for including the "complementary ethological measures" analysis in the EPM came from the idea that stress is usually accompanied by activation of the HPA axis and anxiodepressive phenotype, and that corticosterone prepares the organism for future challenges. The complementary measures in the EPM were suggested to represent an evaluation of threatening situations and determination of strategies to cope with that potential threat [21]. Also, in a pharmacological study, intracerebral administration of midazolam the inferior colliculus increased the frequency of end-arm exploration, head dipping, and risk assessment behaviors in the EPM [22]. As such, we found it reasonable to hypothesize that ARS could also impact the anti-predatory strategies. We confirmed our hypothesis by finding that ARS decreased the number of end-arm exploration and head dipping, and increased risk assessment behaviors. Besides expanding the behavioral characterization of the ARS model in the EPM, our data also might open a new avenue to a deeper investigation of corticosterone release and complementary measures in the EPM. Lastly, we did not see any effect of ARS observed in the DLB, suggesting that this apparatus may be insensitive to detect the behavioral effects of ARS. In a more general sense, and considering that we performed our analyses several hours after the physical restraint episode, we speculate that our ARS rat model might be developed into a model for PTSD, although it requires further testing and characterization/validation. The consolidation of neurophysiological and behavioral effects of a single exposure to stress in rodents have been subject of discussion as preclinical models of PTSD [27,42,43]. It is important to note the task-specific nature of anxiety-like behaviors in our study. Whereas we observed differences between the groups in the EPM assay both in terms of anxiety and risk-taking, no such differences were observed in the DLB test. This could be due to the fact that no behaviors were recorded in the dark chamber of the DLB, but could also reflect a broader assessment of behavioral tests commonly used to measure anxiety in rodents. While the different tests rely on different aspects of assay validity (predictive, face, convergent, etiological, construct, etc.), they also somewhat vary in their focus. When more than one test is used, it is therefore not uncommon to observe task-specific phenotypes, which can complicate the interpretation of results. It is our opinion that this is mainly an indication of the multidimensional nature of stress and its behavioral manifestations.

Several well-characterized models for the investigation of the interrelationship between stress and pain have been proposed. However, it remains unclear whether or not a single acute restraint stress exposure promotes analgesia/anti-nociception or hyperalgesia/allodynia. It has been proposed that pain perception may be inhibited during stress in favor of adaptive behaviors [44], and some studies may corroborate this hypothesis. For example, a single 6-hour episode of physical restraint promoted increased tail-flick latency in rats [45], and similarly, 6 min of exposure to the forced-swim test was associated with increased analgesic responses in the tail-flick test [46]. On the other hand, rats submitted to 1-hour restraint showed hyperalgesia, observed in the tail-flick test, 6 h after the stress exposure [47]. Finally, the systemic administration of a CRF antagonist was shown to reduce visceral pain in rats [48]. It is noteworthy that that habituation and learning can play an important role in the hot plate test, with rats exhibiting decreased latency with repeated exposure to the hot or room-temperature plate [49]. This decrease is present when testing repetition is daily but also when it is

weekly. Indeed, it appears that habituation is less likely to occur when tests are repeated during a single session [50]. Furthermore, our previous protocol used fixed intervals (~each 10 min), a common procedure that can nonetheless facilitate learning. We therefore modified the protocol to one that uses non-fixed intervals (even with one slightly shorter interval, e.g. 23 min instead of 25 min) so that we could prevent learning in our animals. We have shown in the present investigation that a single 2-hour physical restraint induces hypoalgesia in the hot plate test 24 h after the stressful event. We have also showed that ARS increased blood corticosterone 24 h after the restraint, thereby supporting the possibility that the augmented release of corticosterone might be associated with the antinociceptive effect of the ARS. Although these two events might be correlated, a causal link between the two is not well supported by the literature. For example, the elevated corticosterone levels did not change the antinociceptive response in mice exposed to an aversive, totally open elevated plus maze [51]. Our results further emphasize the diversity of biochemical and behavioral stress-related responses that may be dependent on multiple factors such as the animal species, type of stressor, study time-course, nociceptive measures, etc.

Limitations and conclusions.

The enzyme immunoassay used in our study only detects methylated cytosine at the global scale, which does not necessarily reflect changes in gene-specific methylation patterns. Also, we did not perform an assay for DNMT3a activity, which can show different results from enzyme abundance. Finally, we need to study both male and female rats since recent evidence shows sexual dimorphism of the behavioral, molecular, and neuro-immune correlates of acute stress [52–54], as well as the epigenetic regulation of early life stress [55].

The key findings of this study are that the ARS in the rat is a potent stressor that induces HPA axis activation, increases brain DNMT3a expression, and is associated with anxiety-like behaviors and antinociception in the hot-plate test. Our study provides data of a reliable stress model that might help to provide explanations of how acute stress might deregulate the epigenetic machinery, and how this can lead to neuropsychiatric diseases in clinical populations. This work paves the way for the understanding of the underlying mechanisms that regulate the interrelationship between stress, glucocorticoid release, DNA methylation, and defensive behaviors.

CRediT authorship contribution statement

Richard L. Spinieli: . **Rafael Cazuza:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Amanda J. Sales:** Formal analysis, Investigation, Writing – review & editing. **Ruither Carolino:** Formal analysis, Investigation, Writing – review & editing. **Janete A. Franci:** Investigation, Writing – review & editing. **Maral Tajerian:** Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Christie R.A. Leite-Panissi:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

We thank Patricia Basile and Flávia Salata for their technical assistance. We also thank Dr. Sâmia Joca for generously providing expert knowledge in addition to laboratory reagents and kits. We thank Dr. Chris Baines (University of Missouri) for his expert assessment of the western blots. Finally, we thank Dr. Phoebe Edwards and Sebastian Alvarado for the careful review of our manuscript.

Funding

This study was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES PDSE and Finance Code 001). CRA Leite-Panissi received research fellowship from CNPq (#304215/2016-3 and #309215/2019-1). M Tajerian was funded through the National Institute of Health (1SC2 GM135114-01).

References

[1] S.J. Lupien, F. Maheu, M. Tu, A. Fiocco, T.E. Schramek. The effects of stress and stress hormones on human cognition: Implications for the field of brain and cognition. Brain Cogn. 65 (2007) 209-237. doi: j.bandc.2007.02.007.
[2] M. Joels, R.A. Sarabdjitsingh, H. Karst, Unraveling the time domains of corticosteroid hormone influences on brain activity: rapid, slow, and chronic modes, Pharmacol. Rev. 64 (2012) 901–938, https://doi.org/10.1124/pr.112.005892.
[3] L. Durieux, K. Herbeaux, C. Borcuk, C. Hildenbrand, V. Andry, Y. Goumon, A. Barbelivien, C. Mathis, D. Bataglia, D. Majchrzak, L. Lecourtier, Functional brain-wide network mapping during acute stress exposure in rats: Interaction between the lateral habenula and cortical, amygdalar, hypothalamic and monoaminergic regions, Eur. J. Neurosci. 56 (2022) 5154–5176, https://doi.org/10.1111/ejn.15803.
[4] C.M. Padovan, F.S. Guimaraes, Restraint-induced hypoactivity in an elevated plus-maze, Braz. J. Med. Biol. Res. 33 (2000) 79–83, https://doi.org/10.1590/s0100-879x2000000100011.
[5] C. Vila-Verde, A.L. Marinho, S.F. Lisboa, F.S. Guimaraes, Nitric oxide in the prelimbic medial prefrontal cortex is involved in the anxiogenic-like effect induced by acute restraint stress in rats, Neuroscience. 320 (2016) 30–42, https://doi.org/10.1016/j.neuroscience.2016.01.040.
[6] S. Singh, S. Tripathy, A. Rawat, D. Dubey, S.A. Siddiqui, R. Ugale, D. Kumar, A. Prakash, Pre-clinical Investigations of Therapeutic Markers Associated with Acute and Chronic Restraint Stress: A Nuclear Magnetic Resonance Based Contrast Metabolic Approach, Nanotheranostics. 7 (2023) 91–101, https://doi.org/10.7150/ntno.76294.
[7] B.C.J. Dirven, J.R. Homberg, T. Kozicz, M. Henckens, Epigenetic programming of the neuroendocrine stress response by adult life stress, J. Mol. Endocrinol. 59 (2017) R11–R31, https://doi.org/10.1530/JME-17-0019.
[8] R.P. de Oliveira, J.S. de Andrade, M. Spina, J.V. Chamon, P.D.D. Silva, A. K. Werder, D. Ortolani, L.S.C. Thomaz, S. Romariz, D.A. Ribeiro, B.M. Longo, R. C. Spadari, M.B. Viana, L. Melo-Thomas, I.C. Cespedes, R.C.B. da Silva, Clozapine prevented social interaction deficits and reduced c-Fos immunoreactivity expression in several brain areas of rats exposed to acute restraint stress, PLoS One. 17 (2022) e0262728.
[9] Y.F. Zhao, Y.F., W.J. Ren, Y. Zhang, J.R. He, H.Y. Yin, Y. Liao, P. Rubini, J.M. Deussing, A. Verkhratsky, Z.Q. Yuan, P. Illes, Y, Tang, Y. High, in Contrast to Low Levels of Acute Stress Induce Depressive-like Behavior by Involving Astrocytic, in Addition to Microglial P2X7 Receptors in the Rodent Hippocampus. Int. J. Mol. Sci. 8: 23 (2022) 1904. doi: 10.3390/ijms23031904.
[10] B.E. Gibb, I. Chelminski, M. Zimmerman, Childhood emotional, physical, and sexual abuse, and diagnoses of depressive and anxiety disorders in adult psychiatric outpatients, Depress. Anxiety. 24 (2007) 256–263, https://doi.org/10.1002/da.20238.
[11] L.L. Musazzi, P. Tornese, N. Sala, M. Popoli, Acute or Chronic? A Stressful Question, Trends Neurosci. 40 (2017) 525–535, https://doi.org/10.1016/j.tins.2017.07.002.
[12] R.L. Spinieli, R.A. Cazuza, A.J. Sales, R.O.G. Carolino, D. Martinez, J. Anselmo-Franci, M. Tajerian, C.R.A. Leite-Panissi. Persistent inflammatory pain is linked with anxiety-like behaviors, increased blood corticosterone, and reduced global DNA methylation in the rat amygdala. Mol. Pain. 18 (2022) 17448069221121307. doi: 10.1177/17448069221121307.
[13] G. M. Jr. Rodrigues, L.V. Toffoli, M.H. Manfredo, J. Francis-Oliveira, A.S. Silva, H. A. Raquel, M.C. Martins-Pinge, E.G. Moreira, K.B. Fernandes, G.G Pelosi, M.V. Gomes. Acute stress affects the global DNA methylation profile in rat brain: modulation by physical exercise. Behav. Brain Res. 279 (2015) 123-128. doi: 10.1016/j.bbr.2014.11.023.
[14] E. Elliott, S. Manashirov, R. Zwang, S. Gil, M. Tsoory, Y. Shemesh, A. Chen, Dnmt3a in the Medial Prefrontal Cortex Regulates Anxiety-Like Behavior in Adult Mice, J. Neurosci. 36 (2016) 730–740, https://doi.org/10.1523/JNEUROSCI.0971-15.2016.
[15] C.R.A. Leite-Panissi, N.C. Coimbra, L. Menescal-de-Oliveira, The cholinergic stimulation of the central amygdala modifying the tonic immobility response and antinociception in guinea pigs depends on the ventrolateral periaqueductal gray, Brain. Res. Bull. 60 (2003) 167–178, https://doi.org/10.1016/s0361-9230(03)00031-5.

[16] L.L. Falconi-Sobrinho, T. dos Anjos-Garcia, N.C. Coimbra, Nitric oxide-mediated defensive and antinociceptive responses organised at the anterior hypothalamus of mice are modulated by glutamatergic inputs from area 24b of the cingulate cortex, J. Psychopharmacol. 35 (2021) 78–90, https://doi.org/10.1177/0269881120967881.
[17] E.B. Vieira-Rasteli, B.B. de Paula, Y.B. de Paiva, N.C. Coimbra, C.R.A. Leite-Panissi, Restricted lesions of the ventrolateral or dorsal columns of the periaqueductal gray promotes distinct effects on tonic immobility and defensive analgesia in guinea pigs, Physiol. Behav. 194 (2018) 538–544, https://doi.org/10.1016/j.physbeh.2018.07.003.
[18] W. Wang, C. Li, Y. Cai, Z.Z. Pan. Pain vulnerability and DNA methyltransferase 3a involved in the affective dimension of chronic pain. Mol. Pain. 13 (2017) 1744806917726713. doi: 10.1177/1744806917726713.
[19] C.M.D. Mota, C. Rodrigues-Santos, R.A.R. Fernández, R.O.G. Carolino, J. Antunes-Rodrigues, J.A. Anselmo-Franci, L.G. Branco, L. g. s., Central serotonin attenuates LPS-induced systemic inflammation. Brain Behav, Immunity. 66 (2017) 372–381, https://doi.org/10.1016/j.bbi.2017.07.010.
[20] G.C. do Nascimento, C.R.A. Leite-Panissi., Time-dependent analysis of nociception and anxiety-like behavior in rats submitted to persistent inflammation of the temporomandibular joint, Physiol. Behav. 125 (2014) 1–7, https://doi.org/10.1016/j.physbeh.2013.11.009.
[21] L. Albrechet-Souza, K.G. Borelli, M.L. Brandao, Activity of the medial prefrontal cortex and amygdala underlies one-trial tolerance of rats in the elevated plus-maze, J. Neurosci. Methods. 169 (2008) 109–118, https://doi.org/10.1016/j.jneumeth.2007.11.025.
[22] V.M. Saito, M.L. Brandao, The benzodiazepine midazolam acts on the expression of the defensive behavior, but not on the processing of aversive information, produced by exposure to the elevated plus maze and electrical stimulations applied to the inferior colliculus of rats, Neuropharmacology. 88 (2015) 180–186, https://doi.org/10.1016/j.neuropharm.2014.07.018.
[23] J. Bouwknecht, R. Paylor, Behavioral and physiological mouse assays for anxiety: a survey in nine mouse strains, Behav. Brain Res. 136 (2002) 489–501, https://doi.org/10.1016/s0166-4328(02)00200-0.
[24] M. Bourin, M. Hascoet, The mouse light/dark box test, Eur. J. Pharmacol. 463 (2003) 55–65, https://doi.org/10.1016/s0014-2999(03)01274-3.
[25] J.R. Deuis, L.S. Dvorakova, I. Vetter, Methods Used to Evaluate Pain Behaviors in Rodents, Front. Mol. Neurosci. 10 (2017), https://doi.org/10.3389/fnmol.2017.00284.
[26] C. Macedo-Souza, S.S. Maisonnette, J.E. Hallak, J.A. Crippa, A.W. Zuardi, J. Landeira-Fernandez, C.R.A. Leite-Panissi, Systemic Chronic Treatment with Cannabidiol in Carioca High- and Low-Conditioned Freezing Rats in the Neuropathic Pain Model: Evaluation of Pain Sensitivity, Pharmaceuticals (basel) 16 (2023) 1003, https://doi.org/10.3390/ph16071003.
[27] X. Belda, S. Fuentes, R. Nadal, A. Armario, A single exposure to immobilization causes longlasting pituitary-adrenal and behavioral sensitization to mild stressors, Horm. Behav. 54 (2008) 654–661, https://doi.org/10.1016/j.yhbeh.2008.07.003.
[28] S. Alvarado, M. Tajerian, M. Suderman, Z. Machnes, S. Pierfelice, M. Millecamps, L. Stone, M. Szyf, An epigenetic hypothesis for the genomic memory of pain, Front. Cell. Neurosci. 9 (2015) 88, https://doi.org/10.3389/fncel.2015.00088.
[29] M. Tajerian, S. Alvarado, J.D. Clark. Differential olfactory bulb methylation and hydroxymethylation are linked to odor location memory bias in injured mice. Mol. Pain. 15 (2019) 1744806919873475. doi: 10.1177/1744806919873475.
[30] M. Tajerian, S. Alvarado, M. Millecamps, P. Vachon, C. Crosby, M.C. Bushnell, M. Szyf, L.S. Stone, Peripheral nerve injury is associated with chronic, reversible changes in global DNA methylation in the mouse prefrontal cortex, PLoS One. 8 (2013) e55259.
[31] A.J. Sales, S.R.L. Joca, Antidepressant administration modulates stress-induced DNA methylation and DNA methyltransferase expression in rat prefrontal cortex and hippocampus, Behav. Brain Res. 343 (2018) 8–15, https://doi.org/10.1016/j.bbr.2018.01.022.
[32] P.A. Melas, M. Rogdaki, A. Lennartsson, K. Bjork, H. Qi, A. Witasp, M. Werme, G. Wegener, A.A. Mathe, P. Svenningsson, C. Lavebratt, Antidepressant treatment is associated with epigenetic alterations in the promoter of P11 in a genetic model of depression, Int. J. Neuropsychopharmacol. 15 (2012) 669–679, https://doi.org/10.1017/S1461145711000940.
[33] J. Wei, J. Cheng, N.J. Waddell, Z.J. Wang, X. Pang, Q. Cao, A. Liu, J.M. Chitaman, K. Abreu, R.S. Jasrotia, L.J. Duffney, J. Zhang, D.M. Dietz, J. Feng, Z. Yan, DNA Methyltransferase 3A Is Involved in the Sustained Effects of Chronic Stress on Synaptic Functions and Behaviors, Cereb. Cortex 31 (2021) 1998–2012, https://doi.org/10.1093/cercor/bhaa337.
[34] Q. Tian, S.B. Stepaniants, M. Mao, L. Weng, M.C. Feetham, M.J. Doyle, E.C. Yi, H. Dai, V. Thorsson, J. Eng, D. Goodlett, J.P. Berger, B. Gunter, P.S. Linseley, R. B. Stoughton, R. Aebersold, S.J. Collins, W.A. Hanlon,E. Hood., Integrated genomic and proteomic analyses of gene expression in Mammalian cells, Mol. Cell Proteomics. 3 (2004) 960–969, https://doi.org/10.1074/mcp.M400055-MCP200.
[35] B. Pradet-Balade, F. Boulme, H. Beug, E.W. Mullner, J.A. Garcia-Sanz, Translation control: bridging the gap between genomics and proteomics? Trends Biochem. Sci. 26 (2001) 225–229, https://doi.org/10.1016/s0968-0004(00)01776-x.
[36] L. Anderson, J. Seilhamer, A comparison of selected mRNA and protein abundances in human liver, Electrophoresis. 18 (1997) 533–537, https://doi.org/10.1002/elps.1150180333.
[37] X. Wang, L. Jiang, W. Ma, X. Zheng, E. He, B. Zhang, M.K. Vashisth, Z. Gong, Maternal separation affects anxiety-like behavior beginning in adolescence and continuing through adulthood and related to Dnmt3a expression, J. Neurophysiol. 128 (2022) 611–618, https://doi.org/10.1152/jn.00247.2022.
[38] J. Dorts, E. Falisse, E. Schoofs, E. Flamion, P. Kestemont, F. Silvestre, DNA methyltransferases and stress-related genes expression in zebrafish larvae after exposure to heat and copper during reprogramming of DNA methylation, Sci. Rep. 6 (2016) 34254, https://doi.org/10.1038/srep34254.
[39] R.M. Kohli, Y. Zhang, TET enzymes, TDG and the dynamics of DNA demethylation, Nature. 502 (2013) 472–479, https://doi.org/10.1038/nature12750.
[40] M. Gutierrez-Arcelus, T. Lappalainen, S.B. Montgomery, A. Buil, H. Ongen, A. Yurovsky, J. Bryois, T. Giger, L. Romano, A. Planchon, E. Falconnet, D. Bielser, M. Gagnebin, I. Padioleau, C. Borel, A. Letourneau, P. Makrythanasis, M. Guipponi, C. Gehrig, S.E. Antonarakis, E.T. Dermitzakis, Passive and active DNA methylation and the interplay with genetic variation in gene regulation, Elife. 2 (2013) e00523.
[41] H. Denis, M.N. Ndlovu, F. Fuks, Regulation of mammalian DNA methyltransferases: a route to new mechanisms, EMBO Rep. 12 (2011) 647–656, https://doi.org/10.1038/embor.2011.110.
[42] M.J. Lisieski, A.L. Eagle, A.C. Conti, I. Liberzon, S.A. Perrine, Single-Prolonged Stress: A Review of Two Decades of Progress in a Rodent Model of Post-traumatic Stress Disorder, Front. Psychiatry. 9 (2018) 196, https://doi.org/10.3389/fpsyt.2018.00196.
[43] X. Belda, S. Fuentes, N. Daviu, R. Nadal, A. Armario, Stress-induced sensitization: the hypothalamic-pituitary-adrenal axis and beyond, Stress. 18 (2015) 269–279, https://doi.org/10.3109/10253890.2015.1067678.
[44] G.K. Ford, D.P. Finn, Clinical correlates of stress-induced analgesia: evidence from pharmacological studies, Pain. 140 (2008) 3–7, https://doi.org/10.1016/j.pain.2008.09.023.
[45] H. Imbe, S. Murakami, K. Okamoto, Y. Iwai-Liao, E. Senba, The effects of acute and chronic restraint stress on activation of ERK in the rostral ventromedial medulla and locus coeruleus, Pain. 112 (2004) 361–371, https://doi.org/10.1016/j.pain.2004.09.015.
[46] K. Askari, S. Oryan, A. Eidi, J. Zaringhalam, A. Haghparast, Modulatory role of the orexin system in stress-induced analgesia: Involvement of the ventral tegmental area, Eur. J. Pain. 25 (2021) 2266–2277, https://doi.org/10.1002/ejp.1840.
[47] X. Ma, W. Bao, X. Wang, Z. Wang, Q. Liu, Z. Yao, D. Zhang, H. Jiang, S. Cui, Role of spinal GABAA receptor reduction induced by stress in rat thermal hyperalgesia, Exp. Brain Res. 232 (2014) 3413–3420, https://doi.org/10.1007/s00221-014-4027-5.
[48] M. Larauche, J. Erchegyi, C. Miller, M.S. Sim, J. Rivier, D. Behan, Y. Tache, Peripheral CRF-R1/CRF-R2 antagonist, astressin C, induces a long-lasting blockade of acute stress-related visceral pain in male and female rats, Peptides. 157 (2022), 170881, https://doi.org/10.1016/j.peptides.2022.170881.
[49] A. Gunn, E.N. Bobeck, C. Weber, M.M. Morgan, The influence of non-nociceptive factors on hot-plate latency in rats, J. Pain 12 (2011) 222–227, https://doi.org/10.1016/j.jpain.2010.06.011.
[50] S. Hunskaar, O.G. Berge, K.A. Hole, A modified hot-plate test sensitive to mild analgesics, Behav. Brain Res. 21 (1986) 101–108, https://doi.org/10.1016/0166-4328(86)90088-4.
[51] J. Mendes-Gomes, T.T. Miguel, V.C. Amaral, R.L. Nunes-de-Souza, Corticosterone does not change open elevated plus maze-induced antinociception in mice, Horm. Behav. 60 (2011) 408–413, https://doi.org/10.1016/j.yhbeh.2011.07.004.
[52] A. Sood, P.R. Chaudhari, P. Tiwari, S. Shah, V.A. Vaidya, Acute immobilization stress evokes sexually dimorphic peripheral and hippocampal neuroimmune responses in adult rats, Neurosci. Lett. 789 (2022), 136871, https://doi.org/10.1016/j.neulet.2022.136871.
[53] H.A. Vecchiarelli, M. Morena, T.T.Y. Lee, A.S. Nastase, R.J. Aukema, K.D. Leitl, J. M. Gray, G.N. Petrie, K.J. Tellez-Monnery, M.N. Hill, Sex and stressor modality influence acute stress-induced dynamic changes in corticolimbic endocannabinoid levels in adult Sprague Dawley rats, Neurobiol. Stress. 20 (2022), 100470, https://doi.org/10.1016/j.ynstr.2022.100470.
[54] L.A. McKibben, Y. Dwivedi, Early life and adult stress promote sex dependent changes in hypothalamic miRNAs and environmental enrichment prevents stress-induced miRNA and gene expression changes in rats, BMC Genomics. 22 (2021) 701, https://doi.org/10.1186/s12864-021-08003-4.
[55] J. Blaze, T.L. Roth, Exposure to caregiver maltreatment alters expression levels of epigenetic regulators in the medial prefrontal cortex, Int. J. Dev. Neurosci. 31 (2013) 804–810, https://doi.org/10.1016/j.ijdevneu.2013.10.001.