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RESEARCH

# The nonpeptide angiotensin-(1–7) mimic AVE0991 attenuates neuroendocrine and behavioral responses to chronic unpredictable stress

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## Abstract

**Rationale** Chronic stress, frequently associated with dysfunction of the hypothalamic–pituitary–adrenal (HPA) axis and reduced neuroplasticity, is a major risk factor for psychiatric disorders such as anxiety and depression.

**Objective** The present study aimed to validate a 21-day chronic unpredictable stress (CUS) model and to investigate the effects of the Mas receptor agonist AVE0991 on stress-induced behavioral and molecular alterations.

**Methods** Male C57BL/6J mice were exposed to a 21-day CUS protocol. Animals were randomly assigned to four groups: control + saline, CUS + saline, control + AVE0991, and CUS + AVE0991 (3 mg/kg, *i.p.*). AVE was administered daily during the last two weeks of the stress protocol. Behavioral tests were performed to evaluate anxiety- and depressive-like behaviors, and plasma corticosterone, blood glucose levels, and brain-derived neurotrophic factor (BDNF) levels in the prefrontal cortex, hippocampus, and hypothalamus were measured.

**Results** CUS exposure significantly increased plasma corticosterone and glucose levels and induced anxiety- and depressive-like behaviors. Stressed animals also showed reduced BDNF levels in the prefrontal cortex, hippocampus, and hypothalamus. Treatment with AVE0991 attenuated the increase in corticosterone and prevented stress-induced hyperglycemia. Moreover, AVE0991 reduced depressive-like behavior, increased latency to immobility, and improved anxiety-related parameters in the elevated plus maze and open field tests without affecting locomotor activity. AVE0991 also prevented the reduction of BDNF levels in stress-exposed animals.

**Conclusion** These findings validate the CUS model and demonstrate that activation of the Mas receptor by AVE0991 exerts anxiolytic, antidepressant, and neuroprotective effects, supporting its potential as a therapeutic strategy for stress-related neuropsychiatric disorders.

**Keywords** Chronic unpredictable stress · Anxiety · Depression · AVE0991 · BDNF

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## Introduction

Chronic, uncontrollable, and unpredictable stress induces physical and psychological changes that promote the development of emotional disorders in humans and animals (de Kloet et al. 2005; Strekalova et al. 2011). In this context, the chronic unpredictable stress (CUS) experimental model simulates the variability and duration of everyday stress by randomly applying different stressors over time, leading to behavioral and physiological changes (Willner 2017). The response to CUS involves activation of the hypothalamic–pituitary–adrenal (HPA) axis and the release of glucocorticoids, whose prolonged exposure increases vulnerability to metabolic, cardiovascular, and neurodegenerative diseases, as well as mood and anxiety disorders (de Kloet 2000; Lupien et al. 2009; McEwen, 1993). Likewise, prolonged stress compromises neuroplasticity and neurotrophic support, particularly through reduced expression of brain-derived neurotrophic factor (BDNF), a molecule essential for neuronal survival, synaptic plasticity, and cognitive performance (Tapia-Arancibia et al. 2004; Duman and Monteggia 2006). Decreased BDNF levels in the hippocampus and prefrontal cortex have been consistently associated with depressive phenotypes, highlighting the close relationship between stress, neurotrophic signaling, and mood regulation (Castrén and Hen 2013).

In addition, chronic stress also triggers responses from the renin–angiotensin system (RAS) (Saavedra and Benicky 2007; Correa et al. 2022). Angiotensin II, acting through $AT_1$ receptors, which are abundant in brain regions associated with the neurobiology of stress, increases HPA axis activity and sympathetic tone (Jezova et al. 1998; Saavedra et al. 2004; Saavedra and Benicky 2007; Lavoie and Sigmund 2003). Conversely, angiotensin-(1–7) [Ang-(1–7)], acting on the Mas receptor, exerts opposing effects, reducing anxiety-like and depression-like behaviors induced by different stressors (Fontes et al. 2016; Moura Santos et al. 2017; Zhu et al. 2020). Interestingly, recent studies have indicated that the Ang-(1–7)/Mas receptor axis can positively modulate BDNF expression and enhance neuroplasticity (Takahashi et al. 2025). In this sense, Mas receptor activation has been associated with increased neurogenesis, reduced oxidative stress, and improved synaptic integrity in brain regions involved in emotional regulation, such as the hippocampus and amygdala (Takahashi et al. 2025). These findings suggest that the protective role of Ang-(1–7) through Mas receptor extends beyond its cardiovascular effects, encompassing significant impacts on neuronal health and behavior under chronic stress conditions.

AVE0991 (5-formyl-4-methoxy-2-phenyl-1-[[4-[2-(ethylaminocarbonylsulfonamido)-5-isobutyl-3-thienyl]-phenyl]-methyl]-imidazole) is a non-peptide analogue of Ang-(1–7), showing potential for therapeutic use since it replicates the effects of Ang (1–7) in various organs, but with a longer half-life and increased stability compared to Ang (1–7) (Santos and Ferreira 2006; Deng et al. 2024).

Preclinical studies have reported beneficial effects of AVE0991 in experimental models of Alzheimer's disease (Jiang et al. 2018; Duan et al. 2021), Parkinson's disease (Duan et al. 2024), and chronic cerebral hypoperfusion (Xue et al. 2021). Moreover, it was described that AVE0991 reduces oxidative stress injury and neuronal apoptosis through the Mas/PKA/p-CREB/UCP-2 pathway after subarachnoid hemorrhage (Mo et al. 2019).

In this study, we validated a model of CUS in mice and explored the possibility that activation of the Mas receptor with AVE0991, a non-peptide analogue of Ang-(1–7), could mitigate CUS effects.

## Materials and methods

### Animals

164 Male C57BL/6J mice (8–10 weeks old) were used, without weight bias, housed in groups of 5 or 6 animals per cage. All animals were obtained from the Animal Facility of our Institute (CEBIO; Biological Sciences Institute; UFMG) and underwent a 1-week acclimation period in the Animal Facility of the Morphology Department (ICB-UFMG) prior to the start of experimental protocols. Animals were maintained under controlled temperature (22–23 °C) and lighting (12-h hour light/dark cycle) conditions, with food and water available *ad libitum*. Experiments were conducted only during the light phase of the day. All experimental procedures were approved by the Institutional Ethics Committee on the Use of Animals at the Universidade Federal de Minas Gerais (CEUA/UFMG) (protocol n°. 223/2020). All experiments were conducted in accordance with the guidelines of the Brazilian National Council for Control of Animal Experimentation (CONCEA-BRAZIL) and complied with the ARRIVE guidelines. The timeline of the experimental protocol is illustrated in Fig. 1.

### Experimental design

#### Chronic unpredictable stress model validation

The CUS protocol, originally described by Willner et al. (1987, 1992), was adapted in this study to induce persistent behavioral changes associated with stress in mice. This model is based on the daily and random exposure to different physical, social, and environmental stressors,

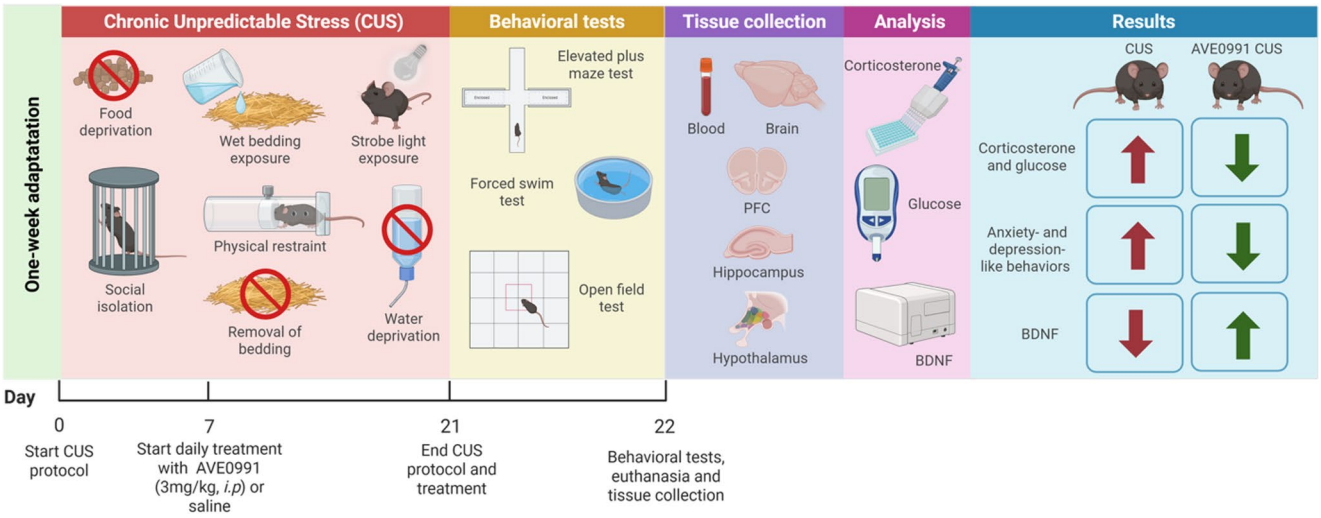



**Fig. 1** Timeline of the experimental protocol used to induce chronic unpredictable stress (CUS), treatment with AVE0991, behavioral tests, euthanasia, tissue collection, molecular analyses and results (created with Biorender.com)

which prevents habituation to stimuli, a common limitation of other chronic stress paradigms (Willner 1997; Mineur et al. 2006; Antoniuk et al. 2019). For 21-days, animals were subjected each day to a single stressor randomly selected from the following: water deprivation for 24 h, food deprivation for 24 h, wet bedding exposure for 6 h, strobe light exposure for 2 h, social isolation for 6 h, physical restraint for 1 h, or removal of bedding for 24 h. Stressors were preferentially applied during the light phase of the circadian cycle (between 8 a.m. and 12 p.m.) to ensure chronobiological consistency and reduce interference from hormonal variations related to the light/dark cycle. Control group animals were handled daily at the same time and in the same environment, but were not exposed to stressors, ensuring control for handling effects without stress induction.

## Mas receptor agonist (AVE0991) treatment

Four experimental groups were used: non-stressed and non-treated (Control), stressed and non-treated (CUS), non-stressed treated with AVE0991 (AVE-Control), and stressed treated with AVE0991 (AVE-CUS). All stressed groups were subjected to the same 21-day CUS protocol. During the last two weeks of the CUS protocol (days 8–21), mice in the treatment groups received daily intraperitoneal (i.p.) injections of AVE0991 MedChemExpress LLC (3 mg/kg; 0.1 mL per animal), the dose was used considering previous studies, including our group (Da Silveira et al. 2010; Jiang et al. 2018; Xue et al. 2021; Deng et al. 2023). In addition, previous reports reported that AVE0991 crosses the blood-brain barrier via intraperitoneal injection route (Jiang et al. 2018; Xue et al. 2021).

The drug was administered immediately after exposure to the daily stressor, at a standardized time between 09:00 and 10:00 h. Control and vehicle groups received the same volume (0.1 mL) of sterile saline following the same administration schedule. At the end of the stress/control protocol, mice underwent behavioral testing, and blood was collected for biochemical analyses.

## Plasma corticosterone

The quantification of plasma corticosterone was performed using mouse corticosterone (CORT) (#E-EL-0161) ELISA kit, Elabsience according to the manufacturer's instructions. For plasma collection, animals were anesthetized with ketamine (100 mg/kg Syntec®) and xylazine (10 mg/kg Syntec®) administered intraperitoneally (i.p.), and blood was obtained via cardiac puncture in the morning hours (between 8 a.m. and 11 a.m.) to minimize circadian variability of corticosterone levels. Samples were immediately transferred to tubes containing EDTA (0.5 M, pH 8.0), centrifuged at 1,500 x *g* for 15 min at 4 °C, and the separated plasma was stored at −80 °C until analysis. All samples were analyzed in duplicate, and the absorbance was measured at 450 nm using a microplate reader (MULTISKAN EX, Thermo scientific). Serum CORT levels were calculated from the standard curve and the results were expressed as ng/mL.

## Blood glucose levels

For fasting glucose levels were measured on the day following completion of the CUS protocol by collecting blood from a puncture at the tip of the mouse tail. Glucose concentrations were determined using reagent strips and a One

Touch Ultra Plus Flex glucometer (Johnson & Johnson®), and the mean of the readings were expressed as mg/dL.

## Behavioral tests

To verify the stress response induced by the CUS model used in our study, mice were subjected to characterized tests to indicate depressive-like behavior, the forced swim and tail suspension tests. Additionally, to investigate anxiety-like behavior, the elevated plus maze and the open field test were carried out. Animals were acclimated to the experimental room with illumination of approximately 30 lx for least 30 min before the beginning of the experiments. The behavioral tests were conducted between 8:00 a.m. and 12:00 p.m on the day following completion of the CUS protocol (day 22 after the start of the experimental assay). Each animal was used for only one behavioral paradigm to avoid carry-over effects.

### Forced swimming test (FST)

The FST was performed as previously described (Becari et al. 2023), each mouse was placed for 6 min in a glass cylinder (20 cm in diameter, 30 cm in height) filled with 20 cm of water, maintained at approximately 25 °C. Each session is video recorded and evaluated by a double-blinded investigator, unaware of the animal group assignment. The latency to immobility, defined by the first immobility with a minimal duration of 2 s, was measured from the start of the test and the total immobility time, defined as the duration the animal remains still performing only minimal movements necessary for floating, was measured during the final four minutes. These parameters were expressed in seconds. To prevent contamination from alarm substances, the water was changed between swimming sessions. The depressive-like behavior is verified when there was an increase in immobility time and a reduction in the latency to immobility.

### Tail suspension test (TST)

The TST was performed according to a previously described (Becari et al. 2023). Mice were suspended by the tail using adhesive tape placed approximately 1 cm from the tip of the tail and attached to a metal rod positioned 50 cm above the floor. Animals were allowed to hang upside-down for 6 min. Immobility time, defined as the absence of any body movements except those related to respiration, was recorded in seconds. Behavioral scoring was performed by an experimenter blinded to the experimental groups. After the test, the adhesive tape was gently removed, and the mice were returned to their home cages.

### Elevated plus maze (EPM) test

The elevated plus maze (EPM) test was performed based on the protocol described by Handley and Mithani (1984). The apparatus consisted of four wooden arms of equal dimensions arranged in a plus shape, including two open arms (30 × 6 cm) and two closed arms (30 × 6 × 5 cm), elevated 50 cm above the floor.

The EPM assesses the conflict between the innate tendency to explore a novel environment (open arms) and the avoidance of potentially aversive exposed areas, which are associated with a perceived risk of predation. Animals were placed in the central area of the maze facing an open arm and allowed to explore the apparatus for 5 min. Behavior was recorded using a camera positioned 1.5 m above the maze, and videos were analyzed using ANY-MAZE software Stoelting Co. (version 4.5), which tracks the animal's position and calculates the percentage of entries into the open arms, the percentage of time spent in the open arms, and the number of entries into the closed arms. An arm entry was considered when all four paws of the animal were inside the arm. The maze was cleaned with 10% ethanol between animals to eliminate olfactory cues.

### Open field test (OFT)

Animals were placed individually in the center of a circular arena (30 cm in diameter and 40 cm in height). The animals' behavior was recorded for 5 min using a video camera positioned 1.5 m above the arena. During this period, the number of crossings that the animal made between the quadrants were quantified, with each crossing only being counted when the animal crossed with all four paws from one quadrant to the other. Animals with anxious-like behavior tend to remain longer on the periphery of the arena, since they are more exposed in the center, the center zone was determined as a 10 cm × 10 cm circle located in the center of the arena. The number of crossings performed by each animal during this period was used as an index of its locomotor and exploratory activity, and the latency in seconds within the central and peripheral areas of the arena was recorded for behavioral analysis. In summary, the x and y coordinates of the animal were extracted from each video frame using the open-source software Bonsai (Lopes et al. 2015). These coordinates were then used to generate a tracking map representing the animal's displacement throughout the arena. The positional data were subsequently imported into MATLAB (2024b), where custom-developed routines were applied for further trajectory analysis.

### BDNF quantification

BDNF levels were quantified in the hypothalamus, prefrontal cortex, and hippocampus regions using a comercial ELISA kit (MyBioSource - MBS2700738), following the manufacturer's instructions. Animals were euthanized, and the brain regions from both hemispheres were quicky dissected and homogenized using an Ultra-Turrax (T10 basic – IKA) in an extraction buffer (100 mg tissue/mL) containing 0.4 M NaCl, 0.05% Tween 20, 0.5% BSA, 0.1 mM phenyl methyl sulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KIU aprotinin. Homogenates were centrifuged at 13,000 x *g* for 10 min at 4 °C, and the supernatants were collected for analysis. BDNF concentrations were determined in duplicates and results were expressed as picograms per 100 mg of tissue.

### Statistical analysis

The results are expressed as mean and standard error of the mean (SEM). The normality of the results was calculated using the Shapiro-Wilk test. Data were analyzed using the Student's t-test for parametric samples and the Wilcoxon test for non-parametric samples according to the normality test. Comparisons between groups were performed using two-way analysis of variance (ANOVA), followed by Tukey`s multiple comparisons test. Tukey's test was used for multiple comparisons because it provides appropriate control of the family-wise Type I error rate while allowing pairwise comparisons among all experimental groups following a significant ANOVA.

The magnitude of effects in ANOVA was estimated with partial eta squared ($\eta p^2$), while Cohen's d was used to assess the effect size of pairwise comparisons. Statistical analyses were conducted using GraphPad Prism software (version 9.0.0), and p-values $\leq 0.05$ were considered statistically significant.

## Results

### Validation of CUS model

As observed in Fig. 2A, mice from the CUS group exhibited significantly higher corticosterone concentrations (269.6 ± 11.71 ng/mL; $n = 12$; $p < 0.0001$; Cohen's d = 2.11; Fig. 2A) as compared to controls (130.8 ± 5.14 ng/mL; $n = 12$; Fig. 2A). Similarly, blood glucose levels were significantly higher (143.3 ± 3.51 mg/dL; $n = 8$; $p < 0.0001$; Cohen's d = 2,06; Fig. 2B) than controls (94.5 ± 2.39 mg/dL; $n = 10$; Fig. 2B).

Behavioral responses in the FST also demonstrated significant stress effects. Latency to first immobility was reduced in CUS mice (33.67 ± 1.92 s; $n = 12$; $p < 0.0001$; Cohen's d = 3.07; Fig. 2C), in comparison to Controls (54 ± 2.07 s; $n = 10$; Fig. 2C). In addition, immobility time in the FST was increased in CUS mice (160.7 ± 3.01 s; $n = 12$; $p < 0.0001$; Cohen's d = 4.74; Fig. 2D) in comparison to controls (111.8 ± 3.20 s; $n = 10$; Fig. 2D). CUS mice also displayed increased immobility in the TST (175.8 ± 4.07 s; $n = 12$; $p = 0.0002$; Cohen's d = 1.95; Fig. 2E) in comparison to controls (Control: 145.2 ± 5.50 s; $n = 10$; Fig. 2E). In the EPM, anxiety-like behavior was affected by stress, with CUS mice spending less time in the open arms (15.07 ± 1.33 s vs. 27.64 ± 2.55 s in Controls; $n = 8$–10; $p = 0.0003$ Cohen's d = 2.19; Fig. 2F). Additionally entering in the open arms less were less frequent (19.38 ± 1.06 vs. 31.9 ± 2.35 in controls; $n = 8$–10; $p = 0.0003$; Cohen's d = 2.15; Fig. 2G). Entries into the enclosed arms were not significantly different in CUS mice (15.6 ± 1.24 vs. 14.88 ± 1.44 in controls; $n = 8$–10; $p = 0.70$; Cohen's d = 0.18; Fig. 2H).

In the OFT, total distance traveled was not affected by CUS (1429 ± 110.3 cm vs. 1241 ± 156 cm in controls; $n = 10$; $p = 0.3391$; Cohen's d = 0.43; $n = 10$; Fig. 2I), whereas time spent in the central zone showed a trend towards decrease (28.47 ± 4.12 s vs. 67.66 ± 17.64 s in controls; $n = 9$–10; $p = 0.0553$; Cohen's d = 0.94; Fig. 2J).

BDNF levels were significantly reduced in the prefrontal cortex (96 ± 6.38 pg/100 mg vs. 154 ± 10.59 pg/100 mg in controls; $n = 10$; $p = 0.0002$; Cohen's d = 2.09; Fig. 2K), hippocampus (180.2 ± 11.41 pg/100 mg vs. 270.7 ± 15.97 pg/100 mg in controls; $n = 10$; $p = 0.0002$; Cohen's d = 2.06; Fig. 2L), and hypothalamus (110.2 ± 4.60 pg/100 mg vs. 175.4 ± 10.48 pg/100 mg in controls; $n = 10$; $p = 0.0001$; Cohen's d = 2.54; Fig. 2M).

### Effect of Mas agonist, AVE0991, treatment in mice subjected to CUS

Plasma corticosterone and blood glucose levels are shown in Fig. 3A–B. Exposure to CUS markedly increased corticosterone concentrations in saline-treated mice (300.0 ± 10.25 ng/mL vs. 123.4 ± 5.76 ng/mL in controls; $p < 0.0001$; $n = 8$; Cohen's d = 7.50; Fig. 3A). Two-way ANOVA revealed significant main effects of stress ($F_{(1,28)} = 294.3$, $p < 0.0001$, $\eta p^2 = 0.91$) and AVE treatment ($F_{(1,28)} = 30.53$, $p < 0.0001$, $\eta p^2 = 0.52$), as well as a significant interaction between stress and treatment ($F_{(1,28)} = 38.27$, $p < 0.0001$, $\eta p^2 = 0.58$). AVE0991 treatment did not alter corticosterone levels in non-stressed animals (128.4 ± 4.53 ng/mL; $n = 8$; $p = 0.9656$; Cohen's d = 0.21). However, in stressed mice, AVE0991 significantly reduced corticosterone concentrations

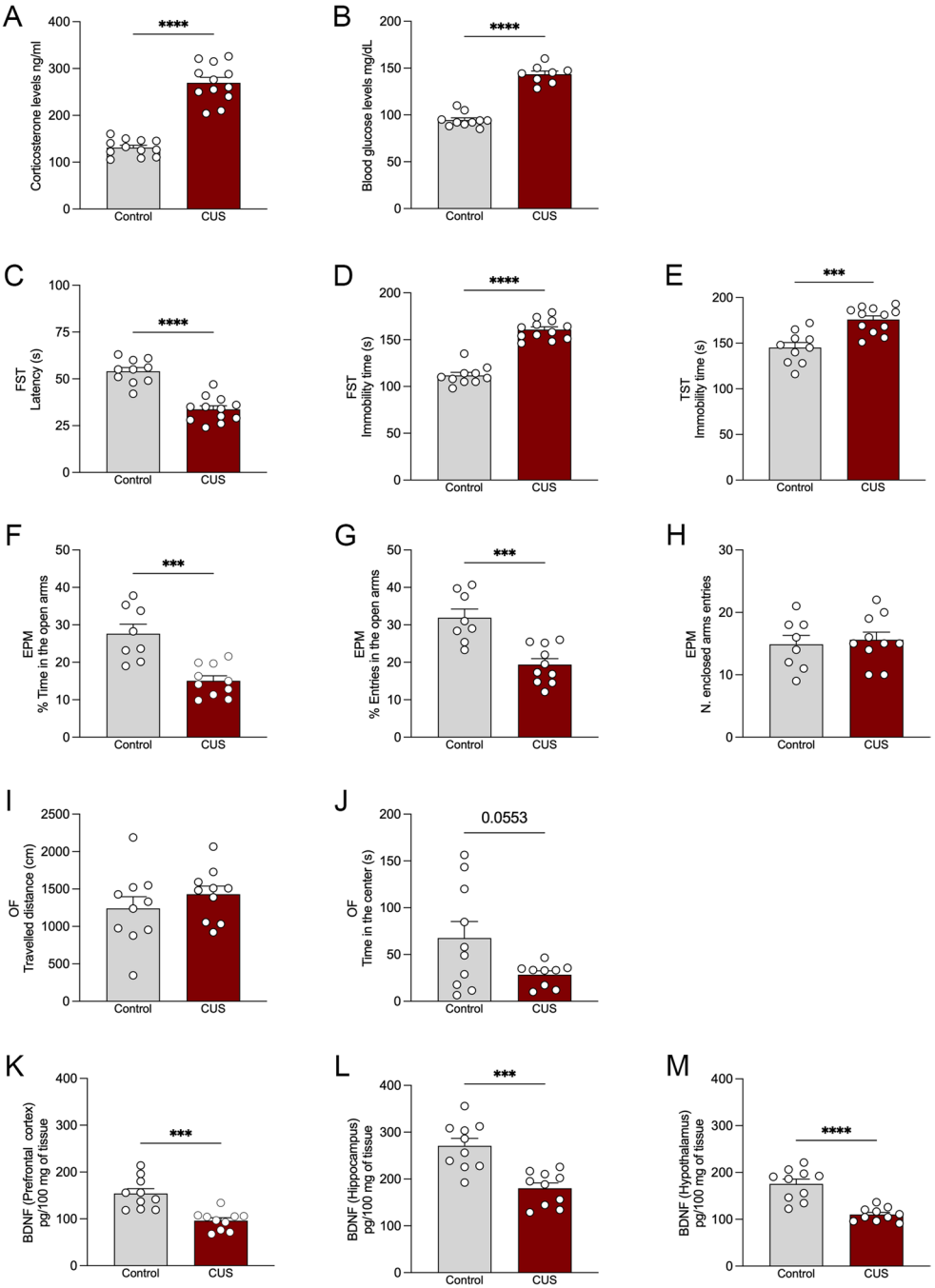



**Fig. 2** Quantification of plasma corticosterone levels (**A**), blood glucose levels (**B**), latency to first immobility in the forced swim test (FST) (**C**), total immobility time in the FST (**D**), immobility time in the tail suspension test (TST) (**E**), time spent in the open arms of the elevated plus maze (EPM) (**F**), number of open arm entries in the EPM (**G**), number of closed arm entries in the EPM (**H**), total distance traveled in the open field test (OFT) (**I**), time spent in the center of the OFT arena (**J**), and BDNF protein levels in the prefrontal cortex (**K**), hippocampus (**L**), and hypothalamus (**M**) in mice exposed to chronic unpredictable stress (CUS) or control conditions. Data are expressed as mean ± SEM ($n = 6–12$ per group depending on the assay). Statistical comparisons between groups were performed using unpaired Student's t-tests. ***$p < 0.001$, ****$p < 0.0001$

(211.4 ± 8.38 ng/mL; $n = 8$; Cohen's d = 3.76) compared with stressed saline-treated animals (300.0 ± 10.25 ng/mL; $n = 8$), although levels remained elevated relative to non-stressed controls (Fig. 3A).

A similar pattern was observed for blood glucose levels. CUS significantly increased glycemia in saline-treated mice (152.5 ± 3.25 mg/dL vs. 101.8 ± 3.37 mg/dL in controls; $n = 8$; $p < 0.0001$; Cohen's d = 5.42; Fig. 3B). Two-way ANOVA revealed significant effects of stress ($F_{(1,30)} = 29.29$, $p < 0.0001$, $\eta p^2 = 0.49$) and AVE treatment ($F_{(1,30)} = 10.94$, $p = 0.0025$, $\eta p^2 = 0.27$), as well as a significant interaction between the two factors ($F_{(1,30)} = 16.66$, $p = 0.0003$, $\eta p^2 = 0.36$). AVE0991 treatment did not significantly affect glucose levels in non-stressed animals (105.9 ± 6.08 mg/dL; $n = 9$; $p = 0.9465$; Cohen's d = 0.14; Fig. 3B). In contrast, AVE0991 administration in stressed mice significantly attenuated stress-induced hyperglycemia (113.0 ± 6.84 mg/dL vs. 152.5 ± 3.24 mg/dL in CUS saline mice; $n = 9$; $p < 0.0001$; Cohen's d = 4.22; Fig. 3B), with glucose levels approaching those observed in non-stressed controls.

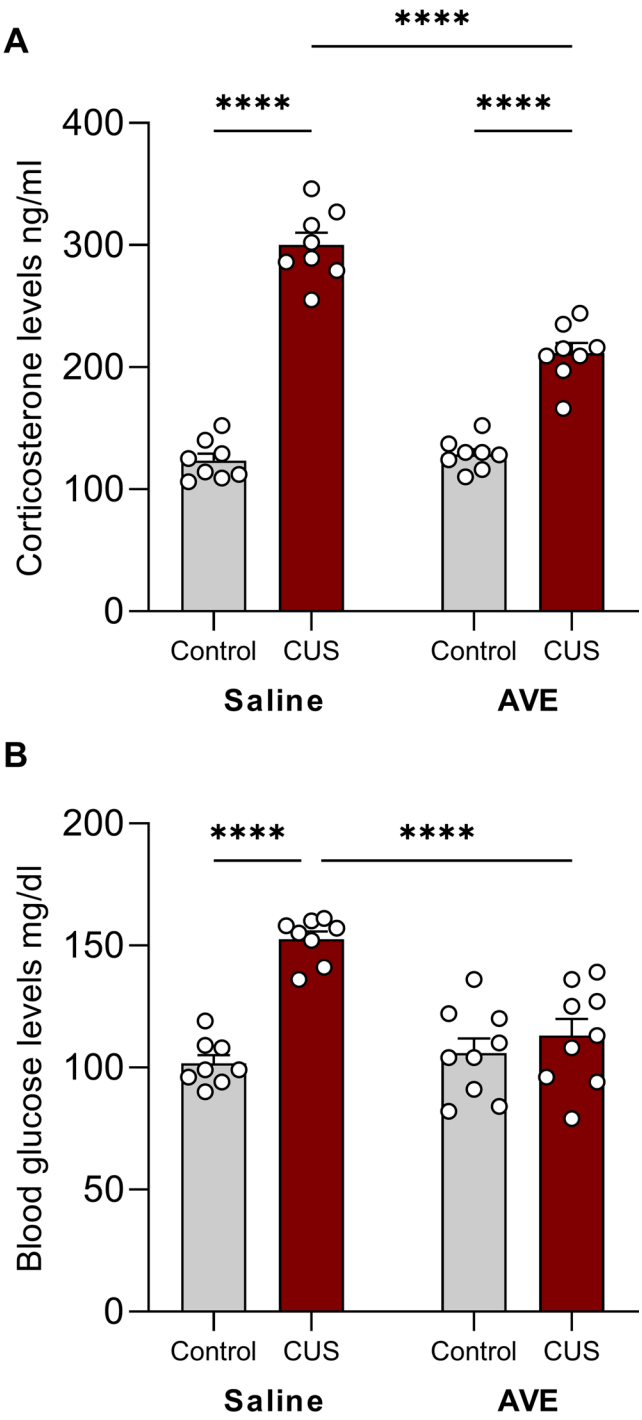



**Fig. 3** Quantification of plasma corticosterone levels (**A**) and blood glucose levels (**B**) in mice under control conditions (Control), exposed to chronic unpredictable stress (CUS), or treated with AVE0991 (3 mg/kg, i.p.) under control conditions (AVE-control) or during CUS exposure (AVE-CUS). Data are expressed as mean ± SEM ($n = 8$ per group for corticosterone; $n = 8$–9 per group for glucose). Statistical analyses were performed using two-way ANOVA followed by Tukey's post hoc test. ****$p < 0.0001$

### Effect of AVE0991 on depressive-like behavior

As shown in Fig. 4, the latency to the first immobility in the forced swim test, two-way ANOVA revealed a significant interaction between stress and treatment ($F_{(1,36)} = 4.90$, $p = 0.0332$, $\eta p^2 = 0.12$). CUS reduced latency in untreated mice (33.8 ± 1.65 s vs. 57.9 ± 7.91 s in controls saline; $n = 10$; $p = 0.0297$; Cohen's d = 1.33). AVE0991 treatment did not significantly affect latency in non-stressed animals (60.0 ± 4.91 s vs. 57.9 ± 7.91 s in controls saline; $n = 10$; $p = 0.9941$; Cohen's d = 0.11; Fig. 4A). In contrast, AVE0991 administration in stressed mice significantly increased latency (61.8 ± 6.88 s vs. 33.8 ± 1.65 s in CUS saline; $n = 10$; $p = 0.0089$; Cohen's d = 1.54; Fig. 4A), restoring values to levels comparable to control animals. A significant main effect of treatment was also observed ($F_{(1,36)} = 6.63$, $p = 0.0143$, $\eta p^2 = 0.16$), whereas the main effect of stress alone did not reach statistical significance ($F_{(1,36)} = 3.63$, $p = 0.0645$, $\eta p^2 = 0.09$). Similarly, analysis of total immobility time in the forced swim test revealed a significant interaction between stress and treatment ($F_{(1,36)} = 9.81$, $p = 0.0034$, $\eta p^2 = 0.21$). CUS markedly increased immobility duration in untreated mice (164.9 ± 5.17 s vs. 104.9 ± 9.23 s in controls saline; $n = 10$; $p < 0.0001$; Cohen's d = 2.42; Fig. 4B). AVE0991 treatment did not alter immobility in non-stressed animals (103.1 ± 6.12 s vs. 104.9 ± 9.23 s in controls saline; $n = 10$; $p = 0.9981$; Cohen's d = 0.07; Fig. 4B). However, in stressed mice, AVE0991 significantly reduced immobility (115.5 ± 8.99 s vs. 164.9 ± 5.17 s in CUS saline; $n = 10$; $p = 0.0003$; Cohen's d = 1.99; Fig. 4B), bringing values closer to those observed in control animals. Significant main effects of stress ($F_{(1,36)} = 22.79$, $p < 0.0001$, $\eta p^2 = 0.39$) and treatment ($F_{(1,36)} = 11.40$, $p = 0.0018$, $\eta p^2 = 0.24$) were also observed.

### Effect of AVE0991 on anxiety-like behavior

In the EPM, CUS significantly reduced both the percentage of time spent in the open arms and the number of entries into the open arms (Fig. 5A-C). Two-way ANOVA revealed significant main effects of stress ($F_{(1,22)} = 21.88$, $p = 0.0001$, $\eta p^2 = 0.50$) and treatment ($F_{(1,22)} = 8.556$, $p = 0.0078$, $\eta p^2 = 0.28$) for time spent in the open arms, with no significant interaction ($F_{(1,22)} = 4.248$, $p = 0.0513$, $\eta p^2 = 0.16$). Stressed untreated animals spent less time in the open arms (12.92 ± 1.36 s vs. 26.77 ± 1.99 s in controls saline; $n = 6$–8; $p = 0.0007$; Cohen's d = 3.32; Fig. 5A) and displayed fewer entries (17.68 ± 1.49 vs. 31.29 ± 1.87 in controls saline; $n = 6$–8; =0.0003; Cohen's d = 3.28; Fig. 5B), indicating anxiety-like behavior. AVE0991 treatment prevented these stress-induced reductions, restoring

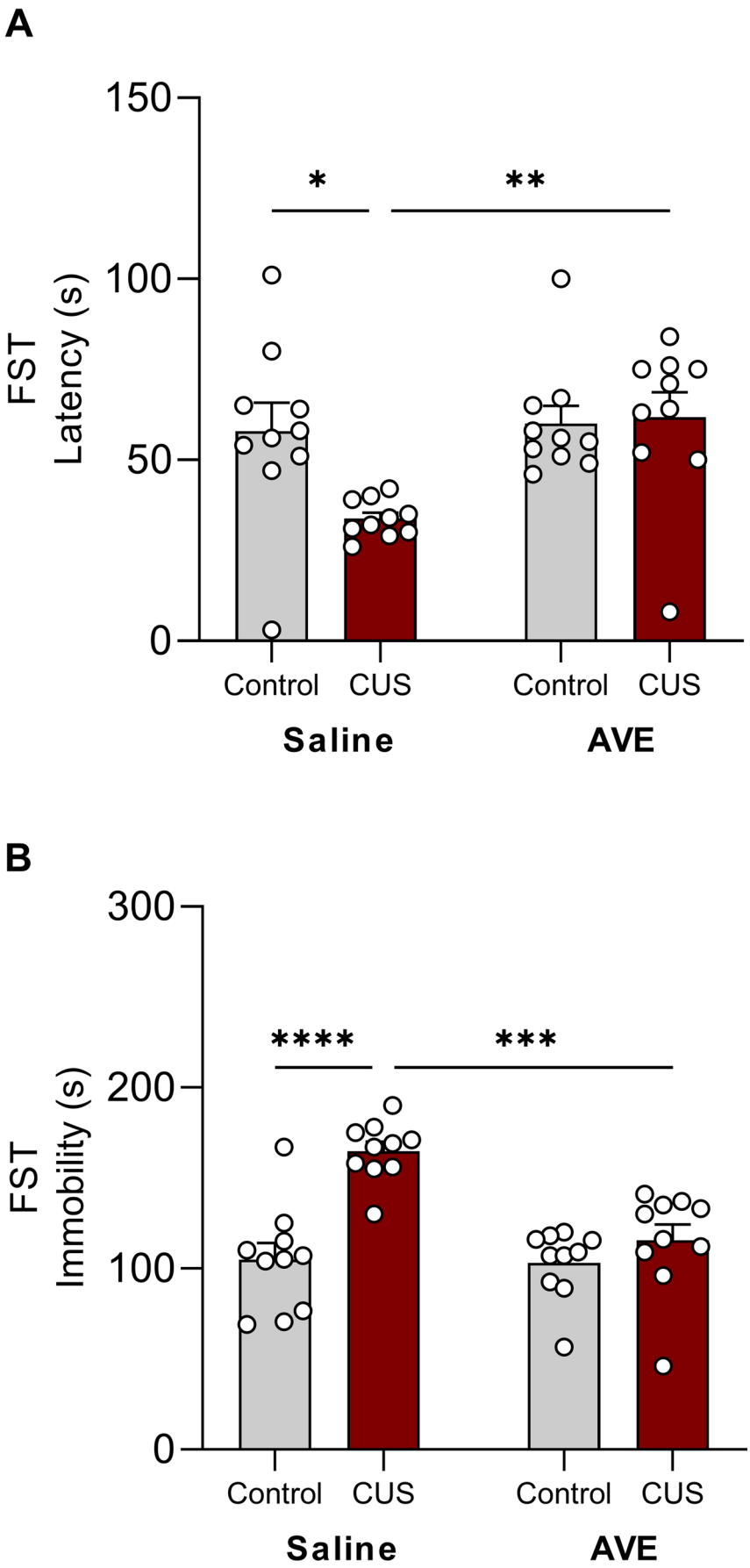



**Fig. 4** Assessment of depressive-like behavior in the forced swim test (FST) in mice under control conditions (Control), exposed to chronic unpredictable stress (CUS), or treated with AVE0991 (3 mg/kg, i.p.) under control conditions (AVE-control) or during CUS exposure (AVE-CUS). (**A**) Latency to first immobility and (**B**) total immobility time. Data are expressed as mean ± SEM ($n = 8$–10 per group). Statistical analyses were performed using two-way ANOVA followed by Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

open arm exploration in stressed mice both in time spent (23.17±2.44 s; $n=8$; $p=0.0072$; Cohen's d=1.79; Fig. 5A) and number of entries (31.17±1.92 s; $n=8$; $p=0.0002$; Cohen's d=2.83; Fig. 5B) to levels comparable to controls saline (Fig. 5). Non-stressed AVE-treated mice did not differ from controls (% time in open arms: 28.55±1.70 s and number of entries: 31.05±2.01; Fig. 5A and B). Analysis of entries into closed arms (Fig. 5C) revealed no significant differences between groups ($p>0.45$), indicating that locomotor activity in the EPM was not affected by stress or treatment. Locomotor activity was further evaluated in the open field test. Total distance traveled (Fig. 5D) did not differ between groups ($F_{(1,22)}=0.0594$, $p=0.8096$), indicating that general locomotion remained intact. However, chronic stress significantly reduced the time spent in the center of the arena ($F_{(1,22)}=9.951$, $p=0.0046$; $\eta p^2= 0.31$; Fig. 5E). Saline-treated stressed mice spent less time in the center (20.18±3.33 s, $n=6$; Fig. 5E) compared to saline controls (46.77±7.65 s; $n=6$; Fig. 5E), also indicating increased anxiety-like behavior. AVE0991 treatment partially restored central exploration in stressed mice (44.32±4.77 s; $n=8$; $p=0.0128$; Cohen's d=2.08; Fig. 5E), while non-stressed AVE-treated mice showed values similar to controls (50.53±3.85 s; $n=6$; Fig. 5E).

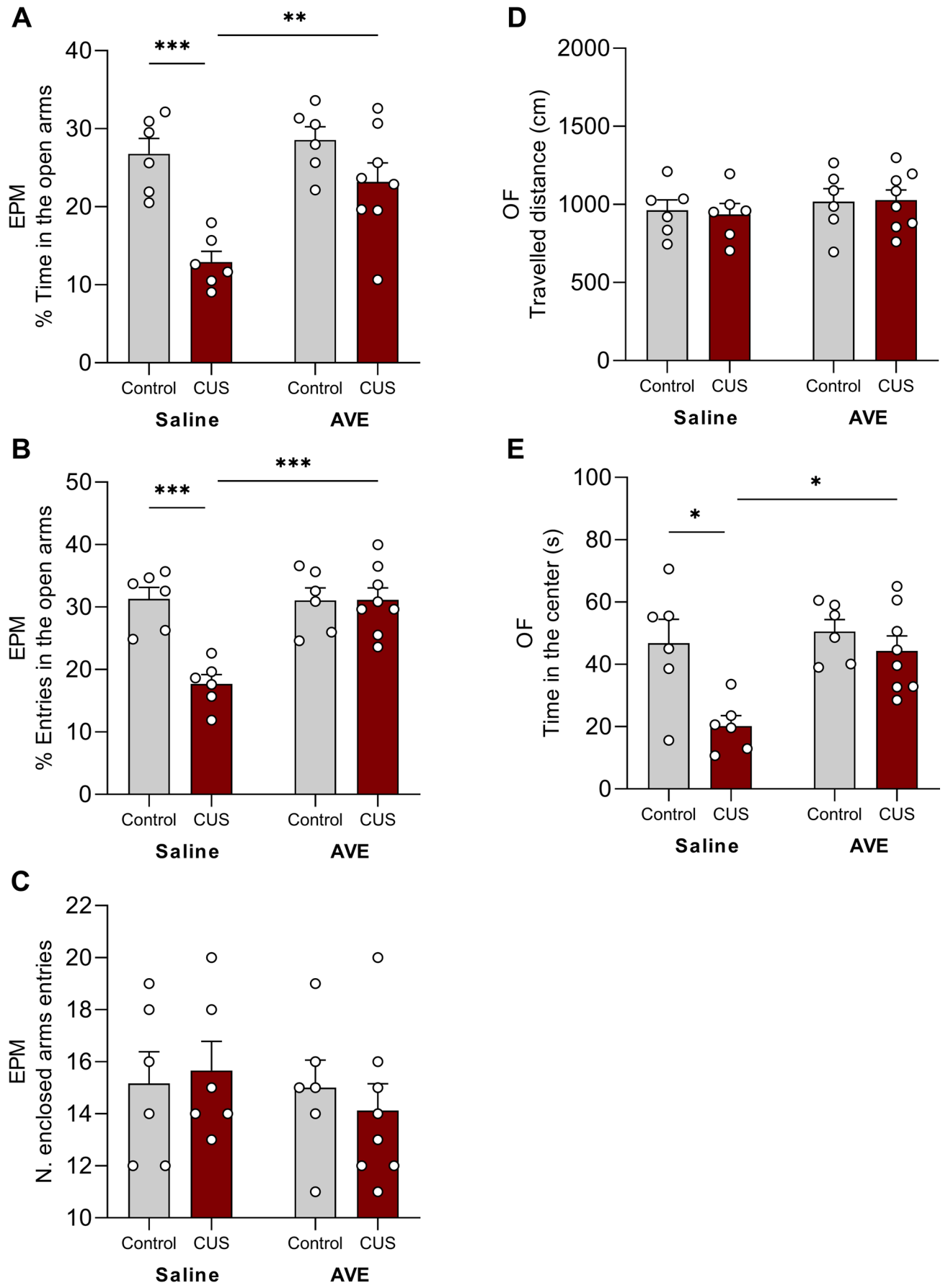
### Effect of AVE0991 in BDNF levels

In the prefrontal cortex, BDNF levels were significantly affected by CUS. Two-way ANOVA revealed a significant main effect of stress ($F_{(1,20)}=37.91$, $p<0.0001$, $\eta p^2= 0.65$). The effect of AVE0991 treatment did not alter low BNDF levels in CUS mice ($F_{(1,20)}=4.32$, $p=0.0508$, $\eta p^2= 0.18$). In addition, there was no significant interaction between stress and treatment ($F_{(1,20)}=3.39$, $p=0.0804$, $\eta p^2= 0.15$). Stressed animals displayed markedly reduced BDNF levels (90.15±5.56 pg/100 mg; $n=6$; Fig. 6A) compared with control mice (152±10.45 pg/100 mg; $n=6$; Fig. 6A). The treatment with AVE0991 in stressed animals partially restored BDNF expression (120.5±6.16 pg/100 mg; $n=6$). Non-stressed animals treated with AVE0991 showed values comparable to saline controls (153.8±7.81 pg/100 mg; $n=6$; Fig. 6A). In hippocampus two-way ANOVA revealed a significant main effect of stress ($F_{(1,20)}=26.45$, $p<0.0001$, $\eta p^2= 0.57$) and treatment ($F_{(1,20)}=6.61$, $p=0.0182$, $\eta p^2= 0.25$), as well as a significant interaction between stress and treatment ($F_{(1,20)}=8.82$, $p=0.0076$, $\eta p^2= 0.31$). Exposure to chronic stress significantly reduced hippocampal BDNF levels (201.6±7.58 pg/100 mg; $n=6$; Fig. 6B) compared with control animals (310.2±13.43 pg/100 mg; $n=6$). Treatment with AVE0991 reversed reduction of BDNF levels in stressed animals (275.8±15.41 pg/100 mg; $n=6$; $p=0.0043$; Cohen's d=2.49). In non-stressed animals, AVE0991 treatment did not significantly alter BDNF levels (304.8±15.53 pg/100 mg; $n=6$) compared with saline-treated controls (310.2±13.43 pg/100 mg; Fig. 6B). In the hypothalamus, two-way ANOVA revealed significant main effects of stress ($F_{(1,20)}=15.02$, $p=0.0009$, $\eta p^2= 0.43$) and treatment ($F_{(1,20)}=7.15$, $p=0.0146$, $\eta p^2= 0.26$), whereas the interaction between stress and treatment was not statistically significant ($F_{(1,20)}=3.01$, $p=0.0981$, $\eta p^2= 0.13$). Chronic stress significantly decreased BDNF levels (111.2±4.15 pg/100 mg; $n=6$; Fig. 6C) compared with control animals (173.3±16.03 pg/100 mg; $n=6$). This effect was reversed by AVE0991 treatment in stressed animals (160±7.25 pg/100 mg; vs. 183.8±12.83 pg/100 mg in controls saline; $n=6$; $p=0.0256$; Cohen's d=3.37). Non-stressed AVE0991-treated mice showed values similar to those observed in the control group (Fig. 6C).

## Discussion

In this study, we initially validated a 21-day CUS protocol, which proved effective in inducing an increase in plasma levels of corticosterone and glucose, accompanied by anxiety-and depressive-like behaviors assessed by the elevated plus-maze, open field, forced swim and tail suspension tests. Additionally, there was a significant reduction in BDNF levels in stressed animals, confirming the effectiveness of the protocol. In a second set of experiments, we evaluated the effects of the AVE0991, a non-peptide analogue of Mas receptor in mice subjected to the CUS model. Treatment with AVE0991 was able to attenuate the increase in corticosterone and glucose, consistent with preventing anxiety and depression behaviors. AVE0991 treatment also inhibits the reduction in BDNF in some brain regions involved in emotional control, demonstrating protective effects against the impacts of the CUS model.

Initially, we observed that our chronic stress protocol was effective, as it significantly elevated plasma corticosterone and glucose levels, widely recognized markers of HPA axis activation and the physiological response to chronic stress (Burchfield et al. 1980; Spiga et al. 2014; Adebeshin et al. 2017; Palumbo et al. 2020; Lee et al. 2021; López López et al. 2021). The increase in corticosterone is considered one of the main indicators of the validity of stress models in rodents, reflecting neuroendocrine hyperactivation similar to that observed in individuals exposed to persistent stress (Spiga et al. 2014; Palumbo et al. 2020). Concurrently, the observed hyperglycemia can be explained by excessive glucocorticoids, which stimulate gluconeogenesis and mobilize energy substrates, characterizing the metabolic adaptation to the stressor (Burchfield et al. 1980; Lee et al. 2021). Therefore, our findings are consistent with previous studies using CUS models (Adebeshin et al. 2017; López López et


A
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% Time in the open arms
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**Fig. 5** Assessment of anxiety-like behavior in mice under control conditions (Control), exposed to chronic unpredictable stress (CUS), or treated with AVE0991 (3 mg/kg, i.p.) under control conditions (AVE-control) or during CUS exposure (AVE-CUS). (**A**) Time spent in the open arms, (**B**) number of open arm entries, and (**C**) number of closed arm entries in the elevated plus maze (EPM); (**D**) total distance traveled and (**E**) time spent in the center of the arena in the open field test (OFT). Data are expressed as mean ± SEM ($n = 8$ per group). Statistical analyses were performed using two-way ANOVA followed by Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

al. 2021) and demonstrate that our protocol induces robust neuroendocrine and metabolic alterations, validating its use as an experimental model of chronic stress. The inclusion of an experimental group treated with AVE0991 resulted in attenuation of corticosterone and glucose levels, indicating a potential modulatory effect on the HPA axis and contributing to the maintenance of metabolic homeostasis.

To our knowledge, this is the first study to evaluate the effects of a Mas receptor agonist AVE0991 in a CUS protocol. However, since this compound is a non-peptidic analog of Ang-(1–7), it is possible to draw parallels for discussing our findings. Although there are no specific reports on Ang-(1–7) administration in the CUS model, several studies have demonstrated its beneficial effects on behavior in different experimental conditions and pathologies, supporting the biological and translational relevance of our results. Ang-(1–7) appears to act on the HPA axis primarily through modulation of hypothalamic nuclei such as the PVN, where the peptide and Mas receptors are abundantly expressed, a critical structure for CRH secretion and subsequent HPA axis activation (Block et al. 1988; Becker et al. 2007). Moreover, studies show that central administration of Ang-(1–7) reduces corticosterone and norepinephrine in stress models, suggesting a direct role in attenuating the endocrine stress response (Zhu et al. 2014). Additionally, the loss of Ang-(1–7)/Mas signaling under stress conditions, with reduced ACE2 and Mas receptor expression in the pituitary and adrenal glands, reinforces the hypothesis that this pathway functions as a physiological counter-regulator of the HPA axis (Nostramo et al. 2015).

Behaviorally, the CUS protocol consistently induced typical anxious-depressive phenotypic changes. Increased immobility time and reduced latency in the FST and TST confirm behavioral despair, while decreased center time in the OFT and time spent in open arms of the EPM indicate heightened anxiety. Importantly, these alterations do not stem from motor deficits but reflect genuine emotional changes. These findings support the predictive and construct validity of the model, in agreement with literature recognizing CUS as one of the most reliable preclinical models for studying mood disorders (de Kloet 2004; Palumbo et al. 2020; Luong et al. 2021; Liang et al. 2022). In contrast, AVE0991 treatment effectively reduced anxious- and depressive-like behaviors in stressed animals, evidenced by decreased immobility time and increased latency in the FST, as well as partial recovery of anxiety parameters in the EPM and OFT, including increased entries and time spent in the open arms and center of the arena. These results align with previous evidence that activation of the Ang-(1–7)/Mas pathway is associated with anxiolytic and antidepressant effects in different experimental contexts. For example, studies have shown that intracerebroventricular administration of Ang-(1–7) increases both time spent and entries into the open arms of the EPM, indicating anxiolytic effects, and improves acquisition and recall of passive avoidance responses, suggesting antidepressant effects (Bild and Ciobica 2013; Kangussu et al. 2013). Additionally, Ang-(1–7) acts on critical limbic regions, such as the amygdala and hippocampus, likely via Mas receptors, promoting neuroplasticity, enhances LTP and regulating HPA axis responses (Albrecht 2007; Zhu et al. 2014; Hellner et al. 2005), supporting the notion that AVE0991 behavioral effects reflect central modulation of stress and neuronal plasticity.

At the molecular level, CUS significantly reduced BDNF levels in the prefrontal cortex, hippocampus, and hypothalamus, essential regions for mood regulation and emotional processing (Tapia-Arancibia et al. 2004; Ping et al. 2014; Badowska-Szalewska et al. 2016; Luong et al. 2021). On the other hand, AVE0991 treatment restored these levels in the hippocampus and hypothalamus, it did not reverse the CUS-induced reduction observed in the PFC, suggesting a possible association between the observed behavioral effects and the maintenance of neuroplasticity-related and neurotrophic signaling markers. The lack of effect in the PFC may be related to the dose of AVE0991 and/or the treatment duration. These findings are consistent with previous studies showing that activation of the counter-regulatory RAS axis may influence neuroprotective, anti-inflammatory, and autophagic processes (Santos et al. 2018; Vian et al. 2017; Jiang et al. 2018). Interestingly, it was recently demonstrated that activation of hippocampal ACE2 prevents the dysbiosis-induced depression-like behavior through modulation of BDNF and Mas receptor activation (Takahashi et al. 2025). Likewise, Odaira-Satoh et al. (2025) have shown that the ACE inhibitor, captopril, attenuates depressive-like behavior by enhancing hippocampal neurogenesis through the activation of the ACE2/Ang-(1–7)/Mas receptor/AMPK/BDNF pathway (Odaira-Satoh et al. 2025).

Importantly, BDNF measurements in brain substrates were performed using a commercially available ELISA kit that detects total BDNF immunoreactivity without discriminating between its precursor form (proBDNF) and mature BDNF (mBDNF). This methodological limitation should be carefully considered when interpreting the present findings. Current evidence indicates that proBDNF and mBDNF

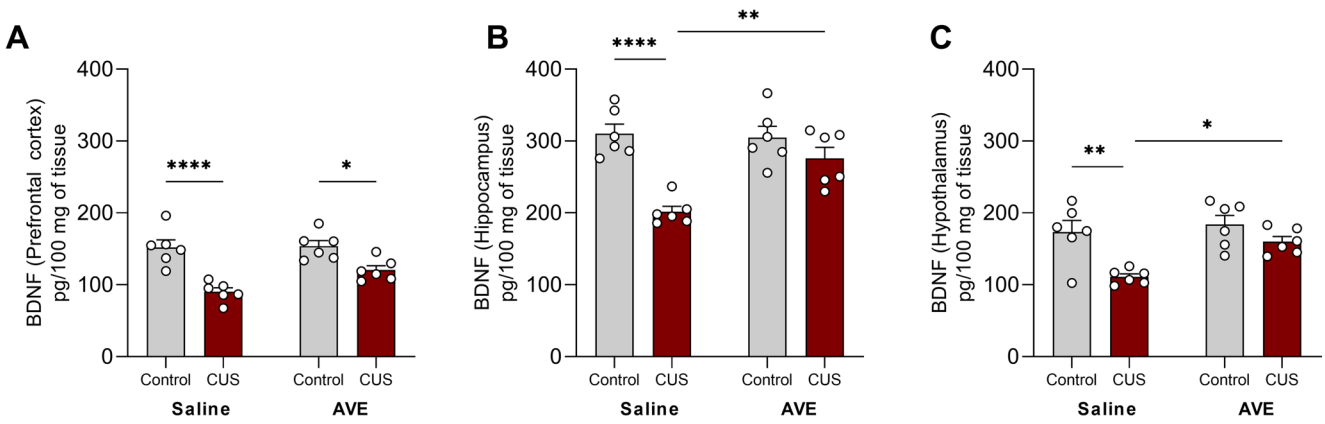


**Fig. 6** Quantification of BDNF protein levels in the prefrontal cortex (**A**), hippocampus (**B**), and hypothalamus (**C**) in mice under control conditions (Control), exposed to chronic unpredictable stress (CUS), or treated with AVE0991 (3 mg/kg, i.p.) under control conditions (AVE-control) or during CUS exposure (AVE-CUS). Data are expressed as mean ± SEM ($n = 6$ per group). Statistical analyses were performed using two-way ANOVA followed by Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$

exert functionally distinct biological effects within the central nervous system (Qiao et al. 2017). While mBDNF is generally associated with synaptic strengthening, neuronal survival, and activity-dependent plasticity through TrkB signaling (Wang et al. 2022), proBDNF has been linked to synaptic weakening, dendritic retraction, and p75NTR-dependent signaling pathways (Yang et al. 2014).

In chronic stress paradigms, studies have reported an imbalance between these neurotrophin isoforms, characterized by increased proBDNF expression accompanied by reductions in mature BDNF signaling in cortical and hippocampal regions (Xue et al. 2022; Qiao et al. 2017).

Despite the methodological constraints associated with the ELISA assay employed in the present study, animals exposed to CUS exhibited substantially reduced total BDNF levels in brain substrates, followed by restoration toward basal values after treatment. Notably, these neurochemical changes occurred in parallel with significant improvements in behavioral performance. However, because the assay does not distinguish between proBDNF and mBDNF isoforms, the present data do not allow conclusions regarding the specific contribution of each signaling pathway to the observed behavioral outcomes.

Although it has not yet been studied in the context of chronic stress, AVE0991 exhibits a broad therapeutic profile already documented in the literature. Its effects include cardiovascular protection, with reductions in fibrosis, inflammation, and hypertrophy, as well as improved cardiac function and baroreflex sensitivity (Ferreira et al. 2007); pulmonary protection, via attenuation of airway remodeling, right ventricular hypertrophy, and inflammation in asthma models (Rodrigues-Machado et al. 2013); and renal effects, including decreased diuresis and increased urinary osmolarity (Cohen-Segev et al. 2023). Additional evidence points to its role in neuroinflammation (Jiang et al. 2018) and neuroprotection, as AVE0991 treatment reduced Aβ deposition, preserved neuronal survival, improved cognitive function, and attenuated astrocyte-mediated inflammation via autophagy activation in APP/PS1 Alzheimer's mice (Jiang et al. 2018; Duan et al. 2021). In this context, AVE0991 significantly alleviated hippocampal synaptic degeneration in rats with chronic cerebral hypoperfusion (CCH). This protection might be achieved by facilitating cerebral blood flow recovery, reducing hippocampal Aβ levels, and suppressing neuroinflammatory responses (Xue et al. 2021).

Our study presents important limitations. The exclusive use of male mice limits the generalizability of the results, to avoid the interference of estrogen, only male animals were used in this study. Moreover, the absence of experimental groups treated with selective Mas antagonist (e.g., A779) limits a direct pharmacological assessment of Mas function. Finally, it is important to mention that recent discussions (Molendijk and de Kloet 2015; de Kloet & Molendijk, 2016; Gururajan et al. 2019) have questioned the usefulness of experimental models for the study of depression, especially FST (it reflects a stress-coping strategy rather - a adaptative mechanism), mainly due to the multifactorial nature and heterogeneity of this disorder. We cannot lose sight, however, of the fact that animal models are not, and never were, designed to represent the human condition in its entirety. Thus, the rodent behavioral tests, such as FST is employed as initial screening before undertaking more complex preclinical test. Moreover, these tests have good predictive validity and allow rapid and economical detection of substances with potential antidepressant-like activity.

The current study provides the first evidence for the neuroprotective effect of AVE0991 against neuropsychiatric

disorders associated with chronic stress. These results expand the previously described scope of action for this compound, whose greater stability and bioavailability relative to the endogenous Ang-(1–7) make it a promising alternative. Our findings suggest that AVE0991 exerts a protective effect against the impacts of chronic stress, modulating behavioral, neuroendocrine, and molecular responses, reinforcing its potential as an anxiolytic, antidepressant, and neuroprotective effector.

**Author contributions** **MLAF** - performed experiments, analyzed data, prepared figures, interpreted results of experiments, drafted manuscript; **LBOA** - performed experiments, analyzed data, prepared figures, interpreted results of experiments, drafted manuscript; **SCAG** - performed experiments, analyzed data, prepared figures, interpreted results of experiments, drafted manuscript; **LAC** - performed experiments, analyzed data; **LBFO** - performed experiments, analyzed data; **FAGM** - analyzed data; interpreted results of experiments, edited and revised manuscript; **MAPF** - interpreted results of experiments, edited and revised manuscript; **MB** - interpreted results of experiments, edited and revised manuscript; **RASS** - interpreted results of experiments, edited and revised manuscript; **MJCS** - interpreted results of experiments, analyzed data, prepared figures, drafted edited and revised manuscript; **LMK** - conception and design of research, analyzed data, supervision, funding acquisition, interpreted results of experiments, prepared figures, drafted edited and revised manuscript. All authors contributed to and approved the final version of manuscript.

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**Data availability** Available with restricted access.

## Declarations

**Competing interests** The authors declare no competing interests.



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