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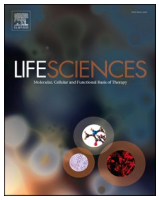

Brain-derived neurotrophic factor modulation in response to oxidative stress and corticosterone: role of scopolamine and mirtazapine

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ABSTRACT

Major Depressive Disorder (MDD) is a very complex disease, challenging to study and manage. The complexities of MDD require extensive research of its mechanisms to develop more effective therapeutic approaches. Crucial in the context of this disease is the role of brain-derived neurotrophic factor (BDNF) signaling pathway.

Aim: This manuscript aims to explore the complex relationship between MDD and BDNF signaling pathway, focusing on how BDNF is modulated in response to oxidative stress and corticosterone, known to be altered in MDD and contributing to the pathology of the disorder, when treated with scopolamine and mirtazapine.

Methods: To assess BDNF levels after the different treatment conditions, rat hippocampal slices and mice primary hippocampus and cortical cell culture were analyzed by immunofluorescence and Western blot.

Key findings: Both mirtazapine and scopolamine under stress conditions induced by hydrogen peroxide (H_2O_2) and corticosterone, had a significant impact on BDNF levels, and this was distinct in different neuronal models. Mirtazapine, especially when combined with H_2O_2, altered BDNF expression. Scopolamine when combined with both stressors also altered BDNF levels. However, its effects varied depending on the specific neuronal model and stress condition. In accordance with BDNF results, phosphorylated tropomyosin receptor kinase B (pTrkB) presented increased activation when neuronal cells subjected to stress were treated with mirtazapine or scopolamine.

Significance: Collectively, this study highlights the complex connection between these compounds, stress conditions, and BDNF/TrkB modulation, supporting the potential therapeutic effects of scopolamine and mirtazapine in modulating BDNF levels, even in stressful conditions.

1. Introduction

The World Health Organization (WHO) reports that approximately 280 million people worldwide suffer from depression [1]. Major depressive disorder (MDD) represents a heterogeneous condition, marked by shifts in mood, anhedonia, and cognitive function alterations, with consequences that can include suicide [2–4]. Challenge in dealing with this disease are the recurrence, treatment ineffectiveness, and

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absence of diagnosis, particularly in low-income countries [1,2,5]. Approximately 30% of individuals affected by MDD do not experience remission, even after multiple treatment approaches [2,6]. This supports further research into this disorder. Nevertheless, studies highlight the influence of various factors, including neurotrophic factors (particularly brain-derived neurotrophic factor, BDNF), neurotransmitters, oxidative stress, endocrine influences, and environmental factors in MDD [7,8].

Neurons are the primary producers of BDNF, initially as proBDNF [9]. ProBDNF undergoes further modifications to become mature, a 14 kDa polypeptide. BDNF forms dimers (28 kDa), that are biologically active, constructed from two identical mature peptide chains held together by noncovalent interactions [10]. This neurotrophic factor acts by interacting with two types of receptors: full length tropomyosin receptor kinase B (TrkB-FL) and the p75 neurotrophic factor receptor (p75 NTR), the latter having a low binding affinity for mature BDNF [11]. Once bound to TrkB-FL, BDNF binds to TrkB, causing receptor dimerization, and induces TrkB autophosphorylation at specific tyrosine residues, enabling protein binding and activation of intracellular signaling cascades [12].

BDNF is involved in diverse functions: promotes the growth and development of neurons during early brain development, contributing to the formation of neuronal connections and circuits [13], regulates synaptic plasticity [14], is implicated in mood regulation and in memory [15,16], promotes neurogenesis [17], and helps to mitigate damage caused by oxidative stress, inflammation, and other harmful processes in the brain [18]. Also, the activity of various neurotransmitters, such as glutamate, is influenced by BDNF, which can modulate the balance and function of neurotransmitter systems [19].

This neurotrophic factor has been associated with psychiatric and neurological conditions that include MDD, anxiety disorders, schizophrenia, Rett syndrome and other neurodegenerative diseases [9,20]. The link between BDNF and MDD is an extensive area of research, with still much left to understand. Nevertheless, the neurotrophic hypothesis of depression highlights the connection between decreased BDNF levels and an increased likelihood of depression [21]. In fact, several antidepressants can enhance BDNF expression, contributing to their effects [22]. However, the literature regarding BDNF assessment presents inconsistencies due to lack of reproducibility reported in many studies [23].

The several pathological mechanisms involved in MDD, such as imbalances in synaptic plasticity, are intensified by high levels of oxidative stress [24,25]. This vulnerability makes oxidative stress a key factor in MDD [25]. Hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis is also one of the most consistent findings in MDD. This hyperactivity results in an increased level of glucocorticoids [26]. In individuals with MDD, the functionality of the glucocorticoid becomes compromised, resulting in a diminished capacity for negative feedback regulation, triggering an increased synthesis of glucocorticoids [27].

Numerous studies suggest that reduced BDNF levels contribute to increased oxidative stress, highlighting a potential protective role for BDNF in mitigating neuronal oxidative damage [28,29]. Other research studies are also trying to understand the complex relationship between the activity of the HPA axis and BDNF. Specifically, stress-induced hyperactivity of the HPA axis and the consequent increase in glucocorticoid levels have been shown to decrease the expression of BDNF. On the other side, BDNF regulates HPA axis, reducing its activity [30].

The aim of this study is to further investigate the effects of mirtazapine and scopolamine on primary hippocampal and cortical cell cultures, and acute hippocampus slices, following initial findings that these compounds attenuated H_2O_2-induced stress but not corticosterone-induced stress in SH-SY5Y human neuroblastoma and HT22 mice hippocampal cell lines [31,32]. Both cortical and hippocampal neurons play significant roles in depression, contributing to its complex pathophysiology. For example, reductions in the size of the hippocampus and medial prefrontal cortex are prominent neural features in the pathology of MDD [33]. The prefrontal cortex regulates mood, decision-making, and social behavior, often showing reduced activity in depression [34]. The hippocampus, vital for emotional regulation, frequently exhibits shrinkage in depressed individuals, contributing to symptoms like emotional instability [35].

Mirtazapine is an antidepressant, more specifically referred to as a noradrenergic and specific serotonergic antidepressant [36]. Primarily, mirtazapine is indicated for the treatment of MDD [37]. Gaining a better understanding of its mechanism of action offers significant advantages for clinical practice. Scopolamine is primarily approved for postoperative nausea and vomiting and motion sickness. However, this competitive antagonist of 5-HT3 serotonin receptors and nonselective muscarinic antagonist has also evidence of antidepressant effects in patients with MDD and bipolar depression [38,39]. When added to antidepressants, scopolamine can effectively relieve the symptoms of patients with severe depression [40]. Despite this reported antidepressant activity, scopolamine also presents contradictory effects, namely regarding its action on BDNF [41]. Thus, studying this drug in this context aimed to provide a clearer understanding of its mechanism of action. Additionally, comparing mirtazapine, a clinically approved antidepressant, with scopolamine, a potential antidepressant that lacks robust clinical evidence, can offer valuable insights and a meaningful comparison.

2. Materials and methods

2.1. Materials

An inventory of the tested compounds, and its solvents are presented in Table S1. Additionally, Table S2 provides a detailed list of the primary and secondary antibodies used in Western blot and immunofluorescence methodologies. Regarding solvents, for individual compounds dissolved in H_2O and DMSO, the maximum solvent concentration was 0.1 %. When combining two drugs in DMSO, the maximum DMSO concentration was 0.2 %. The different biological models used were exposed to the test drugs/combinations or equivalent amount of solvent (controls: vehicle-treated conditions) during 6 h or 24 h. The text points out material suppliers when they are first referenced.

2.2. Animals

For the experiments with hippocampal slices, 4 male adult Wistar rats (about 6–8 weeks old) were used. Wistar rats were purchased from Charles River (Barcelona, Spain) and kept under standardized temperature (22–24 °C), humidity (55 ± 10 %) and lighting conditions, with ad libitum access to water and food at the Rodent Facility of Instituto de Medicina Molecular João Lobo Antunes. This project was also approved by the internal committee (ORBEA) of Instituto de Medicina Molecular João Lobo Antunes, under license number AWB_2016_17, and the Portuguese Animal Ethics Committee (DGAV - Direção Geral de Alimentação e Veterinária), license number 003838\2013. Throughout the experimental work, every effort was made to reduce the number of animals sacrificed. Under profound isoflurane anesthesia, rats were sacrificed by decapitation.

For the experiments with primary neuronal cortical or hippocampal cell cultures, 3C57BL/6 mice embryos at E16.5 were collected through caesarean section of 3 pregnant C57BL/6 mice, euthanized by cervical dislocation. C57BL/6 mice purchased from Charles River (Barcelona, Spain) were maintained under a 12 h light/12 h dark cycle with free access to food and water at the Animal Facility of Instituto de Investigação e Inovação em Saúde (i3S), under the license number APP_2023_18. All procedures were approved by DGAV (license reference 0421/000/000/2017) and carried out with the permission of the i3S's animal ethical committee.

All animals were handled in accordance with Portuguese legislation (DL 113/2013) and European Community Guidelines for Animal Care (European Union Council Directive – 2010/63/ EU). Each animal was

used for each independent experiment.

2.3. Acute hippocampal slices preparation

Acute slices are suited for same-day studies because it is challenging to provide them with the same level of oxygen and nutrients that they would receive in a living brain. They preserve synaptic connections close to their in vivo state at the time of harvest, making them a good choice for these experiments [42]'.Thus, for studying BDNF expression, acute slices were selected, for a period of 6 h of drug exposure.

After decapitation of the animal, the brain was quickly removed and maintained in an ice-cold, oxygenated artificial cerebrospinal fluid (aCSF, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 124 mM NaCl, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 10 mM glucose, with a pH of ~7.4 when aerated with 95 % O_2–5% CO_2) for a few mins. Before further dissections. Then, the hippocampi were isolated and cut into 300-μm thick slices using the McIlwain Tissue Chopper (Campden Instruments, Loughborough, England). The slices were maintained in artificial cerebrospinal for 1 h for energy charge recovery and then exposed for 6 h to the test drugs or equivalent amount of solvent (controls), which were added to the aCSF. Throughout the dissection process, the brain tissue and hippocampal slices were kept in ice-cold, oxygenated aCSF and visualized under a dissecting scope.

2.4. Neuronal cortical and hippocampal cell culture from mice

Neuronal cell culture is also widely used to study the structure and function of neurons in a controlled environment. [43]. In contrast to acute hippocampal slices, these cultures allowed us to explore the effect of the drugs towards only neurons and to expand the drug exposure duration to an extended 24 h timepoint. Additionally, we also tried a period of 48 h drug exposure, but cells began to decline their viability. Consequently, we studied a period of 24 h, as it might, in theory, provide sufficient time to observe some extent of cellular recovery from damage.

Primary neuronal cortical and hippocampal cell cultures were obtained from the cortex and hippocampus of C57BL/6 mice embryos, respectively. More specifically, in this study, prenatal mice at embryonic day 16.5 (E16.5) were used, since at this stage the brain is less susceptible to damage [44].

The isolated cortex and hippocampus were digested with trypsin (1.5 mg/mL), washed with HBSS (Hank's balanced Salt Solution, Gibco® Life Technologies) containing 10 % (*v*/v) inactivated (30 min at 56 °C) fetal bovine serum (FBS) and then washed with HBSS to remove FBS. The tissue was then dissociated in neurobasal medium (Gibco® Life Technologies) using a pipette and cells were plated at a density of 90 × 10^4 cells/cm^2 in pre-coated plates with poly-D-lysin (Sigma-Aldrich). Cells were cultured in neurobasal medium supplemented with 2 % (*v*/v) NeuroCult™ SM1 neuronal supplement (StemCell), GlutaMAX (0.5 mM), glutamate (0.025 mM) and gentamycin (50 μg/mL) (all Gibco® Life Technologies), at 37 °C with 5 % CO_2, for 4 days before the exposure to the different treatments.

2.5. Immunofluorescence analysis

After the exposure to different treatments, primary neuronal hippocampal cell cultures were fixed in 4 % PFA for 10 min, followed by PBS washes and Triton X-100 0.1 % in PBS for permeabilization for 10 min. After that, the cells were blocked with 5 % bovine serum albumin (BSA; NZYtech, Lisbon, Portugal) for 1 h and then treated with primary antibodies overnight at 4 °C. Afterwards, cells were washed again with PBS, and secondary antibodies were added for 1 h in blocking solution. Antibodies used are present in Table S2. Then, Alexa Fluor™ 647 Phalloidin (1:40 dilution; Invitrogen) was added to the cells for 30 min, and the nuclei were stained with Hoechst 33342 (1:5000 dilution; Thermo Fisher Scientific), for 10 min. Finally, mounting medium (Ibidi, Gräfelfing, Germany) was added to each well. Immunostaining assessment was conducted using the Operetta CLS™ High-Content Analysis System (PerkinElmer, Waltham, Massachusetts, USA), using a 20× objective to acquire 25 images for each well. Following image acquisition, fluorescence intensity was quantified through analysis in the Ilastik 1.3.3 and CellProfiler 4.2.6 software.

2.6. Western blot analysis

For the experiment with rat hippocampal slices, after the treatment exposure, total proteins were extracted from these slices using acid-extraction buffer (50 mmol/L sodium acetate, 1 mol/L NaCl, 0.1 % Triton x100, glacial acetic acid until pH 4.0 is reached), containing 1:100 diluted protease inhibitors cocktail (Mini-Complete EDTA-free; Roche Applied Science, Penzberg, Germany). Protein concentrations were measured using DC protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (20 μg) were separated on 15 % polyacrylamide gels and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, UK). After that, membranes were blocked with 3 % BSA in TBS-T (TBS/0.1 % Tween 20) for 1 h, at RT. Protein detection from the membrane was performed with specific primary antibodies, incubated overnight at 4 °C with blocking solution. Then, the membranes were washed with TBS-T and incubated for 1 h (RT) with the secondary antibodies, in blocking solution. Antibodies used are present in Table S2. Finally, membranes were washed with TBS-T and developed with an enhanced chemiluminescence (ECL) Western Blot Detection Reagent (Bio-Rad), visualized using the chemiluminescence detection imager Amersham 800 (GE Healthcare). The relative intensities of protein bands were analyzed using ImageJ Software.

For the experiment with mice neuronal cortical cell culture, after the treatment exposure, total proteins were extracted using RIPA buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS), with 1:100 protease inhibitors cocktail and phosphatase inhibitors (Bimake, Huissen, The Netherlands). Protein concentrations were measured in the supernatant of the protein extracts after 10 min centrifugation at 14,000 rpm, using Pierce BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of protein (15 μg) were separated on 15 % polyacrylamide gels, and subsequently transferred using iBlot™ 2 Dry Blotting System (Thermo Fisher Scientific). After that, membranes were blocked with 5 % BSA in TBS-T (TBS/0.1 % Tween 20) for 1 h, at RT. Protein detection from the membrane was performed with specific primary antibodies, incubated overnight at 4 °C in 1 % BSA blocking solution. Then, the membranes were washed with TBS-T and incubated for 1 h (RT) with the secondary antibody, in 0.5 % blocking solution. Antibodies used are present in Table S2. To finalize, membranes were washed with TBS-T and developed with WesternBright Quantum (Chemiluminescent HRP Substrate, Advansta, San Jose, California, USA), visualized using the chemiluminescence detection imager Chemidoc (Bio-Rad). The relative intensities of protein bands were quantified using ImageJ Software.

2.7. Statistical analysis

The obtained data was quantitatively presented as the mean ± standard error of the mean (SEM), based on three independent experiments, except in rare exceptions where only two independent experiments were possible to be conducted, indicated in the figure legends. Statistical analysis between drug combinations vs. individual stimuli, and between all the tested conditions vs. each respective control was conducted using one-way Analysis of Variance (ANOVA), followed by post hoc analysis via Tukey's multiple comparisons test. Additionally, F values are present in the supplementary table 3 (S3). Statistical analysis, graphical construction, and calculations of IC_{50} values were carried out using software GraphPad Prism 9 (San Diego, California, USA). Statistical significance was considered at $p < 0.05$.

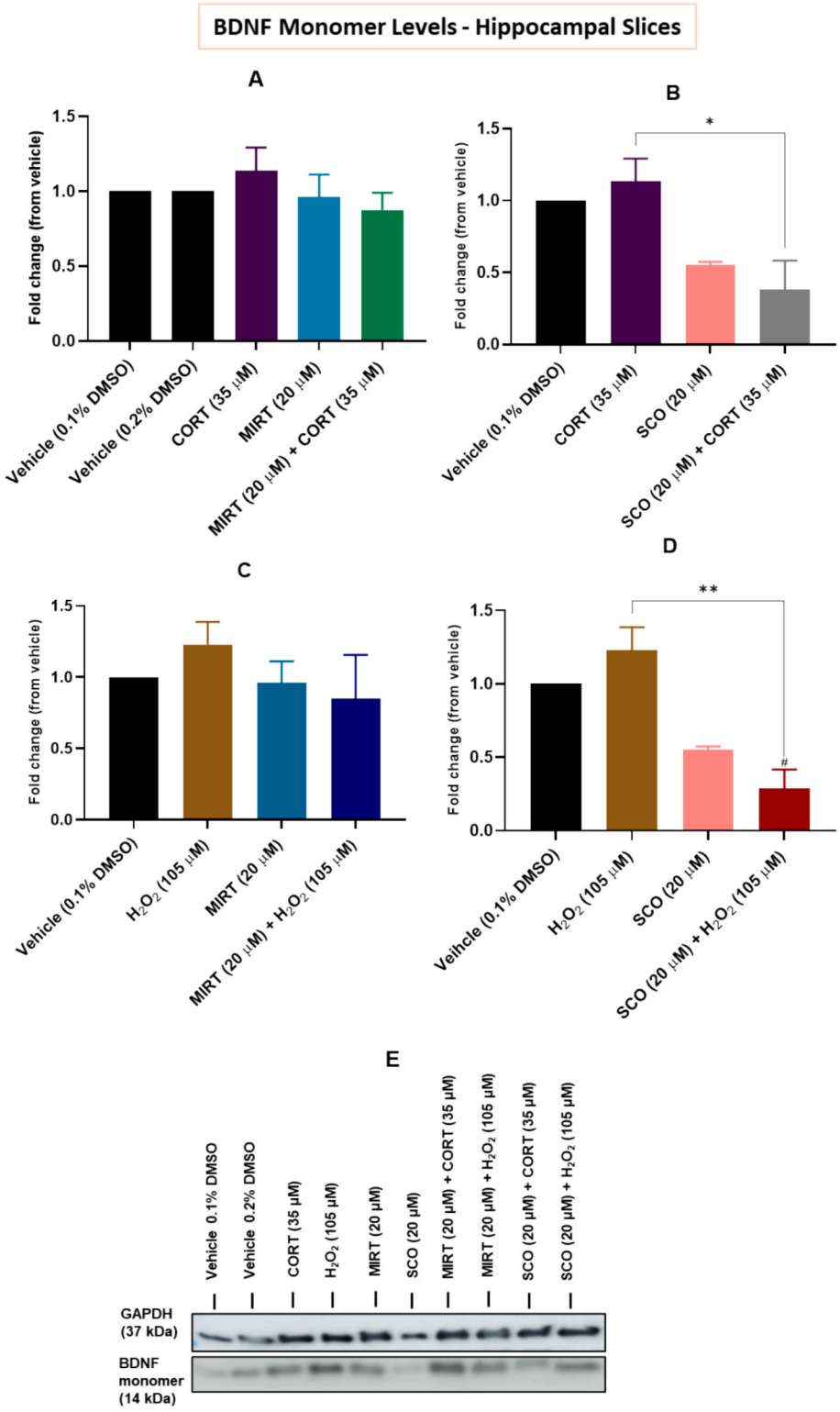


Fig. 1. Western blot analysis of BDNF monomer levels in acute hippocampal slices treated for 6 h with mirtazapine 20 μM combined with (A) corticosterone 35 μM and (C) H_2O_2 105 μM, and scopolamine 20 μM combined with (B) corticosterone 35 μM and (D) H_2O_2 105 μM, after incubation with anti-BDNF primary antibody, previously detailed in the materials and methods section. Representative blot represented in (E). CORT: corticosterone; MIRT: mirtazapine; SCO: scopolamine. For clearer understanding and analysis, the results obtained for H_2O_2, mirtazapine, scopolamine, and corticosterone (for the same timepoint) are repeated in separate graphs. Statistically significant * $p < 0.05$ and ** $p < 0.01$ vs. the aggressor (H_2O_2 105 μM or corticosterone 35 μM), and # $p < 0.05$ vs. vehicle; three independent experiments, except for scopolamine+H_2O_2, as described in materials and methods section. The image shown represents the most representative blots, based on graphical results. Complete sets of blots, organized by independent experiments, are available in the supplementary file for reference.

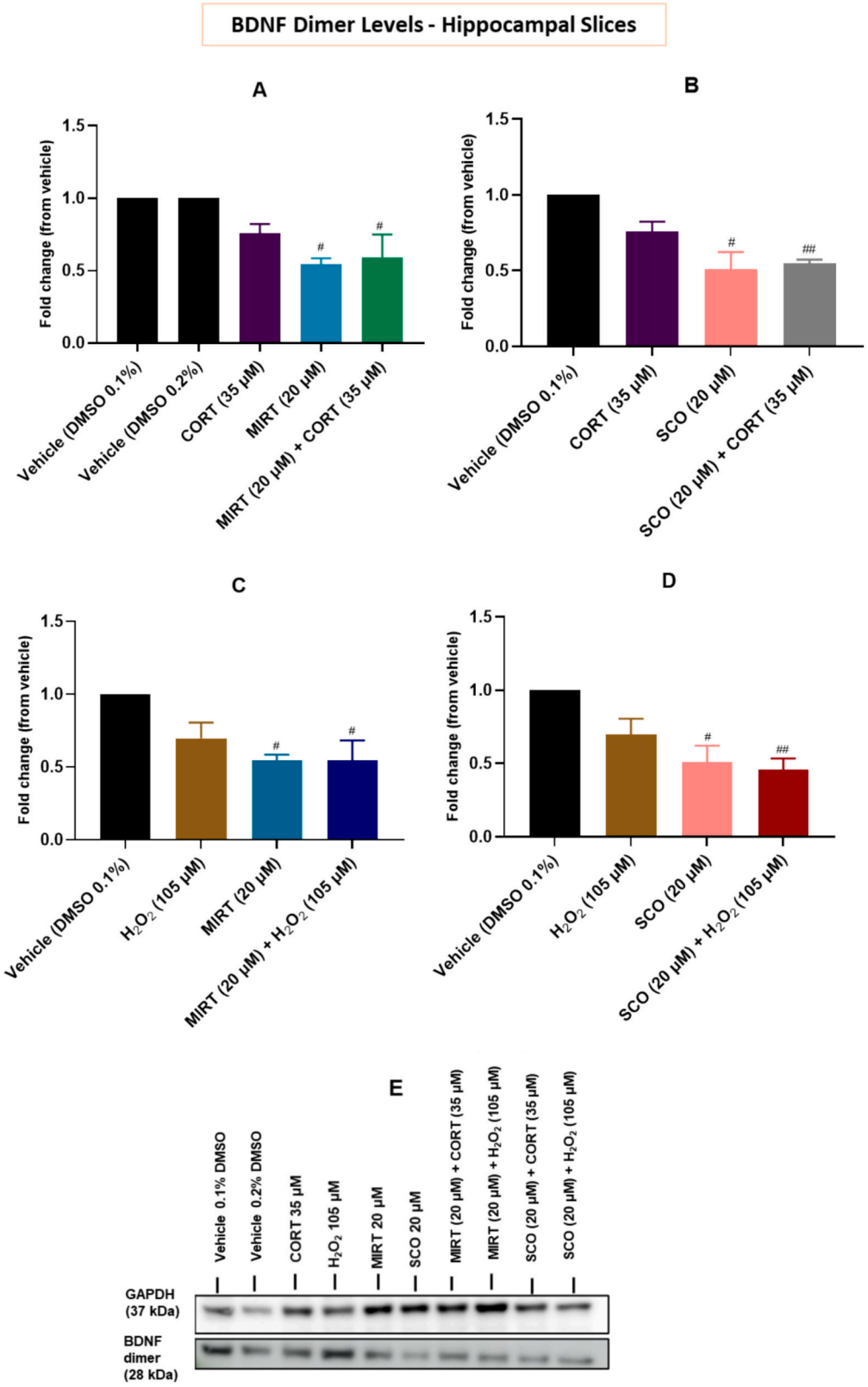


Fig. 2. Western blot analysis of BDNF dimer levels in acute hippocampal slices treated for 6 h with mirtazapine 20 μM combined with (A) corticosterone 35 μM and (C) H_2O_2 105 μM, and scopolamine 20 μM combined with (B) corticosterone 35 μM and (D) H_2O_2 105 μM, after incubation with anti-BDNF primary antibody, previously detailed in the materials and methods section. Representative blot represented in (E). CORT: corticosterone; MIRT: mirtazapine; SCO: scopolamine. For clearer understanding and analysis, the results obtained for H_2O_2, mirtazapine, scopolamine, and corticosterone (for the same timepoint) are repeated in separate graphs. Statistically significant # $p < 0.05$ and ## $p < 0.01$ vs. vehicle; three independent experiments. The image shown represents the most representative blots, based on graphical results. Complete sets of blots, organized by independent experiments, are available in the supplementary file for reference.

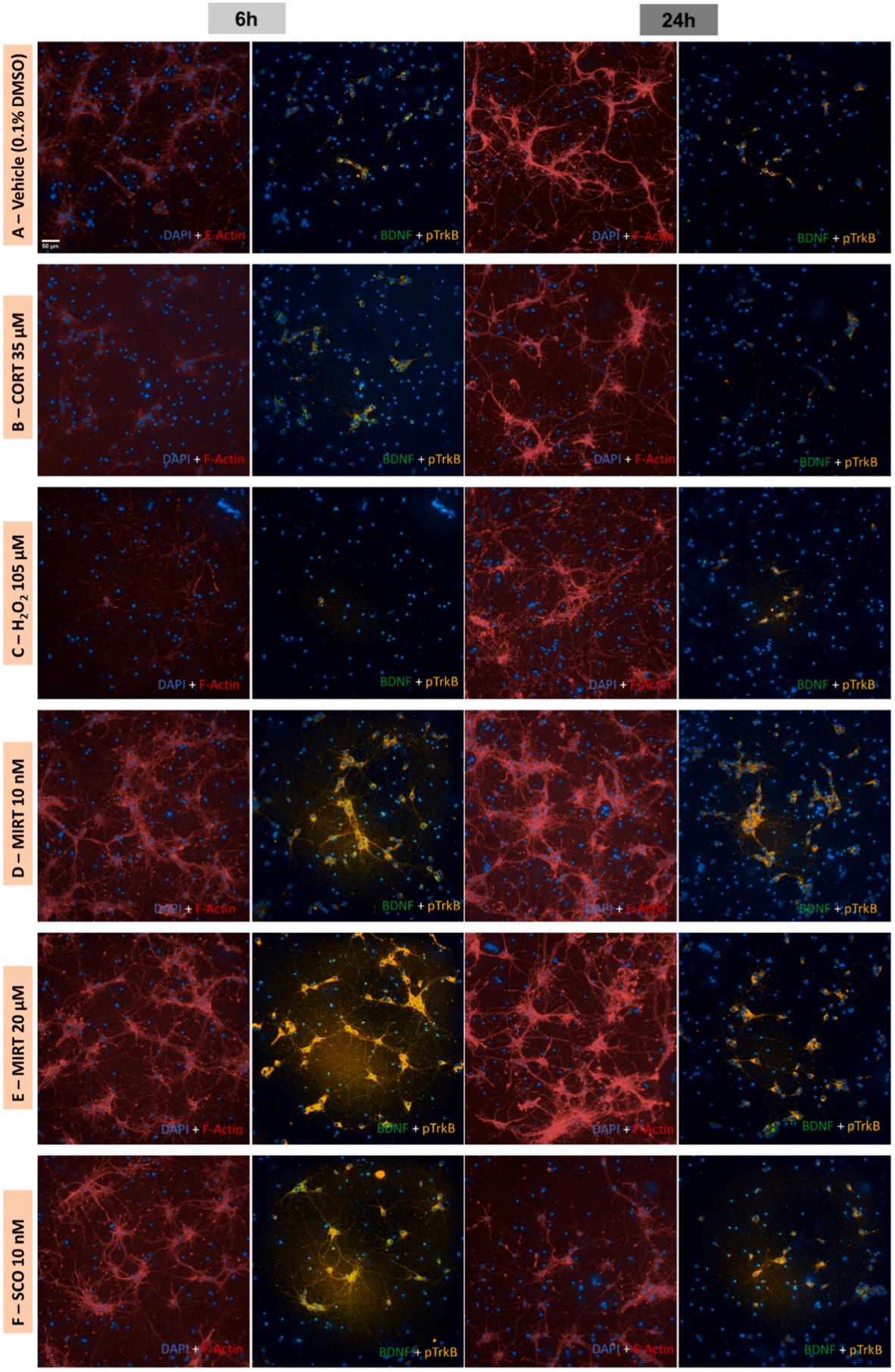

6h
24h
A – Vehicle (0.1% DMSO)
B – CORT 35 µM
C – H_2O_2 105 µM
D – MIRT 10 nM
E – MIRT 20 µM
F – SCO 10 nM
DAPI + F-Actin
BDNF + pTrkB
50 µm

Fig. 3. Representative images (200× total magnification) of neuronal hippocampal cell culture incubated for 6 h (left) or 24 h (right) with (A) vehicle (0.1 % DMSO), (B) corticosterone 35 μM, (C) H_2O_2 105 μM, (D) mirtazapine 10 nM, (E) mirtazapine 20 μM, (F) scopolamine 10 nM, (G) scopolamine 20 μM, (H) corticosterone 35 μM + mirtazapine 10 nM, (I) corticosterone 35 μM + mirtazapine 20 μM, (J) corticosterone 35 μM + scopolamine 10 nM, (K) corticosterone 35 μM + scopolamine 20 μM, (L) H_2O_2 105 μM + mirtazapine 10 nM, (M) H_2O_2 105 μM + mirtazapine 20 μM, (N) H_2O_2 105 μM + scopolamine 10 nM, (O) H_2O_2 105 μM + scopolamine 20 μM, after immunostaining with Hoechst 33342 (blue), Alexa Fluor™ 647 Phalloidin (red), anti-BDNF (conjugated with Alexa Fluor™ 488, green) and anti- pTrkB (conjugated with Alexa Fluor™ 568, orange) primary antibodies. CORT: corticosterone; MIRT: mirtazapine; SCO: scopolamine. Scale bar: 50 μm (present in the first image).

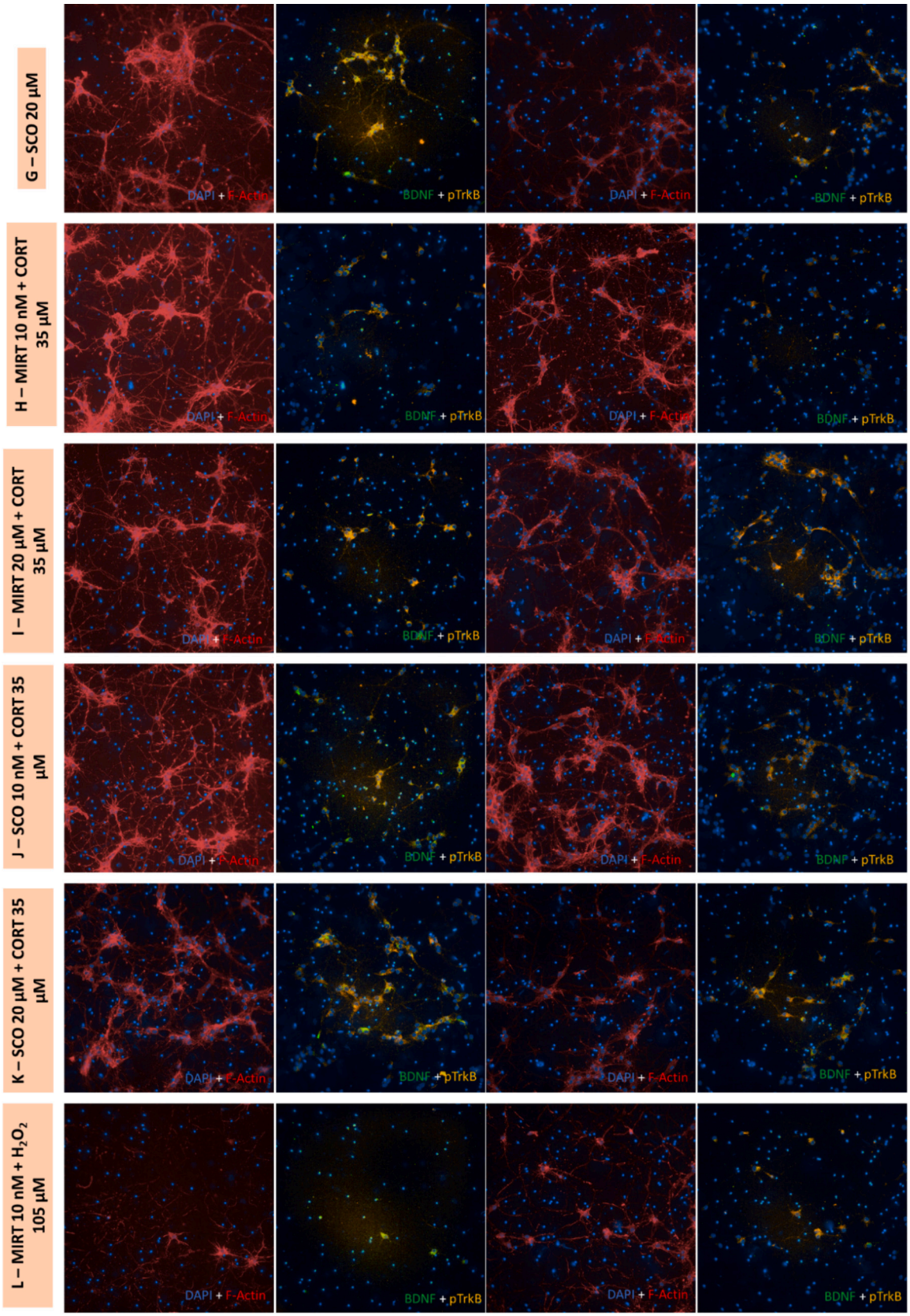


Fig. 3. (*continued*).

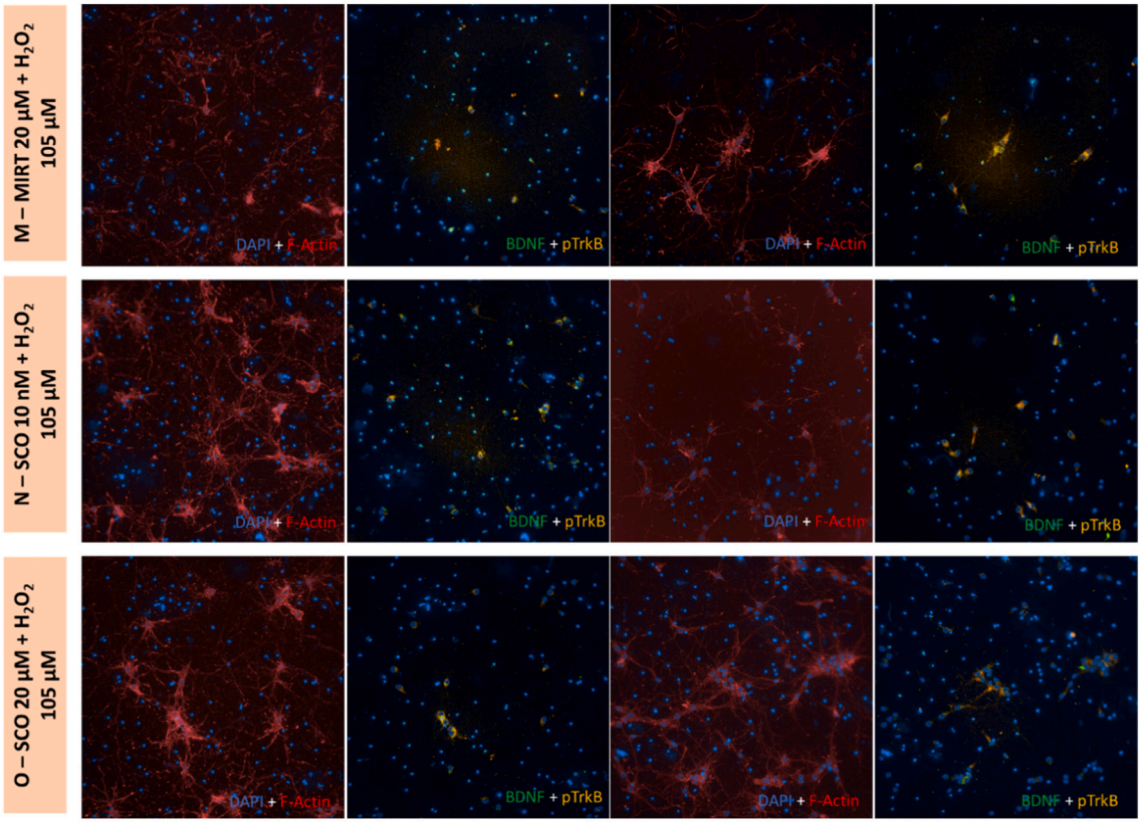


Fig. 3. (*continued*).

3. Results

To elucidate the impact of mirtazapine and scopolamine on BDNF levels, and in the context of corticosterone and H_2O_2 stress stimuli, we used acute hippocampal slices and primary neuronal cell cultures. Specifically, BDNF levels in mice neuronal hippocampal cells were examined using immunofluorescence, while also assessing mice neuronal cortical cells and acute hippocampal slices through Western blot analysis. Additionally, we expanded our study to include the assessment of phosphorylated TrkB (pTrkB) - the BDNF-induced activated form of TrkB - in mice neuronal hippocampal cells using immunofluorescence.

The concentrations of mirtazapine was based on previous studies [32,45–47]. In these studies, no toxicity was observed, being reported beneficial effects such attenuation of cellular viability decrease. Particularly with scopolamine, we aimed to use a range of concentrations similar to the previously tested with mirtazapine, for a better comparison [32,48].

3.1. BDNF levels after mirtazapine, scopolamine, and cellular stress exposure in acute hippocampal slices

To investigate the impact of mirtazapine and scopolamine, alone or after H_2O_2 or corticosterone stimuli on the mature BDNF levels in acute hippocampal slices, the drugs were used at a concentration of 20 μM for 6 h, and H_2O_2 and corticosterone stimuli were applied at concentrations of 105 μM and 35 μM, respectively, corresponding to their medium IC_{50} values in HT22 cellular viability assays, as previously determined [31]. Figs. 1 and 2 present the results of the Western blot analysis for the BDNF monomer and dimer, respectively. In Fig. S1 and S2, the whole Western blots from the quantitative analysis are present.

These findings indicate that scopolamine either alone or combined with corticosterone or H_2O_2 (one-way ANOVA followed by Tukey test; $p = 0.9435$ vs. H_2O_2; $p = 0.0239$ vs. corticosterone), reduced BDNF levels, an effect which was not observed when using corticosterone or H_2O_2 alone (Figs. 1 and 2B, D). However, mirtazapine did not demonstrate a significant impact on BDNF monomer levels (Figs. 1A, C), despite a reduction in BDNF dimer levels (Figs. 2A, C).

3.2. BDNF and phosphorylated TrkB levels after mirtazapine, scopolamine, and cellular stress exposure in neuronal hippocampal cell culture

To explore the 6 h and 24 h effect of mirtazapine and scopolamine, alone or after H_2O_2 and corticosterone stimulus on BDNF (Figs. 3–5) and pTrkB-FL (Figs. 3,6,7) levels in neuronal hippocampal cells, mirtazapine and scopolamine were added to the cells in a concentration of 10 nM and 20 μM. H_2O_2 and corticosterone were added in a concentration of 105 μM and 35 μM, respectively, as explained above. The immunofluorescence results are presented in Figs. 3–7. Specifically, Fig. 3 features representative images of the stained cells, while Figs. 4 to 7 depict the quantitative data derived from the analysis. The images (Fig. 3) were selected from a higher number of images as representative pictures and cannot depict the whole variability commonly observed in these biological systems. This variability is present in the quantitative data.

Regarding the assessment of BDNF levels (Figs. 3–5), these findings indicate that overall, mirtazapine (20 μM) combined with corticosterone or H_2O_2 exhibited a significant increase in BDNF expression when compared to cells with corticosterone (one-way ANOVA followed by Tukey test; $p = 0.0007$ (6 h) and $p \leq 0.0001$ (24 h)) or H_2O_2 (one-way ANOVA followed by Tukey test; $p \leq 0.0001$ (6 h)). Moreover, even at a lower concentration of mirtazapine (10 nM), an enhancement in BDNF expression was observed when compared to the H_2O_2 group (one-way ANOVA followed by Tukey test; $p \leq 0.0001$ (6 h)), but not with corticosterone, suggesting that a higher concentration of mirtazapine is needed to counteract the effects of corticosterone (Figs. 4 A,C). The

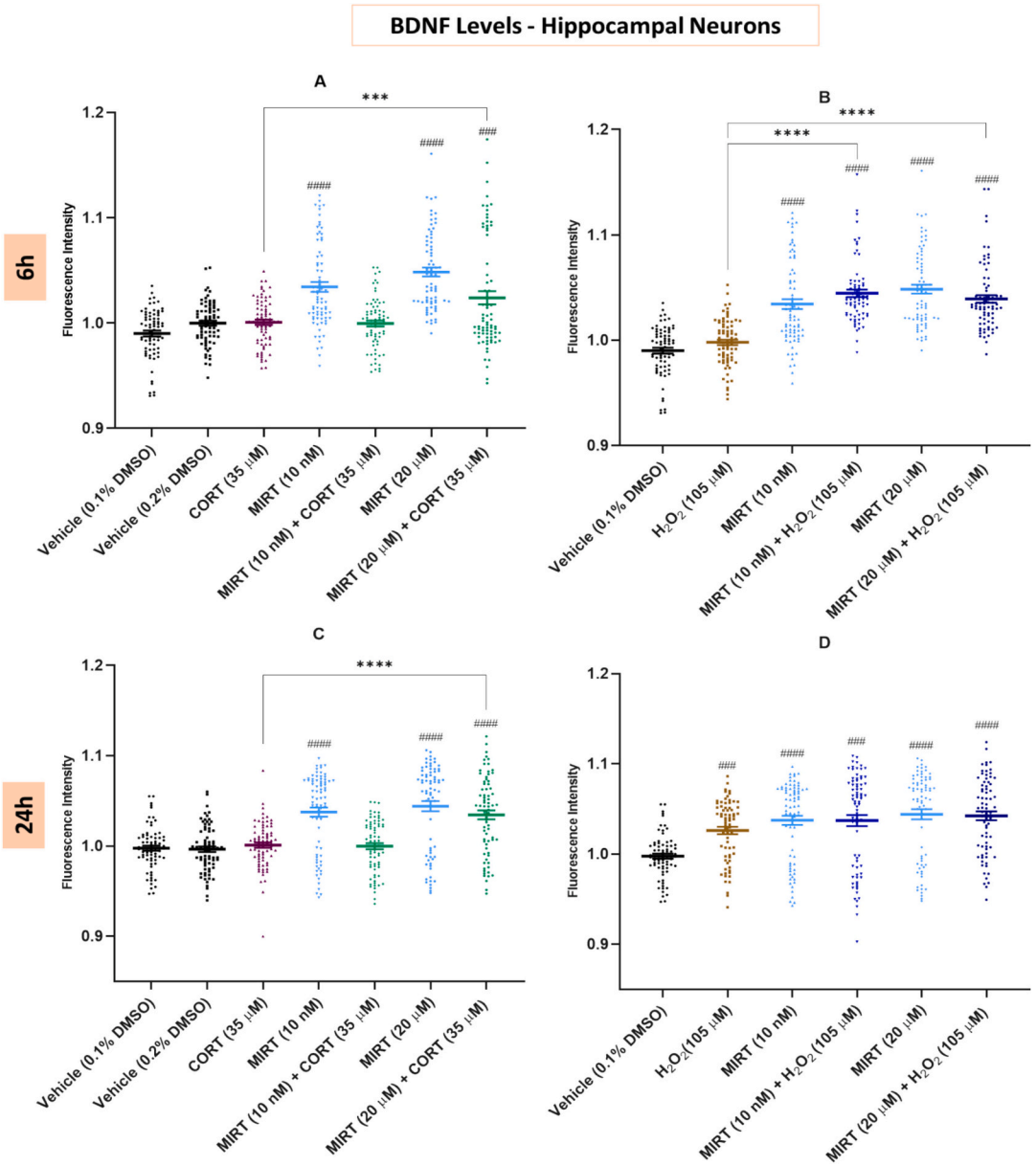


Fig. 4. Fluorescence intensity of BDNF expressed in neuronal hippocampal cell culture incubated for 6 h (A, B) or 24 h (C,D) with mirtazapine 10 nM or 20 μM combined with (A, C) corticosterone 35 μM and (B, D) H_2O_2 105 μM, after immunostaining, as previously detailed in the materials and methods section. For clearer understanding and analysis, the results obtained for H_2O_2, mirtazapine, scopolamine, and corticosterone (for the same timepoint) are repeated in separate graphs. Statistically significant ### $p < 0.001$ and #### $p < 0.0001$ vs. vehicle, *** $p < 0.001$ and **** $p < 0.0001$ vs. the aggressor (H_2O_2 105 μM or corticosterone 35 μM); three independent experiments. CORT: corticosterone; MIRT: mirtazapine; SCO: scopolamine.

enhancement in BDNF expression was particularly pronounced at the 6 h time point for the combination of mirtazapine with H_2O_2 (Fig. 4 B, D). When mirtazapine was used combined with corticosterone, it was noted that there were similar responses at both time points 6 h and 24 h (Fig. 4 A,C). In addition to mirtazapine, scopolamine also enhanced BDNF expression when compared to cells treated with corticosterone (one-way ANOVA followed by Tukey test; $p \leq 0.0001$ (6 h, both concentrations); $p = 0.020$ for 10 nM, and $p < 0.0001$ for 20 μM (24 h)) or H_2O_2 (one-way ANOVA followed by Tukey test; $p \leq 0.0001$ for 10 nM, and $p = 0.0004$ for 20 μM (6 h), and $p = 0.0023$ for 20 μM (24 h)), Fig. 5.

In the assessment of pTrkB levels (Figs. 3, 6, 7), these data revealed that at 6 h, the combination of mirtazapine with either corticosterone or H_2O_2 did not yield a significant enhancement in pTrkB levels compared to the stimulus with corticosterone or H_2O_2 (Fig. 6A,B). When compared to the corticosterone alone, mirtazapine (20 μM) even exhibited a tendency to decrease pTrkB levels, though it did not reach statistical significance (Fig. 6A). In contrast, at 24 h, mirtazapine (20 μM) demonstrated an enhancement in pTrkB levels when compared to the H_2O_2 group (one-way ANOVA followed by Tukey test; $p \leq 0.0001$), Fig. 6D. However, it's noteworthy that mirtazapine, when incubated independently, did indeed enhance pTrkB levels, compared to the control group. In turn, scopolamine enhanced pTrkB levels when compared to corticosterone (one-way ANOVA followed by Tukey test; $p = 0.0009$ for 10 nM and $p < 0.0001$ for 20 μM (6 h); $p < 0.0001$ for both concentrations (24 h)) and H_2O_2 (one-way ANOVA followed by Tukey test; $p = 0.0017$ for 10 nM and $p = 0.0003$ for 20 μM (6 h); $p < 0.0001$ for 20 μM (24 h)), Fig. 7.

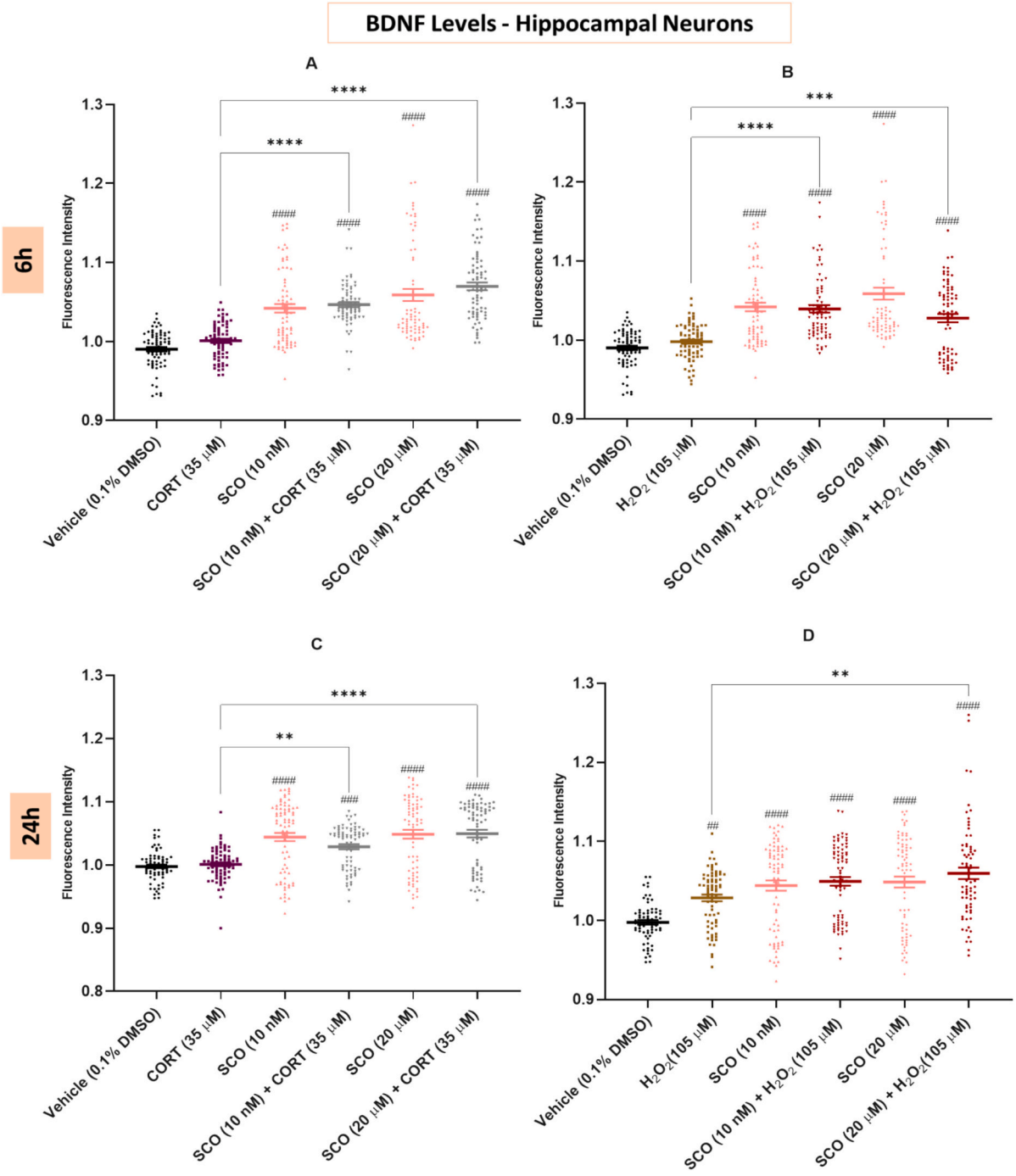


Fig. 5. Fluorescence intensity of BDNF expressed in neuronal hippocampal cell culture incubated for 6 h (A, B) or 24 h (C,D) with scopolamine 10 nM or 20 μM combined with (A, C) corticosterone 35 μM and (B, D) H_2O_2 105 μM, after immunostaining, as previously detailed in the materials and methods section. For clearer understanding and analysis, the results obtained for H_2O_2, mirtazapine, scopolamine, and corticosterone (for the same timepoint) are repeated in separate graphs. Statistically significant ### $p < 0.001$ and #### $p < 0.0001$ vs. vehicle, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ vs. the aggressor (H_2O_2 105 μM or corticosterone 35 μM); three independent experiments. CORT: corticosterone; MIRT: mirtazapine; SCO: scopolamine.

3.3. BDNF levels after mirtazapine, scopolamine, and cellular stress exposure in neuronal cortical cell culture

To further investigate the 6 h and 24 h impact of mirtazapine and scopolamine, alone or after H_2O_2 or corticosterone-induced stress on the mature BDNF levels in neuronal cortical cell culture, mirtazapine and scopolamine were used, once again, at 10 nM and 20 μM, for both compounds. H_2O_2 and corticosterone were applied at concentrations of 105 μM and 35 μM. Fig. 8 presents the results of the Western blot analysis. In Figs. S3-S5, the whole Western blots from the quantitative analysis are present.

These findings highlight that only the combination of mirtazapine at 20 μM with H_2O_2 resulted in a statistically significant increase in BDNF levels at 6 h (one-was ANOVA followed by Tukey test; $p = 0.0069$; Fig. 8B), when compared to H_2O_2 alone. There was also an observable trend at the 24 h time point, where both concentrations of mirtazapine appeared to enhance BDNF levels compared to H_2O_2 alone (Fig. 8D). A similar trend was observed for scopolamine, incubated at both

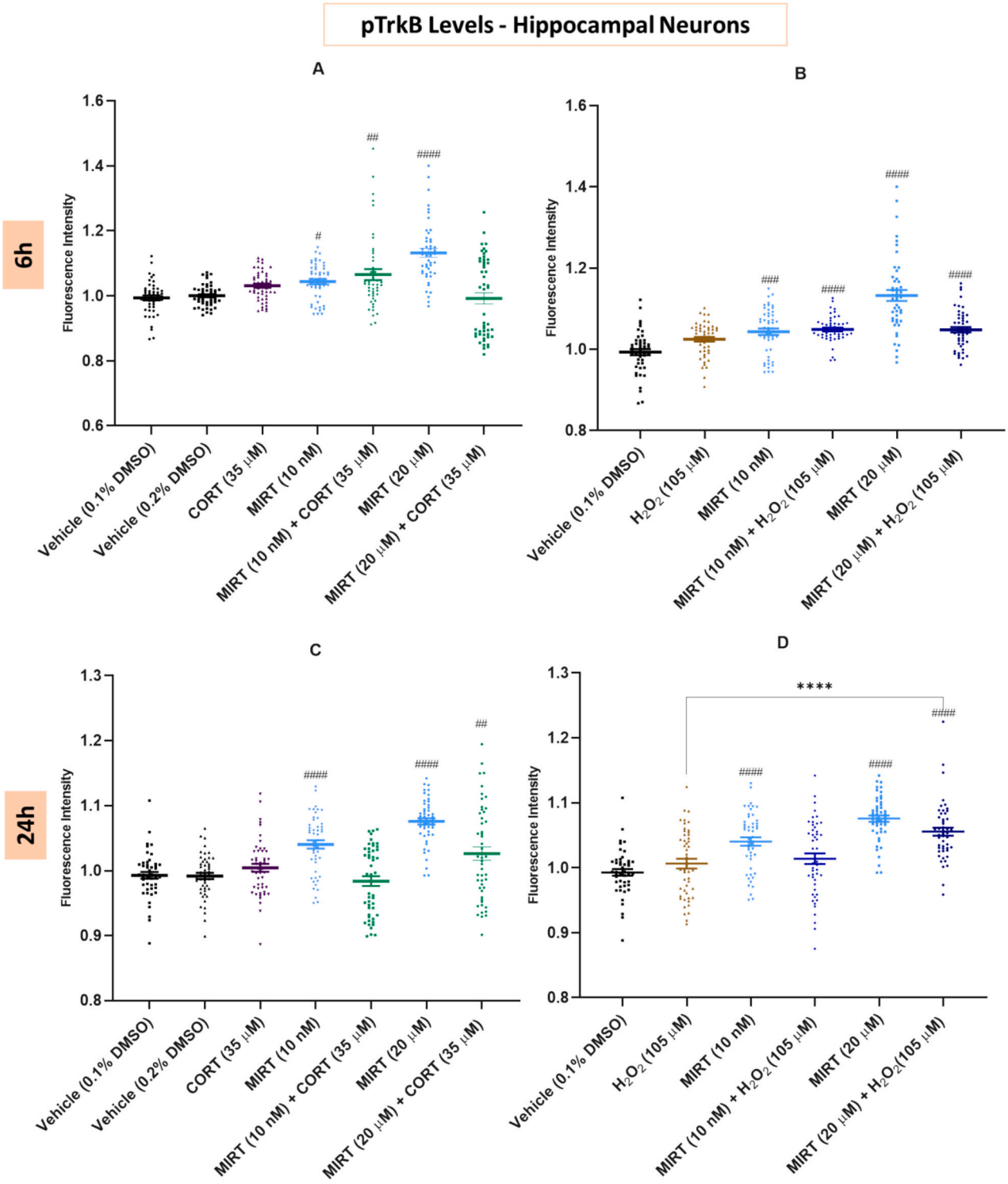


Fig. 6. Fluorescence intensity of pTrkB in neuronal hippocampal cell culture incubated for 6 h (A, B) or 24 h (C,D) with mirtazapine 10 nM or 20 μM combined with (A, C) corticosterone 35 μM and (B, D) H_2O_2 105 μM, after immunostaining, as previously detailed in the materials and methods section. For clearer understanding and analysis, the results obtained for H_2O_2, mirtazapine, scopolamine, and corticosterone (for the same timepoint) are repeated in separate graphs. Statistically significant ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$ vs. vehicle, **** $p < 0.0001$ vs. the aggressor (H_2O_2 105 μM); three independent experiments. CORT: corticosterone; MIRT: mirtazapine; SCO: scopolamine.

concentrations, in combination with H_2O_2 at both 6 h and 24 h (Fig. 8F and H). Indeed, these combinations exhibited an increase in BDNF levels compared to H_2O_2. However, when considering the combination of both mirtazapine and scopolamine with corticosterone at both time points, no particularly notable effects were discerned (Fig. 8A, C, E, G).

4. Discussion

Studying the BDNF/TrkB signaling pathway is highly relevant in the context of depression, aiming to contribute to the advance of our understanding of the biological basis of depression and for developing

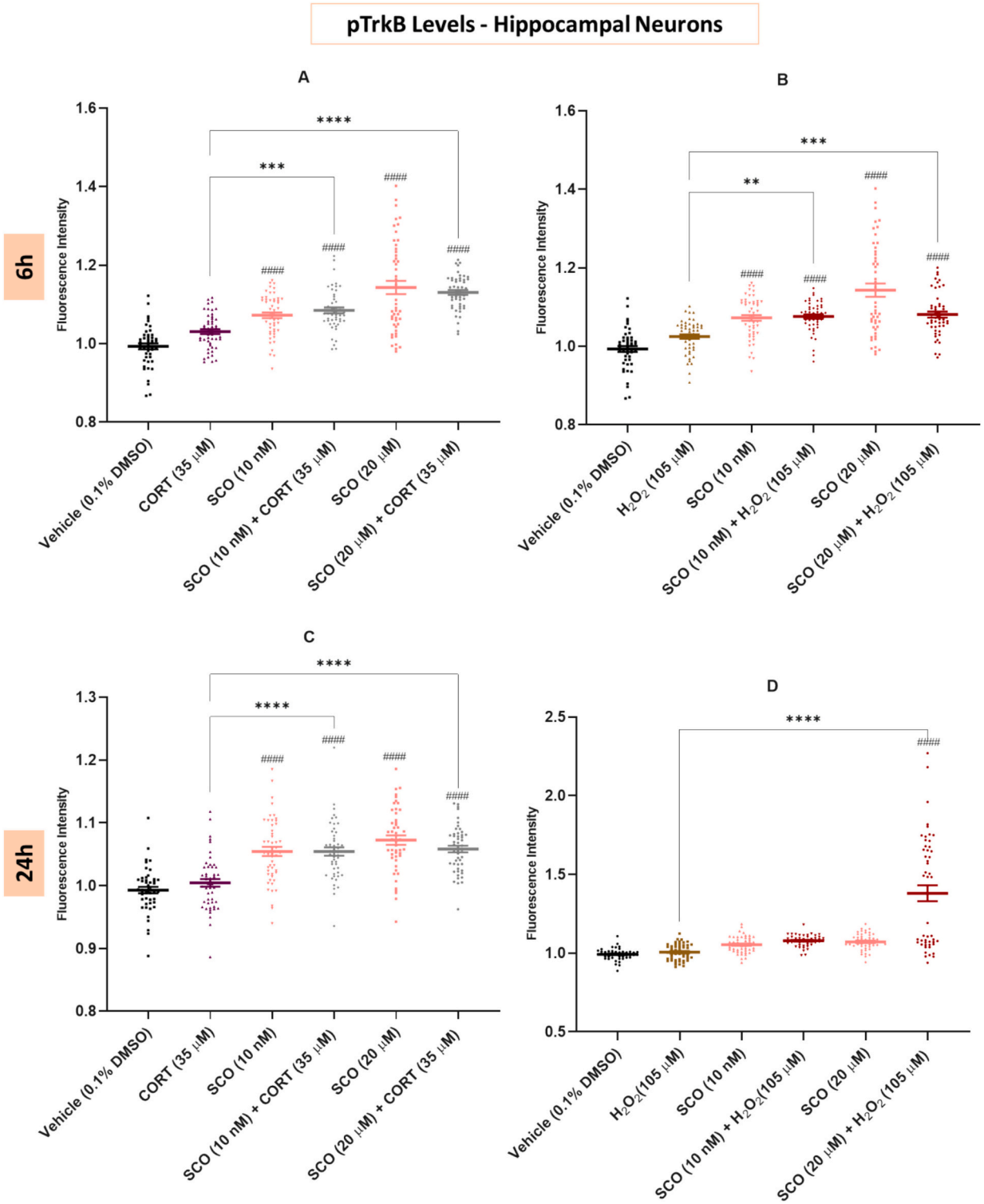
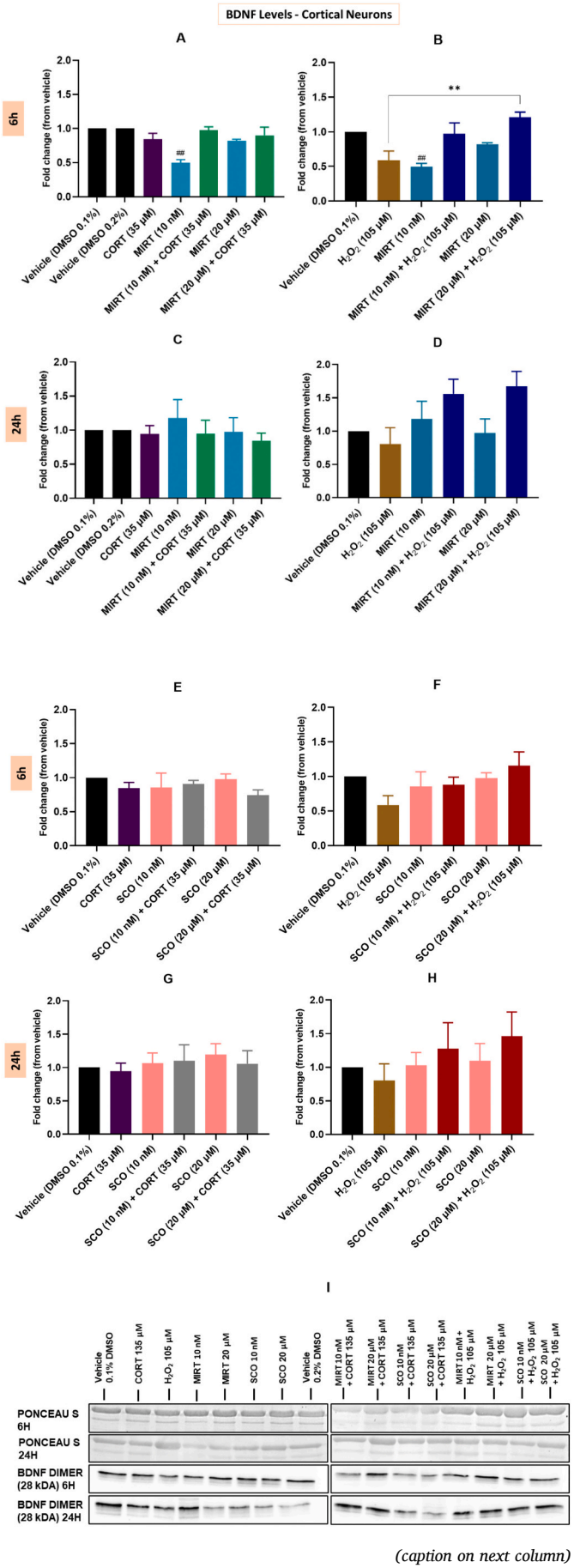


Fig. 7. Fluorescence intensity of pTrkB in neuronal hippocampal cell culture incubated for 6 h (A, B) or 24 h (C,D) with scopolamine 10 nM or 20 μM combined with (A, C) corticosterone 35 μM and (B, D) H_2O_2 105 μM, after immunostaining, as previously detailed in the materials and methods section. For clearer understanding and analysis, the results obtained for H_2O_2, mirtazapine, scopolamine, and corticosterone (for the same timepoint) are repeated in separate graphs. Statistically significant #### $p < 0.0001$ vs. vehicle, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. the aggressor (H_2O_2 105 μM or corticosterone 35 μM); three independent experiments. CORT: corticosterone; MIRT: mirtazapine; SCO: scopolamine.

more effective treatments. Additionally, inconsistences about the mechanism of action of the tested drugs on BDNF signaling, particularly scopolamine, underscores the necessity of this and further investigation.

Both antibodies used in Western blot and immunofluorescence to detect BDNF can detect monomeric and dimeric forms of this protein [49,50]. However, in this study, only in the hippocampal slices was possible to detect the BDNF monomer by Western blot. A possible explanation might be that hippocampal slices contain a more diverse array and amount of cell types, including neurons, glia, and possibly other supporting cells in their native arrangement [51], possibly leading



(caption on next column)

Fig. 8. Western blot analysis of BDNF levels in neuronal cortical cells treated for 6 h (A,B,E,F) or 24 h (C,D,G,H), with mirtazapine 10 nM or 20 µM combined with (A, C) corticosterone 35 µM and (B, D) H_2O_2 105 µM, and scopolamine 10 nM or 20 µM combined with (E,G) corticosterone 35 µM and (F, H) H_2O_2 105 µM, after incubation with anti-BDNF primary antibody, previously detailed in the materials and methods section. Representative blots are presented in image I. The Ponceau lanes are representative of the bands used as the loading control for normalization. CORT: corticosterone; MIRT: mirtazapine; SCO: scopolamine. For clearer understanding and analysis, the results obtained for H_2O_2, mirtazapine, scopolamine, and corticosterone (for the same timepoint) are repeated in separate graphs. Statistically significant ## $p < 0.01$ vs. vehicle, and ** $p < 0.01$ vs. the aggressor (H_2O_2 105 µM); three independent experiments, except for the mirtazapine (10 nM) at 6 h, and control (0.2 % DMSO) at 24 h, as described in materials and methods sections. The images of the blots shown were selected as the most representative based on graphical results and not on independent experiments. Complete sets of blots, organized by independent experiment, are available in the supplementary file for reference.

to the different pattern of BDNF monomer found in the Western blot. In immunofluorescence, as the antibody recognizes both forms, the results can be interpreted as the overall BDNF levels. Distinguishing and quantifying these forms accurately necessitates specific methodologies, such as native polyacrylamide gel (PAGE) or treating the cells with brefeldin A, which should impair dimer formation by inhibiting endoplasmic reticulum-Golgi protein trafficking [52]. In our current study, despite only being able to quantify the BDNF monomer in hippocampal slices, we used methodologies designed to measure overall mature BDNF levels. While there is strong evidence suggesting that the 28 kDa form of BDNF is the dimer (highlighted by previous research [53] and by the obtained strong intensities of the bands), further confirmation would be valuable. This is particularly important because there is a less studied form of BDNF, the 28 kDa cleavage product of the BDNF precursor. However, this form is usually present in residual levels and does not affect the formation of mature BDNF [54,55]. Furthermore, exploring additional data points, adding more independent experiments and varying concentrations would provide a broader understanding of the results.

Numerous studies have reported high levels of oxidative stress with diminished BDNF levels, supporting the potential protective function of BDNF against oxidative harm within neuronal tissues [56]. Interestingly, a study reported that H_2O_2 applied in a range of 100–250 µM initiate PC12 cell death, but also significantly promoted BDNF signaling, contributing to neuroprotection of these cells [57]. Indeed, the obtained immunofluorescence results demonstrated that this compound after 24 h of exposure, enhanced BDNF and pTrkB levels, compared to the untreated condition. This did not occur at 6 h, suggesting a time-dependent response. The cells may initially counteract the oxidative stress, with the effects only becoming apparent after more prolonged exposure. Thus, this suggests that more prolonged time of exposure of H_2O_2 leads to more cytotoxicity, and the enhancement in BDNF levels at 24 h is probably a mechanism of neuroprotection, consistent with literature reports [57].

High levels of corticosterone are also connected to BDNF/TrkB impairments. However, a study in mice suggested that corticosterone has differential effects on proBDNF processing: proBDNF increased in the hippocampus and cerebellum but remained unchanged in the prefrontal cortex and hypothalamus. Both proBDNF and mBDNF increased significantly in the pituitary gland, while proBDNF decreased significantly in the adrenal gland [58]. Moreover, the effect of corticosterone on BDNF might follow an inverted U-shaped curve. Indeed, a study suggested that as cortisol levels increase from low to moderate levels, it may enhance neuroplasticity. However, as cortisol levels continue to rise beyond a certain point, they may start to have a detrimental effect on neuroplasticity. So, the relationship between cortisol and neuroplasticity is not linear; there is an optimal range where cortisol may have a positive influence on plasticity, but excessive levels could be detrimental [59].

The obtained data revealed the complexity of BDNF/TrkB assessment after exposure to corticosterone. Immunofluorescence results, for both time points, demonstrated that corticosterone had no significant effect on BDNF levels nor pTrkB, compared to the untreated condition. This effect on BDNF levels was also observed in cortical cells by Western blot analysis. However, Western blot analysis of hippocampal slices demonstrated a trend to corticosterone upregulation of BDNF levels after corticosterone addition, particularly the BDNF monomer. Possibly, hippocampal slices as a whole, might initially respond to corticosterone adverse stimulus by increasing BDNF production as a protective or adaptive response, being a feedback mechanism Supporting this, a recent study explored the effect of acute and chronic stress in BDNF levels, in humans, revealing that acute psychosocial stress increases serum BDNF and cortisol, whereas chronic stress was linked to lower basal serum BDNF levels [60].

Mirtazapine is known to promote the activation of TrkB receptor [61] and to induce BDNF levels in the serum of depressed patients [62]. Indeed, a study revealed that repeated mirtazapine administration (10 mg/kg) increased BDNF mRNA levels in rat hippocampus and cerebral cortex. In the same study, a lower dose of mirtazapine (5 mg/kg) did not have effects on BDNF mRNA levels in these rats [63]. Other study also highlighted that chronic mirtazapine administration in a chronic unpredictable mild stress rat model, enhanced hippocampal and cortical (prefrontal cortex) BDNF levels, inhibiting also the increase of plasma corticosterone level [64]. A possible mechanism to explain the action of mirtazapine in the tested models might be by being an antagonist of 5-HT3 on GABAergic interneurons, leading to AMPA receptors activation, that culminates in cAMP-response element binding protein (CREB) phosphorylation and transcription of BDNF gene [65].

Our findings demonstrated the impact of mirtazapine on the regulation of mature BDNF and pTrkB levels, both in isolation and in combination with the different stimuli. Notably, our results highlight the dynamic nature of this modulation, with variations in response observed across different time-points and concentrations, consistent with previously reported findings in the literature. Our previous findings indicated that mirtazapine (particularly 20 μM) exhibited a greater capacity to mitigate the adverse effects of oxidative stress, suggesting that the higher concentration may induce more robust neuroprotective effects [31,48]. This effect was also demonstrated in the BDNF levels of hippocampal cells, where it becomes apparent that only the highest concentration of mirtazapine was effective in mitigating the effects of corticosterone, and the effects of H_2O_2 in cortical cells. Regarding pTrkB levels, only 20 μM mirtazapine was also capable of enhance these levels, after H_2O_2 stimulus. These results align with the previously obtained results, supporting that the combination of mirtazapine 20 μM with H_2O_2 seems to exert neuroprotective effects. At 6 h, mirtazapine (10 nM) decreased significantly BDNF levels in cortical cells, returning to values similar to the control group at 24 h, suggesting that the initial decrease may have been a transient effect, and the cells eventually adapted to the presence of mirtazapine. In the case of hippocampal slices, mirtazapine (20 μM) exerted a significant influence on BDNF dimer levels, leading to a reduction in these levels compared to the control group. In these slices, the combination of mirtazapine with H_2O_2 did not affect BDNF levels compared to H_2O_2 alone. In this biological model and in cortical cells, the combination of the antidepressant with corticosterone did not lead to statistically significant effects on BDNF levels when compared to corticosterone alone. However, it did suggest a trend to decreased BDNF levels. Interestingly, the previously obtained results with this combination on cellular viability, extracellular serotonin levels and reactive oxygen species production also revealed that overall, mirtazapine did not attenuate damages induced by corticosterone, but only attenuated damages induced by H_2O_2 [31,32]. Once more, it becomes evident that, despite fluctuations between neuronal systems, this consistent pattern of response underscores the neuroprotective attributes of mirtazapine when confronted with oxidative stress stimulation.

Scopolamine is also reported to modulate BDNF expression and release, essential for its rapid antidepressant effects [66]. A study demonstrated that scopolamine attenuated reserpine-induced depression in mice partially by regulating BDNF (and other components such as SERT) in the hippocampus and prefrontal cortex of mice [67]. However, there are also reports that refer that scopolamine damage mouse memory by downregulating the BDNF/TrkB/Akt pathway [68], highlighting the importance of studies in this regard. Our research findings confirm that scopolamine indeed exerted varying effects on mature BDNF and pTrkB levels. Notably, either alone or when applied in combination with the stimuli, scopolamine overall enhanced BDNF and pTrkB levels in mouse hippocampal cells. However, it conversely reduced BDNF levels in rat hippocampal slices. These apparent divergent outcomes highlight the need for additional research to fully elucidate the underlying mechanisms. In cortical cells, scopolamine demonstrated no effect on BDNF levels. However, when combined with H_2O_2, showed a tendency to increase BDNF levels, particularly at a 20 μM concentration, and at both time points. When scopolamine was combined with corticosterone, it didn't significantly impact BDNF levels compared to corticosterone alone. Collectively, these observations suggest that scopolamine's effect on BDNF and pTrkB is complex and context dependent. Indeed, it highlights the importance of considering the specific type of brain cells and the presence of other stimuli (corticosterone and H_2O_2). For this drug, the antagonism of M2 on glutamatergic neurons leads to a rise in glutamate levels, also culminating in CREB phosphorylation and transcription of BDNF gene may explain its action on BDNF levels [69]. It is particularly evident that like mirtazapine, in cortical and hippocampal cells, scopolamine combined with H_2O_2 also enhanced BDNF levels, compared to H_2O_2 alone. This is consistent with the previous obtained results exploring cell viability in these conditions, highlighting that stress attenuation might involve upregulation of BDNF levels, according with the previous mentioned literature reports. Also, differences between the two drugs were observed. It was particularly notorious in the hippocampal slices, where scopolamine decreased BDNF monomer levels in a pronounced way. A possible explanation might be the presence of glial cells. Indeed, studies demonstrated that after exposure of scopolamine, astrocyte number decreases in rats [70,71]. Additionally, a scopolamine injection promoted neuroinflammation in hippocampus, with microglia activation in mice [72]. Future studies would be valuable to understand these mechanisms.

5. Conclusion

These experiments demonstrated complex and context-dependent responses to the tested compounds, highlighting the complex dynamics of BDNF signaling in different neuronal systems. The findings support the significance of considering different cellular contexts when evaluating the responses of neurons to different treatments and stimulus. Overall, both stimuli, mirtazapine and scopolamine modulated BDNF and pTrkB levels. Globally, and particularly regarding hippocampal slices, mirtazapine seems to be the most promising drug regarding BDNF levels, particularly against H_2O_2 stimulus. Thus, this research aims to contribute with valuable insights into the biological basis of depression and open ways for developing targeted therapies that modulate BDNF/TrkB signaling pathways.

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Declaration of AI and AI-assisted technologies in the writing process

During the preparation of this work, AI was not used to generate new content. ChatGPT 3.5 was only used in some aspects of the grammar/writing process.

CRediT authorship contribution statement

Ana Salomé Correia: Writing – original draft, Software, Methodology, Investigation, Formal analysis. **Marília Torrado:** Methodology, Investigation. **Tiago Costa-Coelho:** Methodology, Investigation. **Eva Daniela Carvalho:** Methodology, Investigation. **Sara Inteiro-Oliveira:** Methodology, Investigation. **Maria José Diógenes:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Ana Paula Pêgo:** Writing – review & editing, Methodology, Investigation. **Sofia Duque Santos:** Writing – review & editing, Methodology, Investigation. **Ana M. Sebastião:** Writing – review & editing, Supervision, Methodology, Investigation. **Nuno Vale:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2024.123133.

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