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Cannabidiol Modulation of Nicotine-Induced Toxicity: Assessing Effects on Behavior, Brain-Derived Neu-Rotrophic Factor, and Oxidative Stress C57BL/6 Mice

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Abstract

High doses of nicotine in rodents are known to induce anxiety, dysregulate brain-derived neu-rotrophic factor (BDNF) levels, contribute to oxidative stress, and promote the secretion of cytokines. Conversely, at lower doses, nicotine exhibits anxiolytic effects. The endocannabinoids and nicotine modu-late several central nervous system processes via their specific receptors, impacting locomotion, anxiety, memory, nociception, and reward. Cannabidiol (CBD), a non-psychoactive active ingredient of Cannabis sativa L., has garnered scientific attention primarily due to its recognized effects, including anxiolytic, an-tioxidant, and anti-inflammatory properties. This work aims to explore the potential anxiety-reducing properties of CBD in a well-established experimental mouse model of anxiety-like behavior induced by high doses of nicotine. In this context, the open field behavioral test was specially conducted to assess CBD's effects on anxiety-like behavior and locomotion. Brain neuronal plasticity, modulated by brain-derived neurotrophic factor (BDNF), along with a diverse array of blood's metabolic markers, was examined as a means of evaluating systemic toxicity under various treatments. Finally, oxidative stress was evaluated through the measurement of GSH, SOD, and MDA, while pro-inflammatory cytokine assessments were conducted to evaluate redox status and immune system function. Our findings indicate that CBD holds promise in alleviating high-dose nicotine-induced anxiety-like behaviors by targeting specific liver en-zymes, maintaining tissue's systemic toxicity (i.e., renal, kidney, and pancreatic), balancing redox reactions (SOD, GSH, and MDA), promoting secretion of pro-inflammatory cytokines (TNF-alpha and IL-6), and mitigating changes in BDNF protein levels.

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1. Introduction

Cannabidiol (CBD) is a naturally occurring compound present in cannabis plants, mainly studied for its diverse array of ben-eficial properties, with historical usage dating back to ancient times. CBD is a non-psychoactive cannabinoid with many pharmacological effects, including anti-inflammatory and antioxidant effects, and belongs to a group of compounds with anxiolytic, antidepressant, antipsychotic, and anticonvulsant properties, among others [1]. Kwee (2022) proposes that CBD holds therapeutic promise for pathological anxiety; however, establishing a clear dosing recommendation remains challenging at present [2]. Another study presents evidence for the neurological basis of CBD's anxiolytic effects, demonstrating that it reduces anxiety in patients with social anxiety disorder and is linked to changes in brain activity [3]. Also, several pre-clinical studies report that high CBD doses of 30-60 mg/kg produced anxiolytic-like effects in behavioral studies in rodents [4][5][6][7][8][9].

It is well-established that nicotine can exert both anxiolytic and anxiogenic effects through β2 nicotinic acetylcholine receptors (nAChRs) in a dose-dependent manner [10][11]. The effect of nicotine on anxiety in both humans and experimental animals is multifactorial, potentially depending on the administration regimen (i.e., acute, chronic, withdrawal), the route of admin-istration (e.g., i.p., s.c., i.v., smoking), and the behavioral state of the experimental subjects (i.e., relaxed, stressed, nicotine deprived, etc.) [11][12]. Studies in experimental animals have shown that during the acute administration of nicotine at low doses (0.05 mg/kg), it acts as an anxiolytic. In contrast, at high doses (0.08-1 mg/kg), it shows anxiogenesis [13][14][15]. Nicotine's anxiolytic and anxiogenic-like effects [16][17] can be studied in animal behavioral model tests such as the el-evated plus-maze and the Open Field Test (OFT) [14][15]. The endocannabinoid system has been linked to the modulation of several central nervous system processes, including locomotion, anxiety, memory, nociception, and reward [18][19][20]. Similarly, nicotine administration affects locomotion, anxiety, memory, and nociception, as well as producing rewarding effects in a variety of animal models [21][22]. The distribution of nAChRs and cannabinoid receptors (i.e., CB1 and CB2) has been found to overlap in several brain regions, including the hippocampus and the amygdala [22] suggesting that these two systems may interact. Previous studies in rodents have shown the anxiogenic effects of nicotine at high doses [14][15][16], while CBD has been found to exert anxiolytic-like effects [23][24][25].

Reduced BDNF expression, along with potential decreases in other growth factors, is linked to depression and anxiety, while the enhancement of BDNF is implicated in the effects of anxiolytics [26]. BDNF has a crucial role in mood disorders that are related to stress [27]. According to Hashimoto's study in 2007, a specific variation of BDNF was discovered to be associated with higher levels of anxiety-related behaviors [28]. In addition, Kalueff (2006) has highlighted the importance of BDNF in mouse social stress, suggesting a correlation between BDNF and anxiety [29]. Furthermore, mice with BDNF signaling alterations have displayed a wide range of behavioral reactions, and animals with diminished BDNF expression exhibit symptoms of anxiety and depression in response to stress [30]. Nicotine can directly cause changes in BDNF

levels [31], as it has been demonstrated that compared to non-smokers, nicotine-dependent smokers have decreased serum BDNF levels [32]. Nevertheless, the correlation between nicotine and BDNF is intricate and requires further comprehensive understanding. Finally, it is noteworthy that according to a recent study, administering a single dose of 30 mg/kg of CBD increased the expression of BDNF in mice [33]. Nicotine toxicity can also be attributed to a disruption in the cell's redox status. It has been shown that nicotine toxicity affects SOD and catalase in male Wistar rats in brain regions by significantly reducing their levels [34]. Another study demonstrated that oral consumption of nicotine resulted in a reduction in SOD, catalase, and GSH levels while simultaneously increasing malondialdehyde (MDA) levels [35].

The acute effects of pure nicotine, distinct from exposure to habitual smoking by cigarette(s) or any electronic nicotine delivery systems, on anxiety-like behavior and toxicity are currently underexplored. To the best of our knowledge, there is no other study examining the coadministration of CBD and nicotine, specifically evaluating their impact on behavior and toxicity biomarkers. The primary objective of the present study is to find out whether cannabidiol's anxiolytic properties can be exploited by the OFT and how they influence the anxiogenic effects induced by nicotine consumption. Additionally, the study aims to investigate whether nicotine can elicit toxicity effects and, if so, whether CBD can mitigate these effects. Thus, our study's experimentation covers the assessment of oxidative stress status, the release of proinflammatory cytokines, the measurement of BDNF changes at the protein level, and the determination of key metabolic molecules and enzymes to fully understand the interactions between CBD and nicotine in the context of behavior and toxicity.

2. Materials and Methods

2.1. Chemicals

Nicotine [(-)-nicotine hydrogen tartrate], DMSO, Tween-80 and all necessary reagents were supplied from Sigma-Aldrich. CBD was supplied from Cayman Chemical (CAS No.13956-29-1). Mouse BDNF (Brain-Derived Neurotrophic Factor) ELISA Kit (MOES00782) (Assay Genie) was used for our experimental purposes.

2.2. Animals

Male C57BL/6 mice [22-30 g body weight (BW)], 3-4-month-old, were housed in standard laboratory polyacrylic cages (3-5 mice per cage), with access to food and water ad libitum. Mice were kept in a room with a controlled temperature (22 ± 1 °C), relative humidity of 50-60%, and a controlled light-dark cycle (12 h/12 h). The handling of animals and the study protocol were in accordance with the Greek Presidential decree 86/2020 for adaptation of Greek legislation to the directive 2010/63/EU of the European Parliament and of the European Council of 22 September 2010 on the protection of animals used for scientific purposes. All efforts were made to minimize the number of animals, optimize their living conditions, and minimize stress during manipulations. …

2.3. Experimental Design

Cannabidiol (CBD) was dissolved in saline containing 5% DMSO and 2% Tween-80, and a stock solution of 50 mg/ml was created. CBD was administered acutely intraperitoneally (i.p.) at a dose of 50 mg/kg in a less than 10 µL/g volume. Nicotine was dissolved in saline to create a stock solution of 0.5 mg/ml, delivered acutely subcutaneously (s.c.) at a dose of 1 mg/kg in a less than 2 µL/g volume. Seven animals were used per group (n=7). Mice were randomly assigned to four groups: Vehicle (VEH) (saline, 5% DMSO, 2% Tween-80), Nicotine, CBD, and Nicotine + CBD (NIC + CBD). The CBD was administered 35 minutes before the behavioral test, while nicotine was administered 30 minutes before. Animals were then euthanized by cervical dislocation. Blood was collected immediately through cardiac puncture in tubes with anticoagulants (K3 EDTA, Sarstedt), and plasma was separated by centrifugation at 1500 g for 10 minutes and stored at –80 °C for further analysis. The liver and brain (cerebral hemispheres (CH) and cerebellum (Ce) were all isolated and stored at -80 °C until homogenization.

2.4. Open Field Test (OFT)

To assess the effects of the CBD and nicotine administrations on behavioral parameters (locomotor activity and anxiety-like behavior), OFT was used. The OFT is based on the physical aversion of the mice to the open field and their natural exploratory behavior in new environments. Moreover, thigmotactism refers to the preference of mice to walk near the walls of an open field apparatus. The experimental apparatus was first described by Simon (1994) [36]. Mice were kept for 1 h in a slightly illuminated room to habituate prior to injections. Each mouse was gently placed in the apparatus, at the center at first, and monitored for 10 min. Thigmotaxis, which is the time spent close to the walls of the apparatus, was recorded as an index of anxiety [37]. All training and testing sessions were conducted during the light phase (8:00 am-2:00 pm). A 42x42x42 cm polyvinyl chloride (PVC) box was used as the test area, and a camera was utilized to track movement into and around the box's inside and exterior [38] (Fig. 1). Changes in locomotion can indicate changes in neurological processes and, as a result, aberrant brain function. Furthermore, this test can assess an animal's overall health and well-being [38], as animals that are not in good health tend to move less. Stressed mice exhibit less activity in the open field and more stereotyped behavior.

Any-Maze software was used for video analysis and the following indexes of anxiety and locomotion were assessed: Anxie-ty-like behavior: Number of entries to the center, Time spent in the periphery (s), and mobility indexes: Total distance traveled (m) and total time mobile (s).

Open Field test setup is presented in Figure 1.

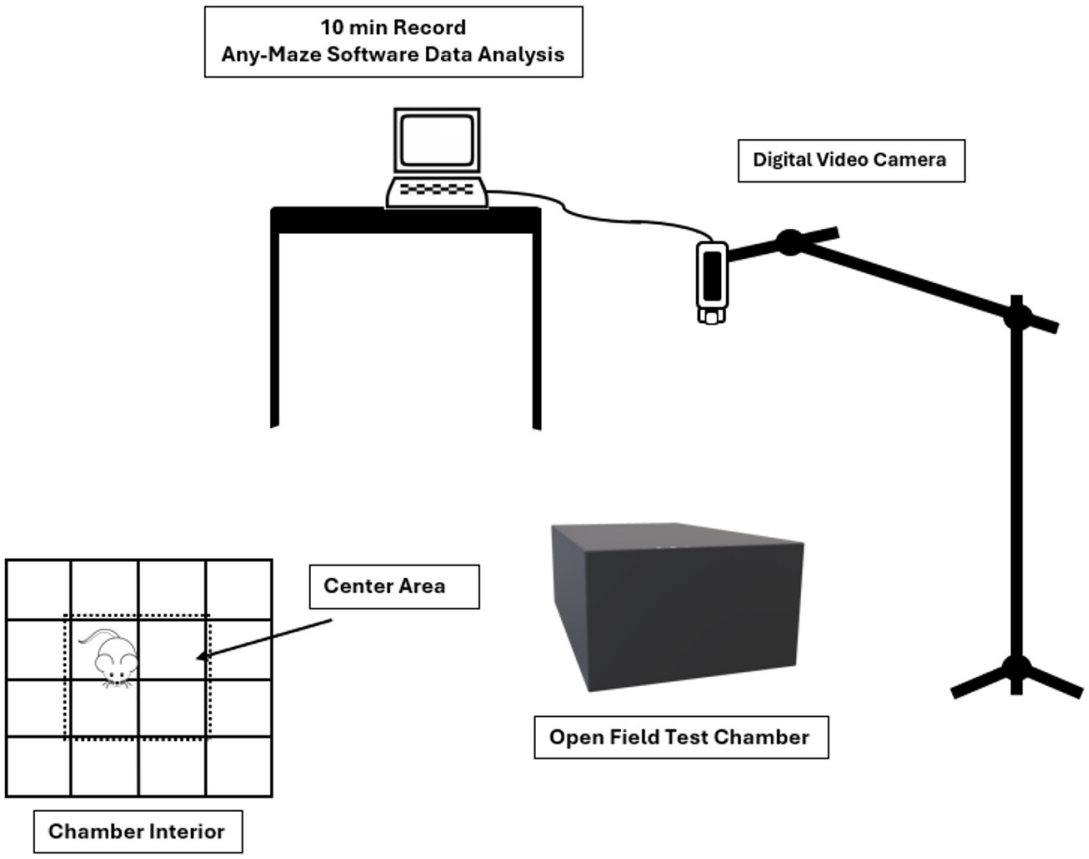

Figure 1. Graphical representation of the Open Field Test set-up.

2.5. Blood Test Analysis

The isolated plasma samples were analyzed using the Samsung PT10V Compact Blood Analyser. The analyzer operates based on a combination of optical and electrochemical detection methods. The following metabolic markers were measured in the plasma samples: glucose (GLU), blood urea nitrogen (BUN), creatinine (CREA), calcium (CA), total protein (TP), albumin (ALB), globulin (Glob), alanine transaminase (ALT), alkaline phosphatase (ALP), bilirubin (TBIL), cholesterol (CHOL), and amylase (AMYL).

2.6. Tissue homogenization

For tissue analysis, samples were prepared by homogenizing them in phosphate-buffered saline containing EDTA-free Protease Inhibitor Cocktail (ROCHE/04693159001) using a glass/Teflon homogenizer while maintaining a cold temperature on ice. The resulting homogenate underwent centrifugation at 15,000 g for 20 minutes at 4°C. The supernatant, containing the desired components, was divided into smaller aliquots and promptly stored at -80°C for future use. Total protein concentration was determined using the Bradford assay[39], and appropriate dilutions were made and tested before each assay.

2.7. Enzyme-linked immunosorbent assay

The quantification of brain-derived neurotrophic factor (BDNF) protein levels was performed with an enzyme-linked immunosorbent assay (ELISA), employing the Mouse BDNF ELISA Kit (Assay Genie, MOFI00015). To ensure accurate meas-urements, BDNF levels were normalized relative to total protein concentrations determined by the Bradford assay. Dilution procedures were carried out and tested to ensure compatibility with the specified range of the kit. The brain regions assessed in the present study included the central hemispheres and the cerebellum.

2.8. Redox markers

Redox markers evaluated in the brain (cerebral hemispheres and cerebellum). The assessment of lipid oxidative stress relied on the production of TBARS during an acid-heating reaction, as previously described [40][41]. Briefly, the samples were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid and then heated in a boiling water bath for 15 minutes. The assessment of TBARS was conducted by measuring absorbance at 535 nm. Measurements are presented as malondialdehyde equivalents per milligram of protein.

For Superoxide Dismutase activity, SOD Assay (Cayman Chemicals, Item No. 706002) was used according to the manufac-turer's instructions. Total SOD Activity was assayed (cytosolic and mitochondrial) and expressed as U/mg of protein. One unit (U) of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical measured in change in absorbance per minute at 25 °C and pH 8.

Reduced Glutathione (GSH), an antioxidant enzyme, was determined using the GSH Assay Kit (Abcam, ab239727), according to the manufacturer's instructions. The results are expressed as umol/mg of protein.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey post hoc comparisons test was employed for statistical analysis in behavioral tests and biochemical assays. GraphPad Prism 5 was used for graph generation and statistical analysis. Data are presented as mean value ± STDEV (Standard Deviation). Probability values less than $p<0.05$ were considered statistically significant.

3. Results

3.1. Biochemical Blood Analysis

Table 1 depicts the biochemical analysis of animal blood samples following different treatments with nicotine, CBD, and coadministration (NIC+CBD). Following nicotine exposure, significant changes were observed in specific liver enzyme activity compared to the control group. Alanine aminotransferase (ALT) levels were found to be statistically increased, while alkaline phosphatase (ALP) levels were substantially reduced. Notably, NIC+CBD demonstrated a potential

protective effect on ALT levels, as seen by the tendency to lower ALT levels. However, this reduction did not reach statistical significance when compared to the Nicotine-alone group. Also, ALP levels remained significantly increased in the coadministration group when compared to the Nicotine-alone group, suggesting a potential modulatory effect of CBD on certain liver enzymes.

Importantly, additional biochemical markers, including Glucose (Glu), Blood Urea Nitrogen (BUN), Creatinine (CREA), Calcium, Total Protein (TP), Albumin (ALB), Globulin (GLOB), Total Bilirubin (TBIL), Cholesterol (CHOL), and Amylase (AMYL), did not show statistically significant differences in any group compared to the control group. These results highlight a specific impact of nicotine and CBD coadministration on select liver enzymes while maintaining a neutral effect on other biochemical markers.

Table 1. Metabolic markers measured in plasma.

	VEH	NICOTINE	CBD	NIC+CBD
GLU (mg/dl)	254.9 ± 39.52	290.7 ± 42.18	249.9 ± 65.36	301.0 ±39.87
BUN (mg/dl)	19.16 ± 3.705	19.80 ± 3.643	19.77 ± 2.848	19.30 ± 1.667
CREA (mg/dl)	0.3000 ± 0.057	0.314 ± 0.089	0.328 ± 0.048	0.357 ± 0.097
CA (mg/dl)	9.857 ± 0.423	9.786 ± 0.337	9.514 ± 0.226	9.517 ± 0.213
TP (g/dl)	5.557 ± 0.395	5.414 ± 0.296	5.400 ± 0.310	5.386 ± 0.367
ALB (g/dl)	2.880 ± 0.044	2.800 ± 0.089	2.740 ± 0.151	2.720 ± 0.164
GLOB (g/dl)	2.714 ± 0.307	2.700 ± 0.238	2.429 ± 0.149	2.500 ± 0.395
ALT (U/L)	66.00 ± 5.164	**78.71 ± 9.163** *	61.86 ± 8.295	72.86 ± 9.924
ALP (U/L)	106.1 ± 4.947	**92.43 ± 4.504** **	106.7 ± 6.264	**104.30 ± 8.287** #
TBIL (mg/dl)	0.1629 ± 0.057	0.1667 ± 0.066	0.1283 ± 0.3817	0.2267 ± 0.1357
CHOL (mg/dl)	82.00 ± 9.309	77.14 ± 8.764	72.71 ± 9.286	71.57 ± 12.37
AMYL (U/L)	1304 ± 114.9	1395 ± 172.7	1302 ± 242.9	1378 ± 197.1

*Note: * p<0.05, ** p<0.01, compared to VEH, # p<0.05 compared to NICOTINE.*

3.2. Open-Field Test

In Figure 2, track plots as captured by Any-Maze software are presented indicatively of one mouse per animal's experimental group. As seen in Figure 3, nicotine administration reduced mobility markers, while CBD coadministration led to a significant increase. Specifically, nicotine administration significantly reduced the total distance travelled (9.152±5.710 m, p<0.001) compared to the VEH group (22.78±4.565m). CBD coadministration (NIC+CBD) resulted in a significant increase (14.20±2.951) compared to the nicotine group. Moreover, nicotine administration reduced the total

time of mobility (177.7±98.54 s), compared to VEH (398.00±42.61 s, $p<0.001$), while in the NIC+CBD group, the time significantly increased (346.8±97.99 s, $p<0.001$).

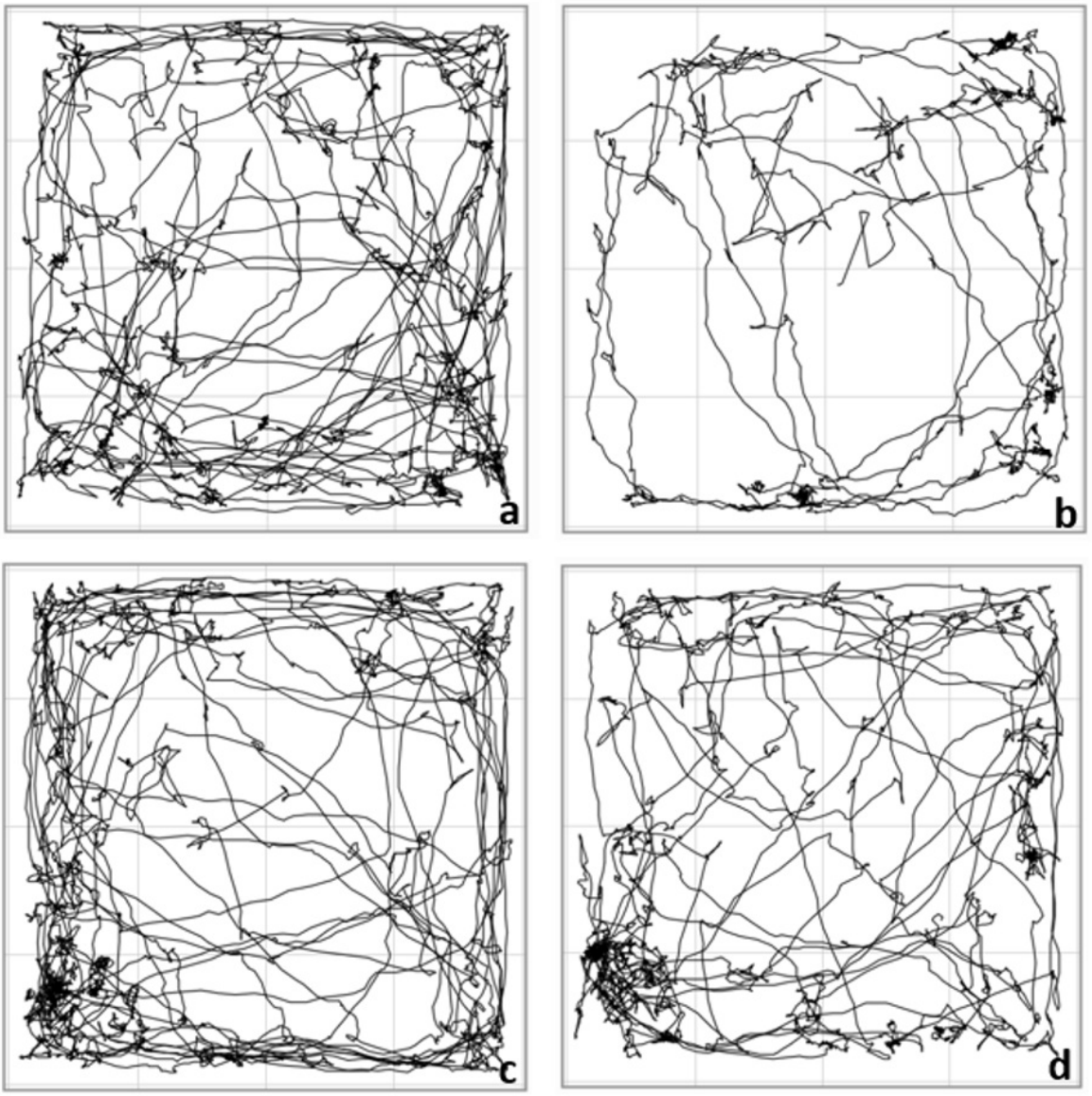

Figure 2. Comparison of animal track paths. Plots show the position of the animal's center point for the entire duration of the test (600 s). Each figure represents one indicative mouse per group as follows: a) VEH, b) CBD, c) NIC, and d) NIC+CBD. The results are extracted and expressed as Total Distance travelled (m).

Nicotine administration induced anxiety-like behavior by increasing thigmotaxis (i.e., time spent in the periphery) and reducing entries to the center, while CBD coadministration (NIC+CBD) reversed these effects. Specifically, nicotine reduced thigmotaxis (555.2±27.06 s, $p<0.05$) compared to VEH (462.5±80.00 s), while CBD coadministration also decreased thigmotaxis (447.7±104.5 s, $p<0.05$). Furthermore, nicotine significantly reduced the number of entries to the center (15.00±3.92) compared to VEH (35.00±11.48), whereas CBD coadministration increased center entries (24.75±4.979).

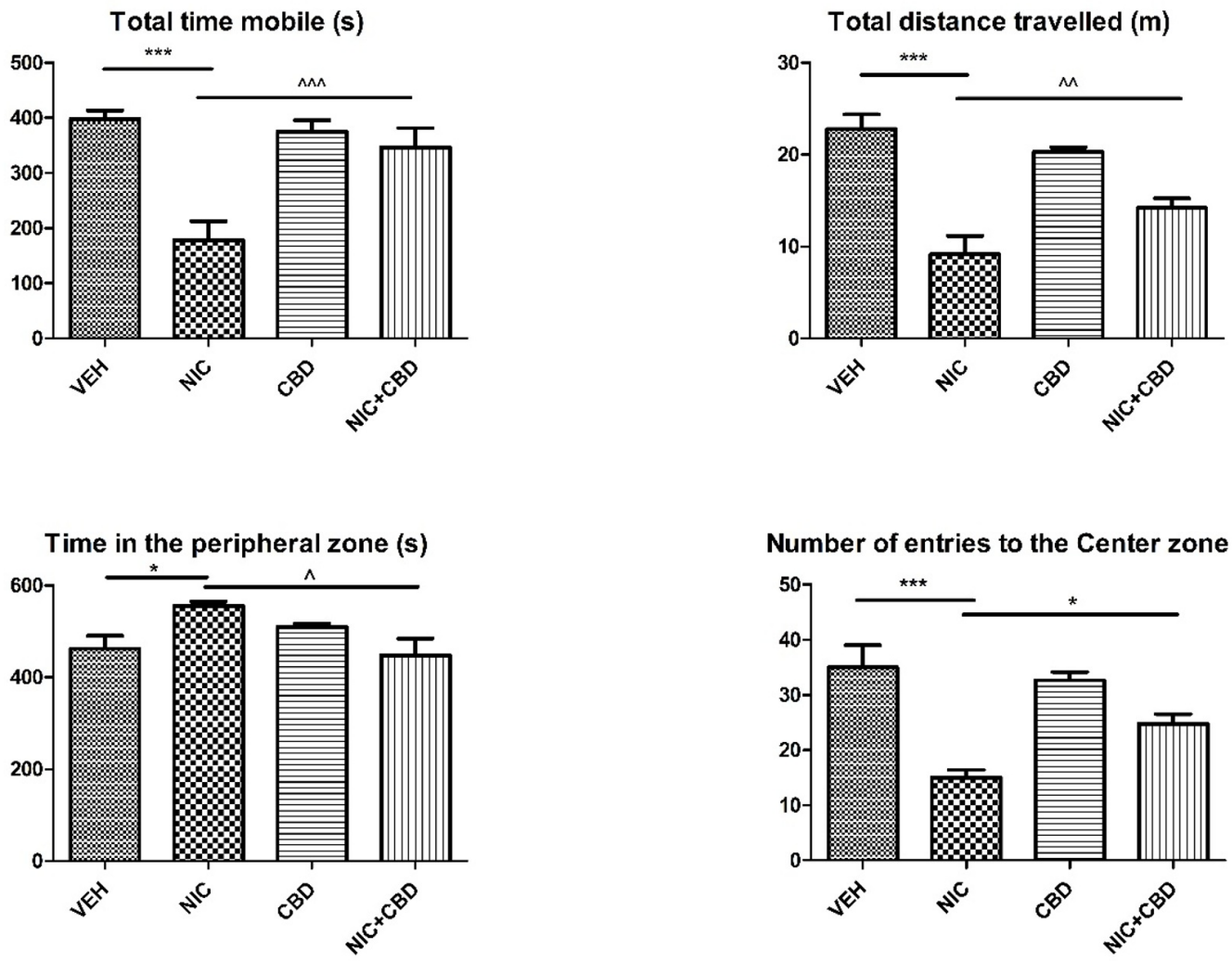

Figure 3. Nicotine, CBD, and co-administration effect on anxiety and mobility according to Open Field Test. The values are presented as MEAN ± STDEV, for n=7 in each group. Time is given as seconds (s), distance as meters (m).* p <0.05, *** p<0.001, compared to VEH, ^^ p <0.01, ^^^ p<0.001, compared to the nicotine group.

3.3. Oxidative Stress

We investigated the impact of nicotine, CBD, and their coadministration on oxidative stress markers, including superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH), in the liver, cerebral hemispheres, and cerebellum. The results in Table 2 demonstrate that nicotine induced substantial oxidative stress by modulating these markers across all regions.

Table 2. Nicotine, CBD, and co-administration effect on SOD, GSH, and MDA in the liver, cerebral hemispheres (CH), and cerebellum (Ce).

		VEH	NIC	CBD	NIC+CBD
SOD (U/mg protein)	CH	3.817±0.639	**2.019±0.382** **	3.024±1.015	3.018±0.552
	Ce	10.56±1.081	**7.219±1.516** *	11.22±1.923	9.907±2.766
	Liver	5.405±1.598	**1.902±1.067** **	4.120±2.242	3.133±1.417
GSH (umol/mg protein)	CH	0.202±0.030	**0.094±0.018** *	0.1905±0.058	**0.1945±0.074** ^
	Ce	0.596±0.045	**0.459±0.043** ***	0.568±0.036	**0.504±0.031** **
	Liver	0.112±0.026	**0.0453±0.020** **	0.086±0.037	**0.1142±0.025** ^^
MDA (pmol/mg protein)	CH	3.952±0.481	**6.135±0.840** ***	3.465±0.508	**4.699±0.881** *,^
	Ce	9.661±4.380	**15.70±1.114** *	8.831±2.287	10.630±3.333
	Liver	2.979±0.407	**3.724±0.220** **	**1.836±0.335** ***	**2.111±0.225** ***,^^^

*The values are presented as MEAN ± STDEV, for n=7 in each group. * p<0.05, ** p<0.01, *** p<0.001, compared to VEH, and ^ p<0.05, ^^ p <0.01, ^^^ p<0.001, compared to nicotine group.*

Superoxide Dismutase (SOD):

Nicotine significantly decreased SOD levels in all three tissue regions compared to the VEH group. Interestingly, coadministration of CBD with nicotine showed a tendency for higher SOD levels, though not statistically different from either VEH or nicotine-alone groups.

Glutathione (GSH):

Nicotine significantly reduced GSH levels compared to VEH, while CBD coadministration effectively reversed this effect. In the NIC+CBD group, GSH levels were significantly elevated in the cerebral hemispheres and liver compared to the nicotine group. However, this upregulation was not observed in the cerebellum, where GSH levels were significantly reduced compared to the VEH group.

Malondialdehyde (MDA):

Nicotine administration significantly increased MDA levels compared to the control group. In contrast, CBD coadministration in the NIC+CBD group significantly lowered MDA levels in the cerebral hemispheres and cerebellum compared to the nico-tine-alone groups. Moreover, the downregulation of MDA in the coadministration group significantly differed from the VEH group in the liver. Additionally, CBD administration alone significantly decreased MDA levels in the liver compared to the VEH.

3.4. Pro-inflammatory Cytokines

The pro-inflammatory cytokines TNF-alpha and IL-6 were assessed following exposure to nicotine, CBD, and their coad-ministration in the CH and Ce and are presented in Figure 4. Nicotine significantly increased both markers in both regions, while CBD effectively reversed this upregulation. Specifically, in the CH, the TNF-alpha levels in the nicotine group were signifi-cantly higher (9.516±3.98 pg/mg of protein, $p<0.001$) compared to the VEH group (3.069±1.752 pg/mg of protein). However, coadministration of CBD in the NIC+CBD group significantly reduced TNF-alpha levels (2.979±1.489 pg/mg of protein, $p<0.001$) compared to the Nicotine group. Moreover, IL-6 at the CH showed a similar upregulation upon nicotine exposure (17.61±6.38 pg/mg of protein, $p<0.001$) compared to VEH (5.13±2.65 pg/mg of protein), while the NIC+CBD group exhibited a significant downregulation (5.75±3.39 pg/mg of protein, $p<0.001$) compared to the Nicotine group.

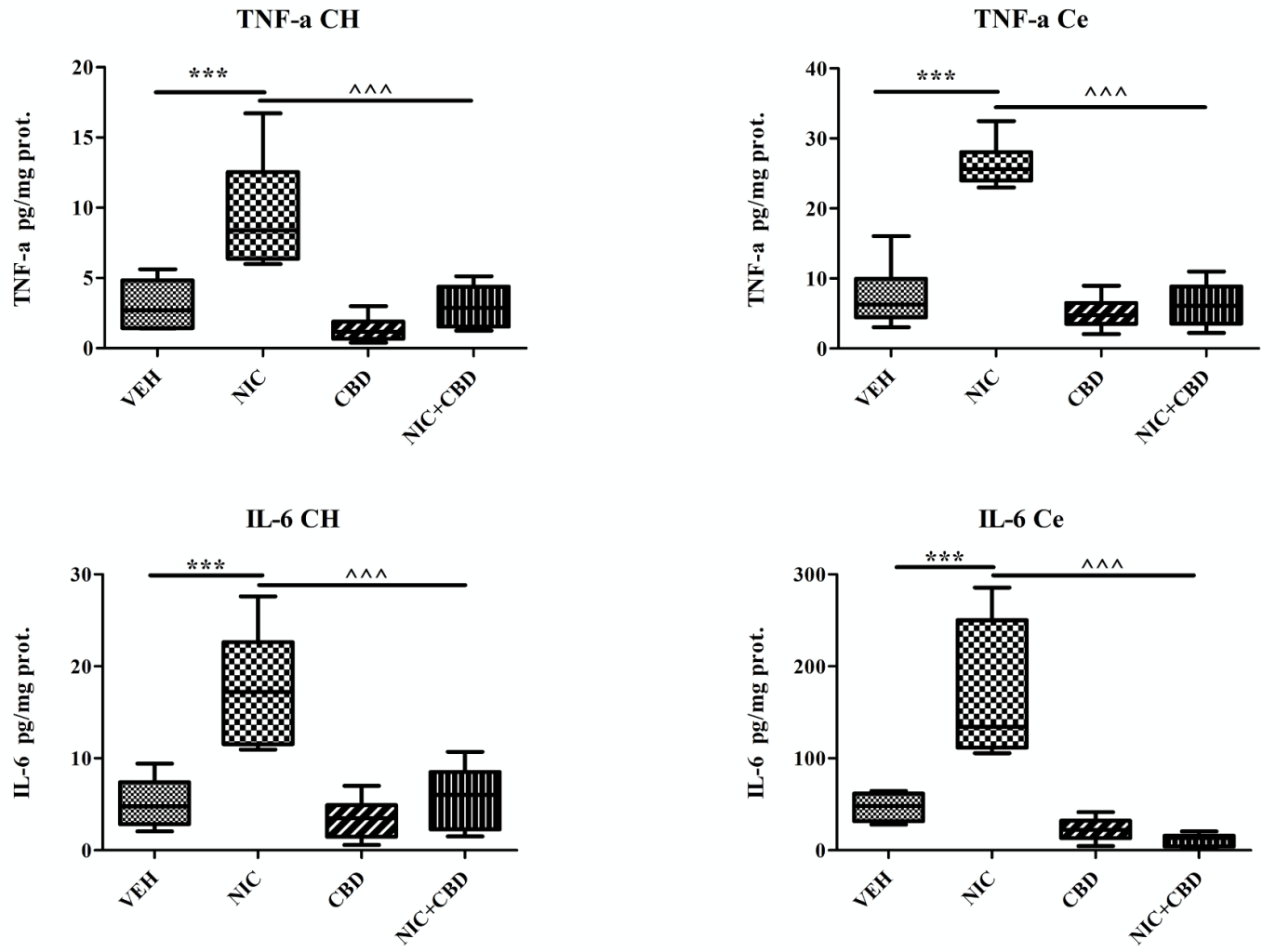

Figure 4. Nicotine, CBD, and co-administration effect on TNF-alpha and IL-6 levels in cerebral hemispheres and Cerebellum. The bars are presented as Whisker: Min to Max, for n=7 in each group. Cytokines are measured as pg/mg of protein. *** $p<0.001$, compared to Vehicle, and ^^^ $p<0.001$, compared to Nicotine group.

The results in the Ce followed the same pattern, with TNF-alpha being significantly higher in the Nicotine group (26.28±3.33 pg/ml) compared to the VEH group (7.42±5.45 pg/ml). At the same time, CBD coadministration reversed this upregulation, with TNF-alpha in the NIC+CBD group being significantly lower (6.26±3.24 pg/ml) compared to the Nicotine group. Similarly, IL-6 presented significantly higher levels in the Nicotine group (168.6±74.86 pg/ml, $p<0.001$) compared to the VEH group (47.02±14.57 pg/ml), while CBD coadministration resulted in significantly lower levels in the NIC+CBD group (11.35±6.73 pg/ml) compared to the Nicotine group.

3.5. BDNF ELISA Measurements

Nicotine effect on the BDNF tested in cerebral hemispheres and cerebellum resulted in a significant decrease in both regions, as seen in Figure 5. More specifically, in CH, BDNF in the nicotine group is significantly reduced (113.3±7.256 pg/mg of protein, $p<0.05$) compared to VEH (202.0±40.11 pg/mg of protein). This reduction is reversed upon nicotine coadministration in the NIC+CBD group (245.4±91.05 pg/mg of protein, $p<0.01$). Nicotine effect on BDNF is also present

at Ce, where nicotine administration reduced BDNF significantly (188.4±74.88 pg/mg of protein, $p<0.05$), compared to VEH (327.8±95.78 pg/mg of protein, $p<0.05$). At the same time, CBD coadministration did not reverse this effect in the NIC+CBD group (173.9±39.06 pg/mg of protein).

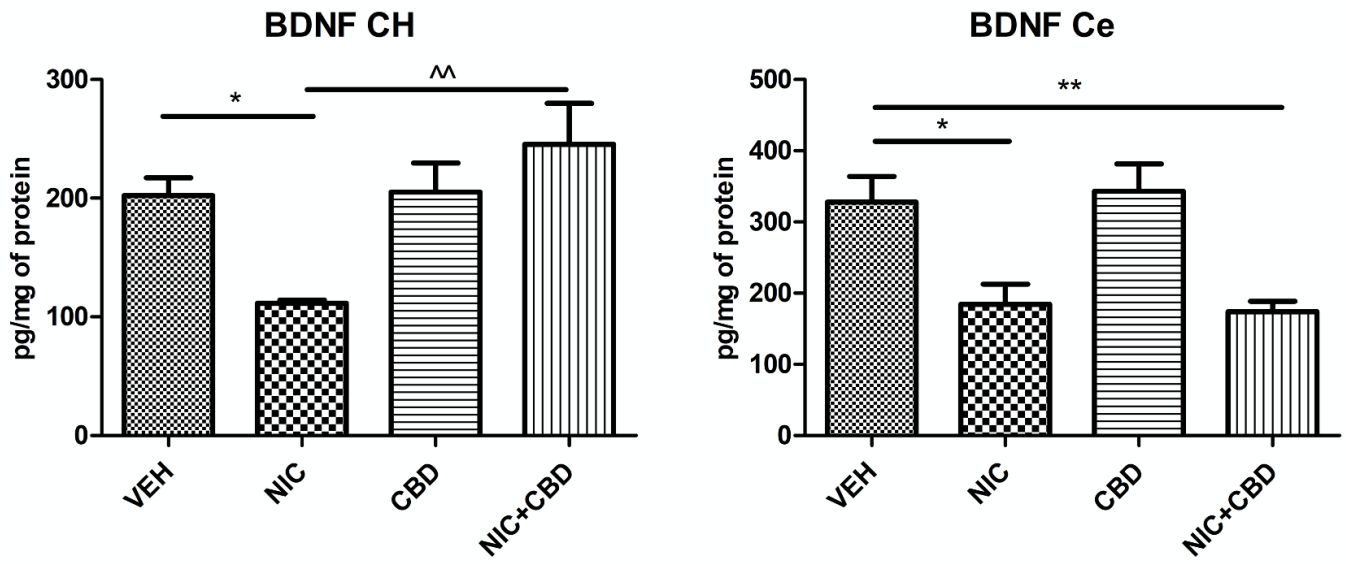

Figure 5. Nicotine, CBD, and co-administration effect on BDNF levels in cerebral hemispheres and cerebellum. The values are presented as MEAN ± STDEV, for n=7 in each group. BDNF is presented as pg/mg of protein. * $p<0.05$, compared to control group, ^ $p<0.05$, compared to nicotine group.

4. Discussion

This paper extends the exploration of nicotine-induced anxiety in mice by exploring several significant associated physiological changes of critical animal organs such as brain function through behavioral tests, toxicity status through renal, kidney, liver, and other blood's metabolic precursors, redox balance through oxidative stress metabolites, neuronal plasticity via BDNF levels, and immune response through the secretion of pro-inflammatory cytokines. Simultaneously, it aims to examine the impact of CBD on these alterations. While acute nicotine administration has been widely employed as a model to study anxiety-like behaviors in mice [13][14][15][42], there is a distinct gap in the literature concerning the simultaneous exploration of acute nicotine toxicity, extending beyond its immediate behavioral effects. By extending the investigation beyond behavioral changes, the study seeks to provide a more comprehensive understanding of the impact of nicotine toxicity. Furthermore, this study aims to investigate whether CBD can mitigate the intoxication markers influenced by nicotine toxicity.

The CBD dose selected was 50 mg/kg, as mentioned above, in accordance with the literature[4][5][6][7][8][9]. According to the human equivalent dose scheme, this dose would be converted to approximately 4 mg/kg in humans [43]. Also, this dose is equivalent and within dose ranges (400-800 mg) that have been tested, resulting in anxiolytic effects in human clinical studies [44][45][46][47][48].

Researchers are currently investigating the potential and applications of CBD in addressing inflammation and oxidative stress [49]. A recent study revealed that CBD attenuates TNF-alpha-induced inflammation in vitro[50]. Another study showed that CBD downregulates TNF-alpha and IL-6 levels upon LPS stimulation in mice [51]. In our study, nicotine induced the secretion of TNF-alpha and IL-6 in the brain across both regions examined. Notably, the coadministration of CBD significantly downregulated these proinflammatory cytokines, implying a protective role of CBD during the early stages of cytokine production. These results align with the literature mentioned above, confirming CBD's ability to regulate TNF-alpha and IL-6 in the context of nicotine's acute toxicity and pro-inflammatory cytokine production. Interestingly, a study examining cytokines and BDNF in mice, upon nicotine injections of 0.5 mg/kg, showed that nicotine may induce both central and systemic inflammatory responses, as well as changes in the BDNF regulation [52].

In the part of oxidative damage, following acute nicotine exposure of 1 mg/kg, the observed decrease in GSH and SOD levels suggests a reduction in the antioxidant capacity of mice, making them more susceptible to oxidative stress. Furthermore, the upregulation of MDA indicates an increase in lipid peroxidation, posing a potential threat to cellular integrity. The findings of nicotine-induced redox imbalance align with a parallel study involving the administration of 0.5 mg/kg nicotine [42]. In that study, nicotine significantly decreased SOD levels in the brain, cerebellum, hippocampus, and cortex, while also leading to MDA upregulation in the brain, hippocampus, and cortex. These findings combined suggest that nicotine can induce a redox imbalance in acute exposure in the brain of mice. In our study, this imbalance is apparent in the brain regions and extends to the liver, implying a potential systemic impact. While CBD treatment shows a tendency to reverse SOD downregulation in the coadministration group, this change is not statistically significant. Interestingly, CBD coadministration effectively reverses nicotine-induced GSH imbalance in the liver and both brain regions, along with reversing MDA imbalances in the liver and cerebral hemispheres. These results suggest that CBD exhibits tissue-specific antioxidant capabilities by modulating GSH and MDA, without significantly impacting SOD activity.

As mentioned above, the significance of BDNF in the context of mouse social stress establishes a crucial connection between BDNF and anxiety. In a previous study, BDNF administration demonstrated a dose-dependent attenuation of induced anxie-ty-like behaviors in a rodent model, as evidenced by increased entries to the center in the OFT [53]. In our study, nicotine administration resulted in a significant reduction in BDNF levels in both brain regions. This decrease suggests a potential connection between nicotine-induced anxiety and BDNF levels in this model during the performance of the OFT. Concurrently, as BDNF is downregulated, anxiety-like behaviors manifest in the OFT, characterized by a reduction in entries to the center and an increase in thigmotaxis. Interestingly, CBD administration demonstrated an anxiolytic effect in the OFT, as evidenced by increased entries to the center and reduced thigmotaxis. Simultaneously, a significant upregulation of BDNF was observed in the coadministration group.

In the context of locomotor activity, nicotine reduced the total time of animals' mobility and total distance traveled, indicating a pronounced impact. Remarkably, CBD coadministration mitigated nicotine's effects, resulting in an improvement in animals' locomotor activity. While research specifically addressing the impact of CBD on nicotine-induced hypolocomotion is currently lacking, various studies involving rodents have explored the effects of single doses of CBD on locomotor alterations induced by other substances. In a model examining the reduction of activity induced by haloperidol,

CBD single doses of 3, 5, and 10 mg/kg were tested. The findings revealed that CBD at a dose of 5 mg/kg effectively antagonized hypolocomotion induced by haloperidol [54]. The results regarding locomotor improvement are controversial in several other studies employing various models. Some studies demonstrate that CBD enhances locomotor activity [55][56], while others report no significant impact of CBD on it[57][58][59]. To our knowledge, our study is the first one examining the impact of CBD on nicotine-induced hypolocomotion.

Finally, nicotine administration led to an imbalance in hepatic enzymes, ALT and ALP, with oxidative stress evident in the liver, as mentioned above, suggesting a potential hepatotoxicity capability of nicotine. CBD administration was well-tolerated, and none of the blood test markers were affected. Interestingly, in the coadministration group, CBD significantly reversed nicotine's impact on ALP, though it did not seem to affect ALT significantly.

5. Conclusions

The acute behavioral changes induced by nicotine, characterized by anxiety-like behavior and reduced locomotor activity, are accompanied by notable biochemical alterations in the brain and liver. Specifically, oxidative damage is evident with an im-balance in GSH, SOD, and MDA, while proinflammatory cytokines TNF-alpha and IL-6 are elevated in CH and Ce. BDNF is affected, showing downregulation in the CH and Ce, suggesting a potential link between behavioral alterations and BDNF levels. Interestingly, CBD demonstrated a reduction in anxiety-like behavior and an improvement in locomotor activity. Ad-ditionally, CBD upregulated the altered BDNF levels. The protective effects of CBD extend to oxidative stress, cytokine production, and ALP levels. These findings collectively suggest that CBD has the potential to alleviate nicotine-induced anxiety-like behaviors by mitigating its systemic toxicity.

Statements and Declarations

Author Contributions: Conceptualization, K.M. and K.P.; methodology, K.M..; software, K.M. and K.A.; formal analysis, K.M. and K.A.; investigation, K.M..; writing—original draft preparation, K.M..; writ-ing—review and editing, K.A., G.L., M.M. and K.P..; visualization, K.M. and K.A..; supervision, K.P.; pro-ject administration, K.P. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: The handling of animals and the study protocol were in accordance with the Greek Presidential decree 86/2020 for adaptation of Greek legislation to the directive 2010/63/EU of the European Parliament and of the European Council of 22 September 2010 on the protection of animals used for scientific purposes. The mice study protocol was approved by the National Veterinary Administra-tion authorities (License.: ΠΔΕ/ΔΚ/923/3, approval date 18 January 2021).

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