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Inducible Nitric Oxide Synthase Inhibition in the Medial Prefrontal Cortex Attenuates the Anxiogenic-Like Effect of Acute Restraint Stress *via* CB_1 Receptors

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Stress exposure can result in several proinflammatory alterations in the brain, including overexpression of the inducible isoform of nitric oxide synthase (iNOS) in the medial prefrontal cortex (mPFC). These changes may be involved in the development of many psychiatric conditions. However, it is unknown if iNOS in mPFC plays a significant role in stress-induced behavioral changes. The endocannabinoid (ECB) system is also influenced by stress. Its activation seems to be a counter regulatory mechanism to prevent or decrease the stress-mediated neuroinflammatory consequences. However, it is unclear if the ECB system and iNOS interact to influence stress consequences. This study aimed to test the hypothesis that the anti-stress effect of iNOS inhibition in mPFC involves the local ECB system, particularly the CB_1 cannabinoid receptors. Male Wistar rats with guide cannula aimed at the mPFC were submitted to acute restraint stress (RS) for 2 h. In the following morning, rats received bilateral microinjections of vehicle, AM251 (CB_1 antagonist; 100 pmol), and/or 1400W (iNOS selective inhibitor; 10^{-4}, 10^{-3}, or 10^{-2} nmol) into the prelimbic area of mPFC (PL-mPFC) before being tested in the elevated plus-maze (EPM). iNOS inhibition by 1400W prevented the anxiogenic-like effect observed in animals submitted to RS. The drug did not promote behavior changes in naive animals, demonstrating a stress-dependent effect. The 1400W-anti-stress effect was prevented by local pretreatment with AM251. Our data suggest that iNOS inhibition may facilitate the local endocannabinoid signaling, attenuating stress effects.

Keywords: iNOS, CB_1 receptor, medial prefrontal cortex, stress, anxiety

INTRODUCTION

Stress exposure is linked to a persistent stage of low inflammatory levels in the periphery and central nervous system, predisposing the individual to develop several pathologies like diabetes, cancer, and cardiovascular diseases (1–4). In this context, the disbalance of immunological components, combined with dysfunctional neuroendocrine and neurotransmitter systems, appears to contribute to psychiatric disorders, such as major depression, anxiety, schizophrenia, and

posttraumatic stress disorder (PTSD) (4–11). Due to such relevance, many researchers worldwide turned their eyes to study the relationship between stress, neuroinflammation, and the development of psychiatric diseases.

Different stressors release danger/damage-associated molecular patterns (DAMPs) or "alarmins" involved in sterile inflammation, which can alter mood, increasing the risk of psychiatric disorders (12, 13). DAMPs, such as ATP, heat shock proteins, and high mobility group box1 (HMGB1) acting through pattern recognition receptors (PPRs), and proinflammatory mediators such as IL-1β, activate the transcription factor nuclear factor Kappa-B (NF-κB) (14). NF-κB is a significant regulator of the inducible nitric oxide synthase (iNOS) isoform (15). iNOS expression and activation increase nitric oxide (NO) synthesis (15), potentially resulting in oxidative/nitrosative stress and neurotoxicity (16).

NMDA receptors activation by glutamate release, which increases in the medial prefrontal cortex (mPFC) during and after stress (17–19), also activates NF-κB (20). Mice exposure to restraint stress (RS) associated with acoustic stress augments iNOS expression in the mPFC (21). Similarly, RS alone increased iNOS activity in the cortex of rats (20). Besides, genetic deletion of iNOS and its systemic pharmacological inhibition reduced stress-elicited behavioral consequences (22). Despite this evidence, the role of iNOS specifically in the mPFC in developing stress-related behaviors is not entirely understood. Previously we demonstrated that the inhibition of neuronal NOS (nNOS) in the prelimbic region of mPFC (PL-mPFC) reverted the anxiogenic-like effect induced by acute stress (23). However, the role of iNOS in this region in stress modulation is still poorly explored. Thus, our first aim is to test the hypothesis that, similar to nNOS, the local inhibition of iNOS in the PL-mPFC reduces the anxiogenic-like effect induced by acute stress.

Exposure to stressful events may also stimulate the endocannabinoid (ECB) system (24). This system can act through retrograde neuromodulators, the endocannabinoids (ECBs), which include anandamide (AEA) and 2-arachidonoylglycerol (2-AG). These two ECBs act on presynaptic CB_1 receptors, reducing the Ca^{2+} influx and decreasing glutamate release (25, 26). They can also activate CB_2 receptors, found predominantly in immune cells, like microglia (27), and show postsynaptic expression in neurons (28).

The ECB system is considered a stress-buffer system, preventing or attenuating stress-related emotional consequences. This neuromodulatory system also regulates proinflammatory mediators (29–32). Evidence suggests a functional interaction between the ECB and nitrergic systems in brain regions engaged by exposure to aversive stimuli (33–35), including in the mPFC (36). Moreover, CB_1 KO mice have increased NOS activity in the brain (37) and pharmacological or genetic inhibition of cannabinoid receptors increases iNOS expression in the PFC after stress (31, 38). Therefore, iNOS inhibition may facilitate stress adaptation by favoring the ECB signaling. Thus, the second aim of this study is to test if the anti-stress effect of iNOS inhibition in the PL-mPFC depends on the CB_1 signaling.

EXPERIMENTAL PROCEDURES

Animals

In the first part of this study (Experiment 1), male Wistar rats (240–300 g, 7–8 weeks old) were acquired from the animal facility of the University of São Paulo, campus Ribeirão Preto. However, this strain production was discontinued at Ribeirão Preto. Therefore, for Experiment 2 Wistar rats were obtained from the Anilab company (Paulinia, São Paulo, Brazil). Animals from different suppliers were not used in the same experiment. Rats were kept in the animal care unit of the Pharmacology Department (FMRP/USP). Rats were housed in groups of four animals per cage (acrylic boxes - 49 × 34 × 26 cm) in a temperature-controlled room (24 ± 1°C) under standard laboratory conditions: 12-h light/dark cycle (lights on at 06:00 a.m.), humidity of 50–55%, with free access to food (Nuvital, Nuvilab, Brazil) and filtered water. All experimental procedures, summarized in **Figure 1**, were approved by the Ethical Review Committee of the Medical School of Ribeirão Preto (protocol no. 224/2017). All efforts were made to reduce the suffering and the number of animals used in this study.

Drugs

The selective, potent, and irreversible inhibitor of the iNOS enzyme, N-([3-(Aminomethyl)phenyl]methyl)ethanimidamide dihydrochloride (1400 W; Sigma-Aldrich, USA), at the doses of 10^{-4}, 10^{-3}, and 10^{-2} nmol/0.2 μL/side, was dissolved in sterile saline. 1400 W doses were calculated based on the Ki for nNOS of a selective nNOS inhibitor, n-propyl-l-arginine (NPLA; 0.01 nmol/0.2 μl;). The dose of 0.01 nmol/0.2 μl of NPLA in the mPFC attenuates immobility time in the forced swimming test (39). Based on this dose and the Ki of NPA (Ki = 57 nM) and 1400 W (Ki = 2,000 nM) for nNOS, we calculated the dose of 1400 W that would be equipotent to the dose of NPA to inhibit nNOS (∼0.35 nmol). Considering that the Ki of 1400 W for inhibiting iNOS is around 286 times lower than that for inhibiting nNOS (7 nM), we calculated the dose of 0.0012 nmol (1.2×10^{-3} nmol) of 1400 W would selectively inhibit iNOS. Higher and lower 1400 W doses were also employed based on this last dose.

The CB_1 receptor antagonist, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251, Tocris, USA), 100 pmol/0.2 μL/side, was dissolved in 10% dimethyl sulfoxide (DMSO) in saline (0.9% NaCl). This dose was based on previous data (40–43). Drugs were freshly prepared before use, kept on ice, and protected from light during experimental sessions. Each control group received the corresponding vehicle of the drug being tested (1400 W: saline, 0.2 μL/side, and AM251: 10% DMSO in saline, 0.2 μL/side).

For the stereotaxic surgery, we administered the general anesthetic 2,2,2-Tribromoethanol (2.5%, 1 ml/kg-Sigma-Aldrich, USA), the local anesthetic lidocaine (2%-Dentsply, Brazil), the poly antibiotic preparation of streptomycin and penicillin (1.200.000 UI−0.2 ml/rat; Pentabiotic-Fort Dodge, Brazil), and

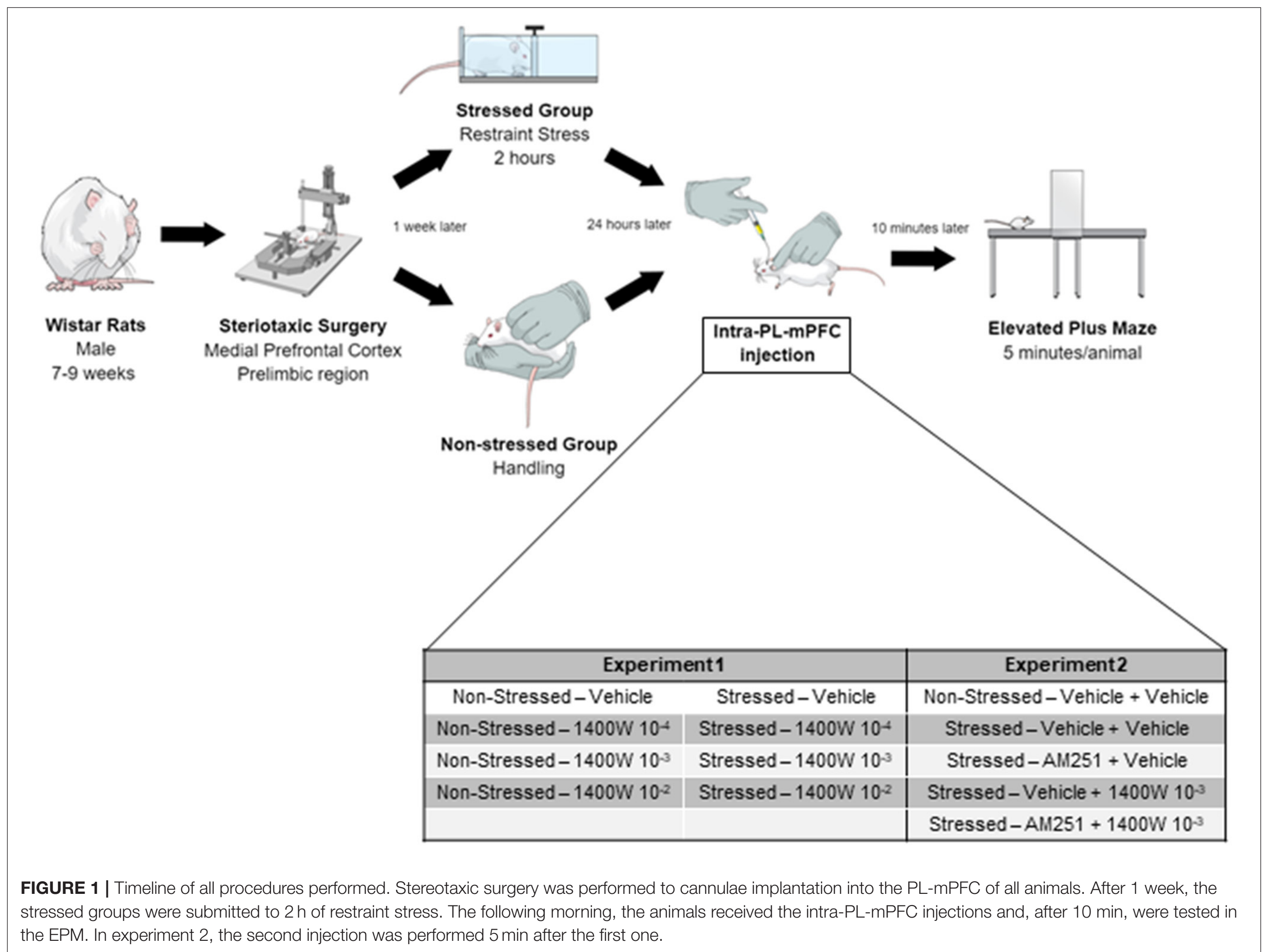

FIGURE 1 | Timeline of all procedures performed. Stereotaxic surgery was performed to cannulae implantation into the PL-mPFC of all animals. After 1 week, the stressed groups were submitted to 2 h of restraint stress. The following morning, the animals received the intra-PL-mPFC injections and, after 10 min, were tested in the EPM. In experiment 2, the second injection was performed 5 min after the first one.

the anti-inflammatory flunixin meglumine (s.c., 0.025 g/kg; Banamine®-Schering Plough, Brazil) for postoperative analgesia.

Stereotaxic Surgery

Five to seven days before the stress procedure, stereotaxic surgeries were performed in rodents to implant stainless steel guide cannula (11 mm, 0.6 mm outer diameter-OD) bilaterally into the pre-limbic region of the medial prefrontal cortex (PL-mPFC). Animals were previously anesthetized with 2,2,2-Tribromoethanol intraperitoneally (i.p.) and placed in the stereotaxic frame (Stoelting, USA). The skull was surgically exposed for cannulae implantation after scalp anesthesia with 2% lidocaine subcutaneous (s.c.). Based on the rat brain atlas (44), stereotaxic coordinates used were: AP: +3.3 mm from Bregma, L: +1.9 mm from the medial suture, V: −2.4 mm of the skull, and cannulae angle was 22°. The position of the incisor bar was adjusted to −3.2 mm. Guide cannulae were fixed to the skull with acrylic cement and a metal screw; a stylet inside the cannulae prevented obstruction. The animals received antibiotic (intramuscular – i.m. – Pentabiotic) and anti-inflammatory (s.c.-Banamine) injections during the surgery to avoid infections and induce analgesia.

Restraint-Stress (RS)

One day before the behavioral test, animals were submitted to the restraint-stress (RS). Rodents were immobilized in metallic tubes (20 × 7 × 4 cm) with adjustable roof and ventilation holes for 2 h. The non-stressed animals received handling procedures during the restraint session. The protocol was conducted in a sound-attenuated and temperature-controlled (24°C) room. At the end of the RS, the animals (stressed and non-stressed) were individually housed in acrylic cages (36 × 25 × 24 cm) until the behavioral test. Handling and restraint stress were performed in the morning.

Drug Microinjection

Animals received bilateral injections (0.2 µL each) into the PL-mPFC using a dental needle (33 G/0.3 mm OD), 1 mm longer than the guide cannula, connected via polyethylene tubing (PE-10) to a 10-µl syringe (7001-KH; Hamilton Co., USA). Needles were carefully inserted into the guide cannula, and the solution

was infused for over 30 s with the help of an infusion pump (KD Scientific, Massachusetts, USA). The needles remained in the cannulae for an additional 30 s to prevent reflux.

Elevated Plus Maze (EPM) and Experimental Design

All experiments were carried out between 7 a.m. and 2 p.m. Animals were transported to a sound-attenuated and temperature-controlled (24°C) room for habituation before starting experimental procedures. The injections were performed as described in the previous section. Ten minutes after the last injection, animals were subjected to the Elevated Plus Maze (EPM). The wood apparatus consists of two opposite open arms (50 cm length × 10 cm width), crossed at a right angle by two closed arms with the exact dimensions, enclosed by 40 cm high walls with no roof. The maze is 50 cm from the ground. The ANY-Maze^{TM} software (version 4.7, Stoelting) (RRID: SCR_014289) performed the behavior analysis of the movies recorded through a video camera. This software detects the animal's position within the maze by contrasting the animal's color with the maze floor and calculates the number of entries onto the arms and the time spent in the open arms. Each test lasted 5 min, and the apparatus was cleaned with an alcohol solution between trials.

All animals were habituated for at least 1 h in the experimental room. In the first experiment, rats received microinjections of vehicle (saline - 0.9% NaCl) or 1400 W (10^{-4}, 10^{-3}, or 10^{-2} nmol) into the PL-mPFC. 10 min later, they were tested on the EPM. Similarly, in the second experiment, animals received a first local injection of vehicle (10% DMSO in saline) or AM251 (100 pmol) followed, 5 min later, by the second injection of vehicle (saline-0.9% NaCl) or 1400 W (10^{-3} nmol) into the PL-mPFC. They were tested on the EPM 10 min later.

Histological Procedure

In the afternoon that followed the behavioral tests, the rats were anesthetized with chloral hydrate 5% (1 mL/kg, i.p.) and perfused with isotonic saline followed by 10% formalin solution. As a site marker, 0.2 μL of 1% Evan's blue dye was bilaterally injected into the PL-mPFC. The brains were postfixed in 10% formalin solution for 24 h, and sections of 40 mm were cut using a cryostat (CM-1900; Leica, Wetzlar, Germany). The injection sites were identified with the help of the rat brain atlas (44). Representative photomicrography of the injection sites into the PL-mPFC can be seen in **Figure 2**. Rats receiving injections outside the aimed area were excluded from the analyses.

Statistical Analysis

The anxiety index was calculated for each animal as in equation 1 (45):

$$\textit{Anxiety index} = 100 - \left[\frac{\%\ \textit{entries in the open arms} + \%\ \textit{time in the open arms}}{2}\right]$$

The percentage of entries and time in the open arms of the maze were plotted and analyzed separately.

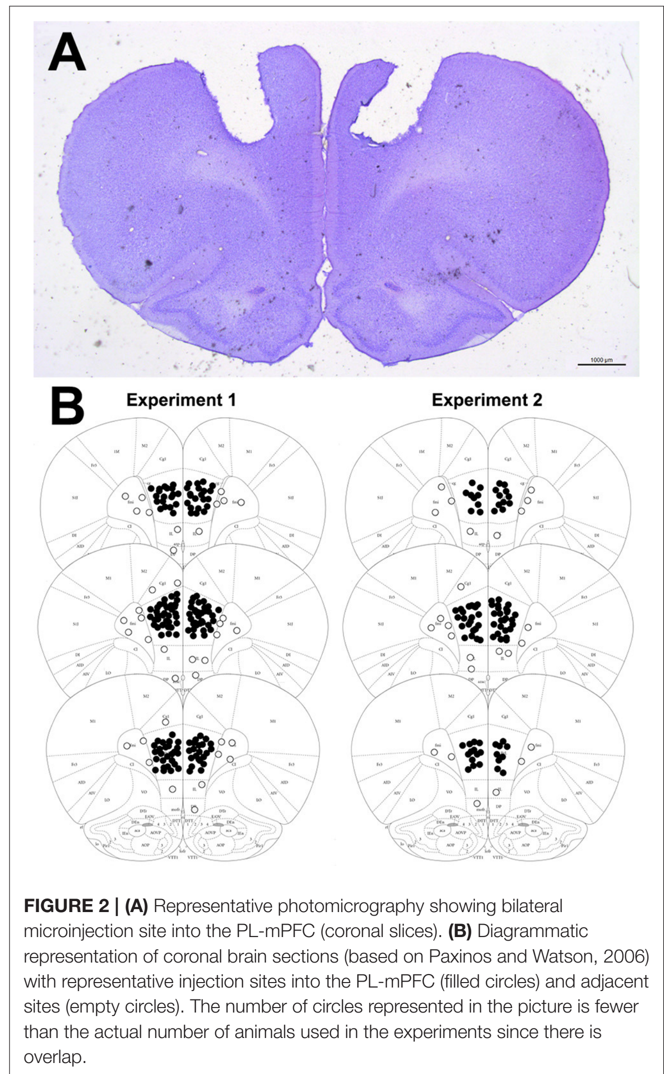

FIGURE 2 | (A) Representative photomicrography showing bilateral microinjection site into the PL-mPFC (coronal slices). **(B)** Diagrammatic representation of coronal brain sections (based on Paxinos and Watson, 2006) with representative injection sites into the PL-mPFC (filled circles) and adjacent sites (empty circles). The number of circles represented in the picture is fewer than the actual number of animals used in the experiments since there is overlap.

All data were tested for normality and homogeneity of variance by the Kolmogorov-Smirnov and Levene tests, respectively. Welch's correction was performed to adjust the calculations of F and t ratios and degrees of freedom to adjust for heterogeneity of within-group variances. Behavioral parameters were expressed as the means ± standard error of the mean (SEM). For the first experiment, the anxiety index (percentage of entries and time in the open arm) and the number of enclosed arms entries were analyzed. First, all groups were analyzed by two-way Analysis of Variance (ANOVA), considering stress (condition) and treatment as factors to be analyzed. After that, naïve and stressed rats that received vehicle were compared by unpaired Student's *t*-test to verify stress interference. Then, each condition's data (naïve and stressed animals) were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. For the second experiment, there was no drug administration in naïve rats. Therefore, naive and stressed-vehicle groups were compared by unpaired Student's *t*-test. After that, data from the stressed group were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. Statistical differences were considered

significant when $p < 0.05$. Statistical tendency was considered if $p = 0.06$–0.1.

RESULTS

Intra-PL-mPFC Injection of 1400 W Reversed the Stress-Induced Anxiogenic-Like Effect in the Elevated Plus-Maze

In the anxiety index, a two-way ANOVA indicated a significant interaction between the factors (condition × treatment) [$F_{(3,79)} = 2.866$, $p = 0.041$, two-way ANOVA]. Comparing naïve-vehicle and stressed-vehicle, we observed that acute restraint stress (RS) induced an anxiogenic-like effect in the EPM 24 h later, increasing the anxiety index [$t_{(23.35)} = 3.344$, $p = 0.002$, unpaired *t*-test with Welch's correction] (**Figure 3A**). One-way ANOVA of stressed groups revealed that 1400 W (10^{-3} and 10^{-2} nmol) reversed the stress-induced anxiogenic-like effect [$F_{(3,32)} = 6.399$, $p = 0.001$, one-way ANOVA;10^{-3} nmol: $p = 0.007$; 10^{-2} nmol: $p = 0.004$, Tukey's *post hoc*]. A dose-response curve was generated by non-linear regression ($r^2 = 0.374$, $df = 33$) (**Figure 3D**). 1400 W did not alter behavior in non-stressed animals [$F_{(3,47)} = 0.173$, $p = 0.914$, one-way ANOVA] (**Figure 3C**). In addition, no changes were observed in the enclosed arm entries, suggesting that neither acute stress nor 1400 W affected basal motor activity ($p > 0.05$) (**Figure 3B**).

The analysis of the percentage of time and entries in the open arms of the EPM indicated the same conclusions. In the percentage of time spent in the open arms, there was an effect of condition [$F_{(1,79)} = 5.820$, $p = 0.018$] and treatment [$F_{(3,79)} = 2.756$, $p < 0.049$]. Moreover, there was a tendency for an interaction between the factors (condition × treatment) in the percentage of time [$F_{(3,79)} = 2.450$, $p = 0.069$] and entries [$F_{(3,79)} = 2.545$, $p = 0.062$] in the open arms. When comparing vehicle-treated groups, RS reduced the percentage of entries [$t_{(32)} = 2.669$, $p = 0.011$, unpaired *t*-test] and time [$t_{(21,55)} = 4.253$, $p = 0.0003$, unpaired *t*-test with Welch's correction] in the open arms, suggesting an anxiogenic-like effect. Doses of 10^{-3} and 10^{-2} reversed the reduction in the percentage of entries [$F_{(3,32)} = 4.383$, $p = 0.010$, one-way ANOVA] [10^{-3} nmol: $p = 0.036$; 10^{-2} nmol: $p = 0.022$, Tukey's *post hoc*] (**Figure 3E**), and percentage of time [$F_{(3,32)} = 6.611$, $p = 0.001$, one-way ANOVA] [10^{-3} nmol: $p = 0.005$; 10^{-2} nmol: $p = 0.005$, Tukey's *post hoc*] (**Figure 3F**) in the open arms of the EPM. None of the doses affected the non-stressed animals ($p > 0.05$).

A CB_1 Receptor Antagonist Injected Into the PL-mPFC Prevented 1400 W Effects on Stress-Induced Behavioral Consequences in the Elevated Plus-Maze

Once again, RS induced an anxiogenic-like effect in the EPM, evidenced by the increase in the anxiety index [$t_{(20)} = 2.206$, $p = 0.039$]. One-way ANOVA also revealed a significant effect of treatment in stressed animals [$F_{(3,35)} = 4.584$, $p = 0.008$]. Confirming our previous result, the 1400 W injection (lower effective dose: 10^{-3} nmol) reversed the stress effect ($p = 0.012$, Tukey's *post hoc*). Prior intra-PL-mPFC injection of the CB_1 receptor antagonist AM251 blocked the 1400 W effect ($p > 0.999$ compared with Stress–Veh+Veh group; and $p = 0.021$ compared with Stress–Veh+1400 W group, Tukey *post hoc*). AM251 did not induce any effect *per se* ($p = 0.939$, Tukey *post hoc*) (**Figure 4A**). No significant difference was observed in the enclosed arm entries ($p > 0.05$) (**Figure 4B**).

Similar conclusions were reached from the analysis of the percentage of entries and time in the open arms of the maze individually. RS increased the percentage of entries [$t_{(20)} = 2.148$, $p = 0.044$] and tended to increase the percentage of time [$t_{(20)} = 2.075$, $p = 0.051$] in the open arms of the EPM. The one-way ANOVA revealed a significant effect of treatment in stressed animals [%entries: $F_{(3,35)} = 5.004$, $p = 0.005$; %time: $F_{(3,35)} = 3.686$, $p = 0.020$]. 1400 W reversed the stress-induced anxiogenic-like effect (%entries: $p = 0.008$; %time: $p = 0.030$, Tukey's *post hoc*). The previous administration of AM251 blocked 1400 W effect (%entries: $p = 0.997$; %time: $p > 0.999$, Tukey's *post hoc*). AM251 had no effect *per se* (%entries: $p = 0.957$; %time: $p = 0.930$, Tukey's *post hoc*) (**Figures 4C,D**).

DISCUSSION

Our study confirms previous data showing that acute RS in rats induced anxiogenic-like behavior (23, 46–48). The administration of the selective inhibitor of iNOS 1400 W into the PL-mPFC reversed this anxiogenic effect but did not affect unstressed animals, suggesting that iNOS was activated by stress. Furthermore, we observed that the anti-stress effect of 1400 W is blocked by the antagonist AM251, indicating that it depends on the endocannabinoid signaling, particularly CB_1 receptors.

RS induces several neurotransmitter changes. It increases glutamate levels (17, 48), alters the release of corticosterone (49, 50), and changes proinflammatory cytokines levels by activating NF-?B (51, 52). In addition, depending on the duration and the number of episodes, stress induces behavioral changes, including depressive- (51) and anxious-like (23, 46–48) behaviors. Our results showed that 2-h of RS decreased the exploration of open arms of EPM (percentage of entries and time), increasing the anxiety index. Also, stress did not alter the number of entries in the closed arms of EPM, suggesting that this was not a motor effect.

The PFC is connected to several brain areas and promotes the integration between stimuli, playing a pivotal role in decision making, goal-directed behaviors, and working memory (53, 54). However, the precise role of each of its subdivisions in these processes is still under debate. The PL-mPFC projects to the "stress circuit areas," such as the basolateral amygdala (54, 55). It seems to be involved with stress response control and anxiety-related behaviors (55, 56). Stress exposure can promote plastic changes in the PL-mPFC (57, 58), increasing the expression of c-fos protein (59) or the firing rate of local neurons during acute stress (60), for example. Furthermore, stress increases monoamines, glutamate, and glucocorticoid release (17, 61, 62) and alters the dendritic morphology of PL (63). Finally,

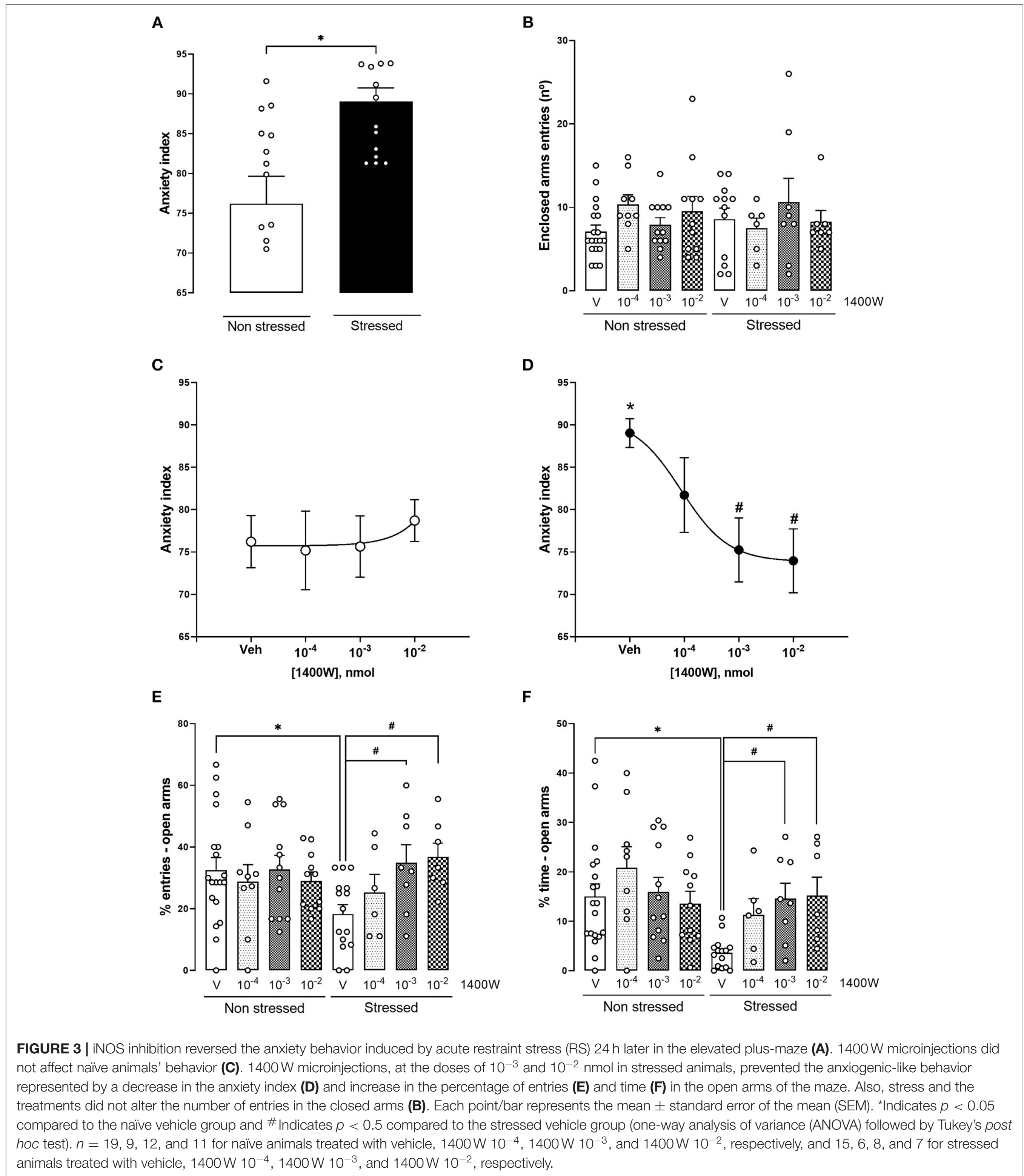

FIGURE 3 | iNOS inhibition reversed the anxiety behavior induced by acute restraint stress (RS) 24 h later in the elevated plus-maze **(A)**. 1400 W microinjections did not affect naïve animals' behavior **(C)**. 1400 W microinjections, at the doses of 10^{-3} and 10^{-2} nmol in stressed animals, prevented the anxiogenic-like behavior represented by a decrease in the anxiety index **(D)** and increase in the percentage of entries **(E)** and time **(F)** in the open arms of the maze. Also, stress and the treatments did not alter the number of entries in the closed arms **(B)**. Each point/bar represents the mean ± standard error of the mean (SEM). *Indicates $p < 0.05$ compared to the naïve vehicle group and #Indicates $p < 0.5$ compared to the stressed vehicle group (one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test). $n = 19$, 9, 12, and 11 for naïve animals treated with vehicle, 1400 W 10^{-4}, 1400 W 10^{-3}, and 1400 W 10^{-2}, respectively, and 15, 6, 8, and 7 for stressed animals treated with vehicle, 1400 W 10^{-4}, 1400 W 10^{-3}, and 1400 W 10^{-2}, respectively.

stressful events increase the expression and activity of iNOS in the mPFC (21, 64, 65). These findings suggest that PL-mPFC hyperactivation in response to stress could influence behavior and contribute to the anxiogenic effect. Moreover, as observed in the present work with iNOS, modulation of the local changes induced by stress could reverse the behavioral consequences

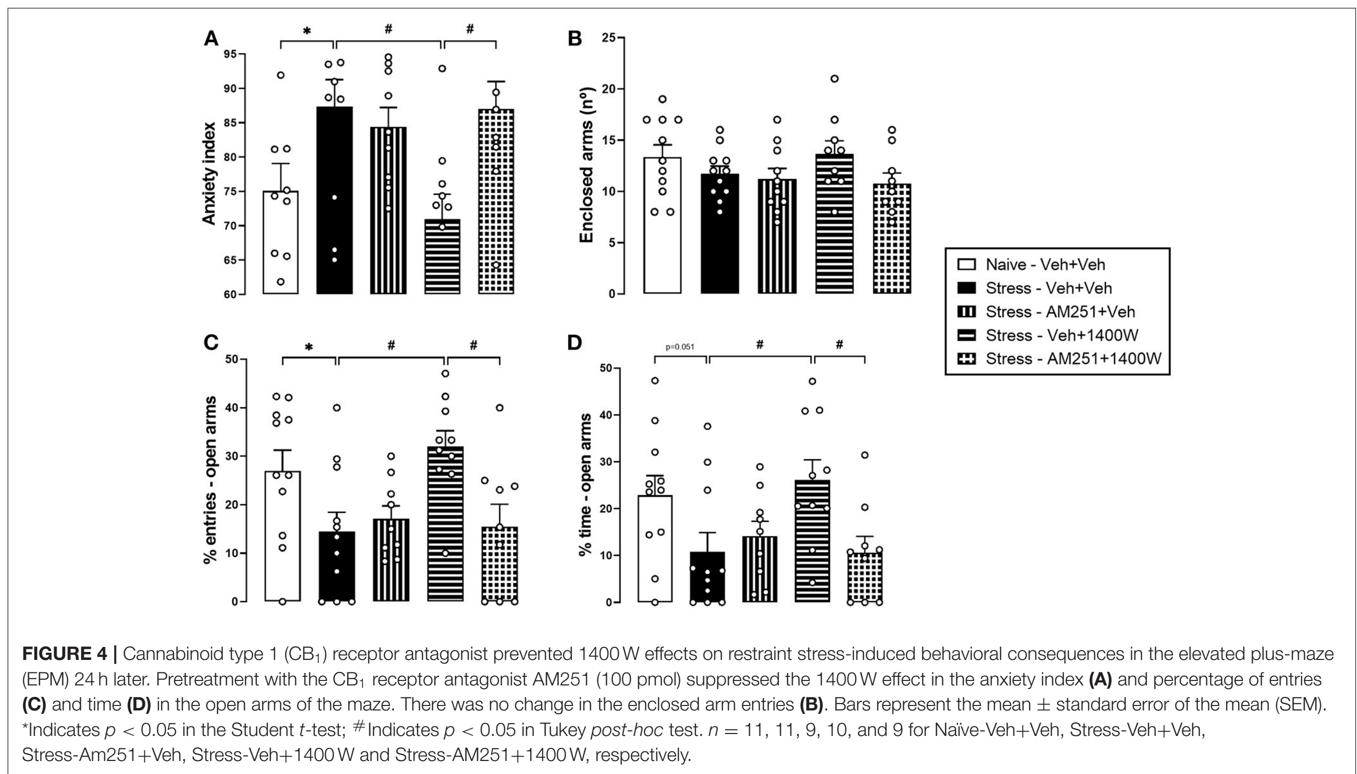

FIGURE 4 | Cannabinoid type 1 (CB_1) receptor antagonist prevented 1400 W effects on restraint stress-induced behavioral consequences in the elevated plus-maze (EPM) 24 h later. Pretreatment with the CB_1 receptor antagonist AM251 (100 pmol) suppressed the 1400 W effect in the anxiety index **(A)** and percentage of entries **(C)** and time **(D)** in the open arms of the maze. There was no change in the enclosed arm entries **(B)**. Bars represent the mean ± standard error of the mean (SEM). *Indicates $p < 0.05$ in the Student *t*-test; #Indicates $p < 0.05$ in Tukey *post-hoc* test. $n = 11, 11, 9, 10$, and 9 for Naïve-Veh+Veh, Stress-Veh+Veh, Stress-Am251+Veh, Stress-Veh+1400 W and Stress-AM251+1400 W, respectively.

of stress exposure. Previous studies (66, 67), including from our group (68), showed that systemic administration of iNOS inhibitors could induce anxiolytic-like effects, attenuate stress effects, or induce antidepressant-like effects. Altogether, the present results suggest that the mPFC could be a possible target of these drugs.

NO modulates several functions in the CNS, such as neurotransmitter release, synaptic plasticity, and neuroprotection (69–71). However, when at high concentrations, it can produce harmful actions (72). Increased NO levels, as observed in stressful situations, positively modulate glutamate release, which could create positive feedback and facilitate excitotoxicity (73, 74). Furthermore, NO can promote S-nitrosylation of cysteine thiol groups of several proteins, interfering with their functions (72, 75, 76). For example, the S-nitrosylation of protein-disulphide isomerase (PDI) in patients with neurodegenerative disorders inhibits its enzymatic activity, leading to the accumulation of polyubiquitinated proteins in the brain (77). Moreover, S-nitrosylation can result in protein misfolding, mitochondrial dysfunction, synaptic damage, and neuronal cell death (77). Overall, these changes induced by NO could negatively impact behavior.

Acute intense stress increased NO metabolites (NOx) levels in the PL-mPFC and induced anxiety-like behavior (78). Also, anxiety-like behavior caused by acute restraint stress was reverted by intra-PL-mPFC injection of an nNOS inhibitor (23). Moreover, intra-hippocampal injection of aminoguanidine, an iNOS inhibitor, prevented depressive behaviors induced by chronic stress (79). As far as we know, this is the first study demonstrating that, similarly to the latter study, inhibition of the iNOS enzyme directly in the PL-mPFC reverses the stress-induced anxiogenic-like effect. Considering previous evidence observed with nNOS inhibitors administered into the same brain region (23), the present data adds more complexity to NO's regulatory mechanisms in the mPFC, implicating iNOS involvement in the anxiogenic-like effect induced by stress. It is worth mentioning that the calculated dose of 1400W was based on its Ki values, which is almost 300 times lower to inhibit iNOS than for inhibiting nNOS. Therefore, considering 1400 W high selectivity for iNOS, it is unlikely that the doses used promote nNOS inhibition, strengthening the involvement of iNOS in the present study. The complexity of NO signaling could be attributed in part to the expression of NOS isoforms by different cell types, but also by several mechanisms by which NO can affect proteins and systems function (80). For future studies, it would be interesting to perform a combination of subeffective doses of the different isoform inhibitors to evaluate the possible attenuation of the stress effect.

The effect of 1400 W in the PL-mPFC was only observed in stressed rats, suggesting that even if iNOS is constitutively expressed in some brain areas in the healthy brain (81), stress exposure increases its expression or activity, which would be necessary to observe the behavioral changes. Therefore, the 1400 W effect is dependent on stress-induced molecular alterations.

The NO signaling can regulate and be regulated by the ECB system (33–35, 74, 80, 82). For example, it is well-established that CB_1 receptor activation reduces glutamatergic neurotransmission, which could be important to avoid the previously mentioned excitotoxicity induced by NO and glutamate (74). Moreover, activation of CB receptors by anandamide in microglia attenuates iNOS activation via activation of the MAPK phosphatase-1 (MKP-1) (83). In addition, the overexpression of CB_1 receptors in the spinal cord reduced the expression of NF-κB, TLR4, and IL-17 in this region (84). On the other hand, NO can regulate the activity of G protein-coupled receptors via S-nitrosylation of cysteine residues (75, 76). It was shown in a study with brain slices that after treatment with S-nitrosothiols, CB_1 receptor agonists had reduced efficacy in ^{35}S GTP Binding Assay (85). Therefore, we speculate that stress-induced iNOS activation and increase in NO levels could S-nitrosylate CB_1 receptors, impairing its signaling in the mPFC. This possibility, however, still needs to be addressed.

The CB_1 receptor is expressed in the mPFC (40, 86), and its activation usually attenuates anxiety-like behaviors in stressful conditions (38, 40). Moreover, a CB_1 agonist decreased stress-induced iNOS expression in the cortex of mice (87). Finally, CB_1 KO mice have increased NOS activity in the hippocampus (37). Together, these results suggest that the nitrergic and endocannabinoid systems could play opposite roles in regulating stress-induced anxiety behavior in the PL-mPFC. Corroborating this proposal, we found that the anti-stress effect of 1400 W in this region was blocked by the previous administration of the CB_1 antagonist AM251.

iNOS is constitutively expressed in neurons in some brain regions (81) and astrocytes in cortical tissue (88). However, its expression increases significantly in microglia after inflammatory stimuli (81). Psychological stressors activate microglia (89) and increase iNOS expression in the brain, particularly in the PFC (64). Increased NO production is involved in stress response (23, 29, 78). Therefore, it is possible that the anti-stress effect of 1400 W results from inhibition of iNOS activity and NO production in microglial cells, reducing the neuronal impact of stress. However, we cannot exclude that iNOS inhibition also occurs in neurons.

Microglia cells synthesize and release ECBs (90, 91), and stress activates the ECB system (98). Then, iNOS inhibition may facilitate the ECB signaling, buffering the stress response. Accordingly, AM251 reversed the anti-stress effect of 1400 W. Considering that CB_1 receptors are predominantly expressed by neurons in the brain and that a CB_1 antagonist reversed

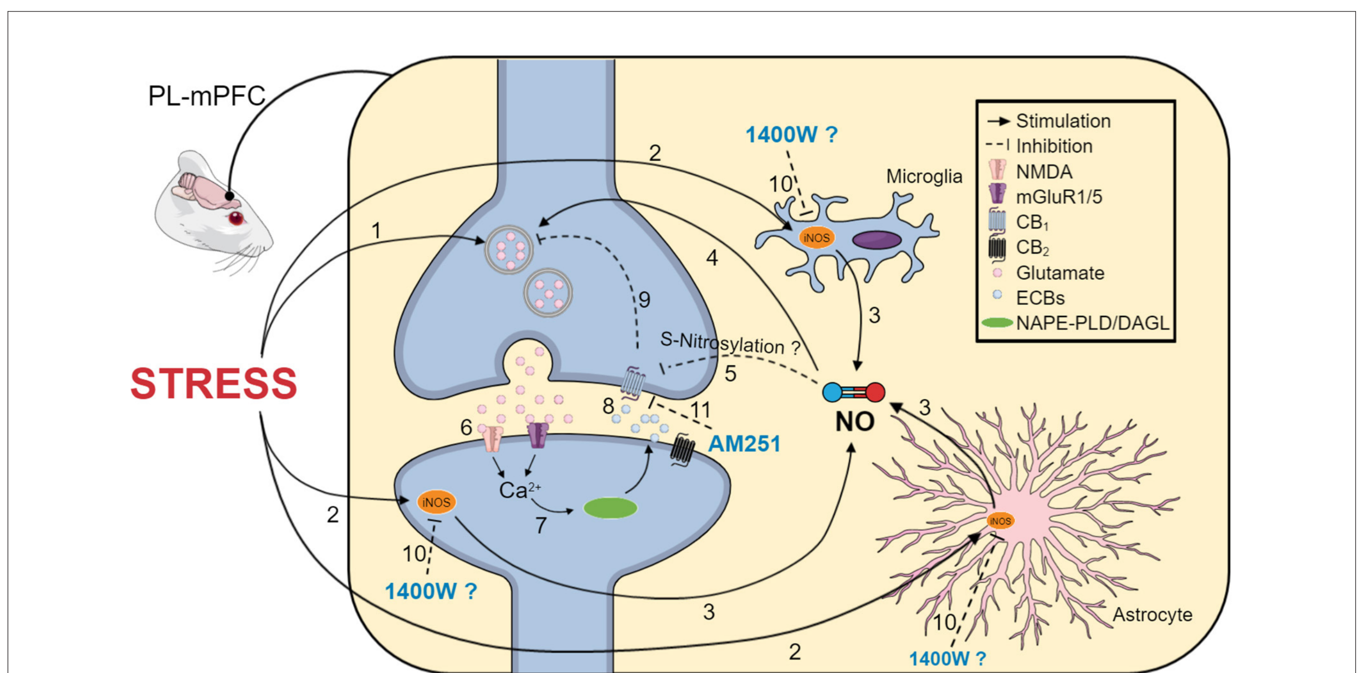

FIGURE 5 | Schematic representation of the proposed mechanisms involved in the anti-stress effect of 1400 W in the PL-mPFC. (1) Acute stress induces glutamate release (2) and activates iNOS, which could be expressed in microglia, astrocytes, and neurons. (3) Increased iNOS activity increases NO levels, (4) which can potentiate glutamatergic transmission. (5) NO may promote protein S-nitrosylation, including the CB_1 receptor. Therefore, it could inhibit its function, impairing neurotransmission control. (6) Glutamate also activates mGlu5 and NMDA receptors, increasing intracellular calcium levels, (7) resulting in activation of the ECB synthesizing enzymes, NAPE-PLD and DAGL-a, increasing ECB levels. (8) Anandamide and 2-AG could act on $CB_{1/2}$ receptors (9), regulating neuronal excitability. In the context of stress, NO could overcome ECBs' stress-buffer actions, favoring the release of glutamate, excessive excitability, and anxiety-like behaviors. In this way, (10) iNOS inhibition by 1400W could attenuate this positive feedback in neurons and reduce inflammatory consequences of iNOS activation in glial cells. ECBs acting at CB_1 receptors contributes to the anxiolytic effect of 1400 W because (11) blocking CB_1 receptors with AM251 prevented the anti-stress effect of 1400 W. We propose that the anti-stress effect of pharmacological iNOS inhibition in the PL-mPFC is dependent on the local ECB signaling, mediated at least by the CB_1 receptors. The figure was simplified. Therefore, not all cells and components of synapses and glial cells are depicted.

the 1400 W effect, we suggest that, in the presence of an iNOS inhibition, local ECBs could regulate neuronal excitability through CB_1 receptors and prevent the manifestation of the stress response in the EPM.

In conclusion, our results indicate that the iNOS in the PL-mPFC is also involved in the behavioral consequences of acute stress exposure. Moreover, the present data strengthens the proposal that there is a crosstalk between the nitrergic and endocannabinoid systems, particularly CB_1 receptors, in modulating stress and anxiety behaviors. As summarized in **Figure 5**, we propose that the behavioral effects after restraint stress may be caused by increased glutamate release, which is potentiated by NO synthesis from iNOS. NO could promote S-nitrosylation of several proteins, including CB_1. In this case, it could inhibit CB_1 function, impairing neurotransmission control. Under homeostatic conditions, the activation of the endocannabinoid system, resulting in the release of anandamide and 2-AG, could be able to counteract the stress effects. However, when this signaling is impaired, the anxiogenic effect prevails. By inhibiting iNOS, we can attenuate the positive feedback mentioned before, allowing the endocannabinoid activity to regulate the synaptic neurotransmission, resulting in an anxiolytic effect.

NOMENCLATURE

Resource Identification Initiative ANY-maze (RRID: SCR_014289).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Review Committee, Ribeirão Preto Medical School (protocol no. 224/2017).

AUTHOR CONTRIBUTIONS

SL and FG designed the study. AC, CV-V, AS, DU, and LB performed the experiments and developed the data analysis. AC and CV-V wrote the manuscript. All authors approved the final version.

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