Title Page

Cannabidiol as an add-on therapy to overcome the slow-onset and, possibly, resistance to antidepressant treatment: involvement of NAPE-PLD in the medial prefrontal cortex.

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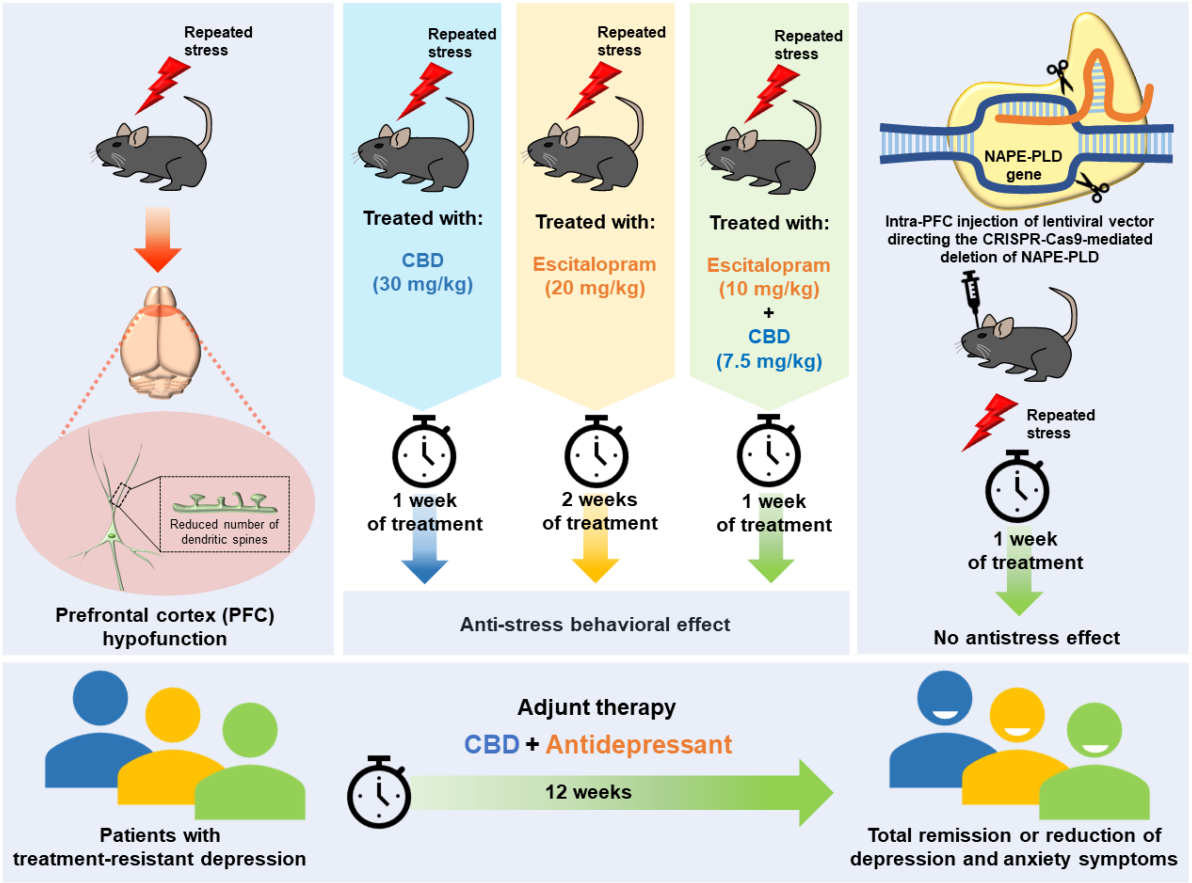
Highlights

- In mice, cannabidiol (CBD), but not escitalopram, induced a fast-onset anti-stress action.
- Combinations of sub-effective doses of CBD and escitalopram produce anti-stress effects after only 7 days.
- The Escitalopram + CBD treatment modulated synaptic protein markers in the medial prefrontal cortex.
- CRISPR-Cas9-mediated knockdown of NAPE-PLD in the medial PFC prevents the anti-stress effect of the Escitalopram + CBD.
- Adding CBD to an antidepressants regimen successfully treated three patients with treatment resistant depression.

Graphical abstract



Abstract

Antidepressants such as serotonin uptake inhibitors are the first-line pharmacological treatment for chronic stress-related psychiatric disorders. However, their late-onset therapeutic action and frequent side effects, however, are important challenges for clinicians and patients. Besides, around 30% of major depression patients are considered treatment-resistant. Cannabidiol (CBD) is a non-psychotomimetic phytocannabinoid with a wide range of psychopharmacological effects, but its mechanism of action remains unclear. Here, we found that in male mice submitted to two different repeated stress protocols (chronic unpredictable and social defeat stress), low doses of CBD (7.5mg/Kg) caused an early-onset behavioral effect when combined to the antidepressant escitalopram (ESC-10mg/Kg). The behavioral effects of the ESC+CBD combination depended on the expression/activity of the N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD, responsible for synthesizing the endocannabinoid anandamide), but not the DAGLα, enzyme in the ventromedial prefrontal cortex. In addition, we described a case series with three treatment-resistant depression that were successfully treated with CBD as adjuvant therapy, as evaluated by standardized clinical rating scales. After 12 weeks of treatment, two patients were considered depression remitted (MADRS score lower than 10) while one patient successfully responded to CBD as add-on treatment (more than 50% decrease from the baseline MADRS). Our results suggest that CBD might be useful as an add-on therapy for optimizing the action of antidepressants. They also suggest that CBD's beneficial actions depends on the facilitation of N-acylethanolamines actions in the medial prefrontal cortex.

Keywords: cannabidiol; antidepressant; chronic stress; NAPE-PLD; late-onset; treatment-resistant

Introduction

Antidepressants, especially the selective serotonin reuptake inhibitors (SSRIs), are widely prescribed to treat psychiatric disorders such as major depression and anxiety. However, their late-onset action and associated side-effects are vexing clinical problems, contributing to low adherence to the therapy. Moreover, about 30% of patients are considered treatment-resistant (Caraci et al., 2018). Of great current concern in the clinical practice, treatment-resistant depression (TRD) is an unsatisfactory response to at least two adequate antidepressant medication trials, and is usually linked to significant cognitive function impairment, low quality of life, and higher suicide rates (Mrazek et al., 2014; Thase, 2011).

In clinical practice, combined therapy is a strategy commonly adopted for patients who do not respond appropriately to monotherapy. One advantages of combining agents include decreasing the dose and, as a consequence, diminishing the incidence or the severity of side effects. However, whether the combination with other promising antidepressant drugs might decrease the latency for the onset of action of antidepressants has not yet to be investigated.

The phytocannabinoid cannabidiol (CBD) is a major non-psychotomimetic component of the Cannabis sativa plant. Antidepressant, anxiolytic, sleep regulator, anti-inflammatory, antioxidant, and neuroprotective are among the described properties of this cannabinoid (Crippa et al., 2018). Like clinically used antidepressants, CBD counteracts the behavioral and neuroplastic outcomes of stress exposure in rodents (Campos et al., 2013; Fogaça et al., 2018). Even though the exact mechanisms of this effect remain unclear, the endocannabinoid system has been associated with CBD and SSRI actions (Hill et al., 2008b).

Using the chronic unpredictable stress (CUS) model, we showed attenuation of the stress-induced behavioral and neuroplastic changes by repeated CBD administration depends on CB1 and CB2 receptors (Campos et al., 2013; Fogaça et al., 2018). Chronic treatment with

CBD increased hippocampal levels of anandamide (AEA), but not other endocannabinoids (Campos et al., 2013).

The N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) is considered major biochemical pathway responsible for synthesizing AEA and other N-acylethanolamines (NAESs). Like AEA, NAEs can also modulate neuronal and glial cell function by different mechanisms (interference with inflammation, metabolism, energy homeostasis, etc.). Recently, Leishman et al. (2018) demonstrated that CBD (3mg/Kg) enhanced NAPE-PLD activity and increase NAEs production in the brain (Leishman et al., 2018). Regarding the effects of SSRI on this enzyme, Smaga and colleagues (2014) showed that chronic treatment with escitalopram (10 mg/Kg) increases NAPE-PLD expression in the hippocampus and dorsal striatum (Smaga et al., 2014).

Among the brain regions associated with depression, neuroplastic mechanisms within the prefrontal cortex (PFC) are likely to be involved in the effects of antidepressants, particularly those of fast-acting "antidepressants" such as ketamine and, possibly, CBD (Pothula et al., 2020; Sales et al., 2019; Zhang et al., 2020). Moreover, direct administration of CBD into the PFC eleicits antidepressant-like effect dependent on CB1 receptor activation (Sartim et al., 2016).

Based on these pieces of evidence, the current study aimed at addressing to the following questions: 1. Can the combination of sub-effective doses of CBD and the SSRI escitalopram prevent behavioral effects of chronic stress?; 2. Will these effects be associated with plastic changes in the PFC?; 3. Are endocannabinoids or NAEs involved in CBD's actions?; 4. Would CBD be useful in treatment-resistant depressive patients as an add-on therapy?

Materials and methods

Animals

The experiments involving animals described here were conducted following the current Brazilian guidelines for using laboratory animals (federal law number nº 11.794/08) and in compliance with the ARRIVE guidelines (Piercie du Sert et al., 2020). All protocols were approved by the Ribeirão Preto Medical School Ethics Committee under the numbers: 032/2015-1; 047/2019. C57Bl6 (2-3 months old; 24-30g) and Swiss mice (6 months old) from the local animal facility of the University of São Paulo (Campus Ribeirao Preto) were transferred to the animal care unit of the Pharmacology Department at least one month before the beginning of the experiments. Animals were maintained in a temperature-controlled room (24°C) with water and food available *ad libitum* and under a 12h/12h light/dark cycle (lights on at 6h30). After arrival, they were housed in cages in groups of 3 (for social defeat stress protocols) or 4-5 (for chronic unpredictable stress protocols).

Chronic unpredictable stress

The model was adapted from Willner et al. (1992). Briefly, the animals were submitted daily to one of the following stressors (distributed randomly): forced swimming (15 minutes), restraint stress (2 hours), intermittent dark/light cycle (24h), inverted dark/light cycle (24h), wet bedding (24h), tilted cage (overnight) and food deprivation (24h). The stress procedure was conducted for 7, 10, or 14 days, depending on the protocol. The control (non-stressed) mice were maintained at the animal care unit during all the experimental protocol.

Social defeat stress

The model applied in our study was adapted from Golden et al. (2011). Before the stress procedure, the C57Bl6 mice were housed in three animals per cage for at least a month. During this period, animals established their social hierarchy. After that, the stress procedure took place. It consisted of placing a Swiss intruder mouse (older and bigger) in the cage of the

resident C57Bl6 mice. The goal was to disturb the previously established social hierarchy and generate a socially defeated phenotype in the resident mice. The intruder Swiss mouse was kept for 2 hours a day for ten days in the cage of the C57Bl6 animals. A different intruder was used every day for each of resident cage.

Drug treatments

For the treatment schedule, we used escitalopram oxalate (Pratti-Donaduzzi; 10 and 20 mg/kg) (Anwar et al., 2013; Seo et al., 2017), CBD (BSPG; 7.5 and 30 mg/kg) (Campos et al., 2013) and URB597 (Cayman; 0.1 mg/kg)(Gobbi et al., 2005). Treatments were administered intraperitoneally (i.p.) daily for 7 or 14 days.

Novelty Suppressed Feeding

The test was performed as previously described (Dulawa et al., 2004). Briefly, it consisted of placing 24h food-deprived mice in a rectangular arena (40cmx 40 cm x 30 cm- the novelty component) located in a dark room. In the center of this arena, a food pellet was placed on an illuminated platform. To measure the novelty-induced hypophagia, we assessed the time elapsing before the animals started to eat the food pellet, with the cut-off time of 600 seconds. As a control measure of appetite, right after the test, the food consumption in the mice home-cages was evaluated for 5 minutes.

Viral vectors

Lentiviral vectors were used to express the CRISPR/Cas9 system and guide RNA sequence targeting the NAPE-PLD enzyme (responsible for AEA synthesis) gene encoding or the DAGLα enzyme (responsible for 2 -AG synthesis) gene encoding. In both targets, the vector consisted of a Gecho2 lenti guide All in One system with coexpression of Cas9 and tGPF directed by the EF1α promoter and the expression of the guide RNA directed by the U6 promoter (Gecko2 lentiguide-tGFP, U6-gRNA / EF1a-tGFP; NAPE-PLD Target ID: MM0000610807; NAPE-PLD Exon target: 3; DAGLa Target ID: MM0000628057; DAGLa

Exon target: 10; Sigma-Aldrich). As a control, the CRISPR-lenti lentiviral vector Non-directed control plasmid (Sigma-Aldrich) was used. The system was similar to that used for the NAPE-PLD and DAGLα target deletion, with the difference that the guide RNA consisted of a scrambled sequence, which did not affect any known target in the mouse genome. The control plasmid co-expresses Cas9, GFP, and puromycin directed by the EF1α promoter, and the U6 promoter similarly directs the expression of the sequence. After inducing the CRISPR (Cas9) nuclease expression associated in cells transfected by the viral vector, the nuclease forms a complex with the guide RNA leading to DNA cleavage in the specific target sequence directed by the guide RNA right after a PAM motif (protospacer adapter motif). Cas9 cleavage promotes double-strand breaks, activating DNA repair mechanisms by Non-Homologous End Joining (NHEJ), promoting the mutation of the target site, with consequent gene inactivation (Gao et al., 2020; Liu et al., 2019).

Stereotaxic surgery

Eight-week-old mice were anesthetized with tribromoethanol and placed in the stereotaxic apparatus. The coordinates used to reach the prefrontal cortex were (from Bregma, based on the Franklin and Paxinos, 2008): anteroposterior: (+) 1.5; mediolateral: (±) 0.4; dorsoventral: (-) 2.5. We injected 200µl of vector directing the deletion via CRISPR-Cas9 targeting a sequence for DAGLα, NAPE-PLD, or the scrambled sequence as a guide DNA using a Hamilton syringe coupled to an infusion pump. The injection was performed bilaterally (200µl per hemisphere) at a speed of 20nl/min. The needle was slowly removed from the site 2 minutes after the end of the infusion. Then, the animal was carefully removed from the apparatus. The infusion site was sutured with 4.0 nylon or silk thread. At the end of the surgery, each animal was placed in a post-surgical room for recovery. Mice were monitored for at least 2 hours and 1 hour after awakening from anesthesia. After that, they were monitored on daily-basis in their home-cages until the experiments' beginning.

Histology (stereotaxic surgery)

After the experiments involving stereotaxic surgery, brains were sectioned (20microns, and the samples contained the vmPFC observed under the epifluorescence microscope (BX53, Olympus, Tokyo, Japan) to verify the position of the injection sited according to the presence of tGFP.

Western blot

After euthanasia, the PFC was manually dissected. The brain tissue was homogenated in lysis buffer (Tris-HCl 25mM pH=7,5, NaCl 75mM, Na2H2O7P2 2,5mM, Na3VO4 1mM, Na2MoO4 5mM, 0,5% Triton X-100) with protease inhibitor cocktail (*Protease Inhibitor Cocktail powder*, Cat. P2714, Components: AEBSF, Aprotinin, Bestatin, E-64, EDTA, Leupeptin; Sigma Aldrich, St. Loius, USA) using the bead-beater homogenizer (*TissueLyser LT Adapter*, Qiagen; 40Hz, 90 seconds). After the lysis, the samples were centrifuged (12000 rpm, 10 minutes, 4ºC) the supernatant was collected for another round of centrifugation (12000 rpm, 10 minutes, 4ºC). Bradford's method was used to evaluate total protein concentration against a BSA standard curve and using a NanoDrop (absorbance reading at 450nm -*NanoDrop One Microvolume UV-Vis Spectrophotometer Thermo Scientific Waltham*, Massachusetts, USA*)*. For protein separation, electrophoresis was conducted on a polyacrylamide gel with a gradient density (4-12% - NuPAGETM Invitrogen), with a final sample volume of 20uL and a protein concentration of 1g / L per well. After protein transfer onto a nitrocellulose membrane (Amersham Potran, Little Chalfont, United Kingdom), membranes were blocked in 10% non-fat milk (Bio-Rad) (dissolved in Tris- saline- buffer +0.5% of Tween20 -TBSt) for two hours, at room temperature. After blocking, membranes were rinsed quickly with TBSt to remove the excess of blocking solution and then incubated with the primary antibody at 4°C overnight under constant orbital agitation at the following dilutions: synaptophysin (~33kDa; 1:1000; Rabbit; *Cell Signalling*, Cat. 5461S, Danvers, Massachusetts, USA), synaptotagmin (~60kDa;

1:1000; Rabbit; *Cell Signalling*, Cat. 14558S, Danvers, Massachusetts, USA), synapsin 1a/b (~80-86kDa; 1:1000; Rabbit; *Abcam*, Cat. ab254349, Cambridge, UK), PSD95 (~95kDa; 1:1000; Rabbit; *Cell Signalling*, Cat. 3409S, Danvers, Massachusetts, USA) and α-tubulin (~52kDa; 1:10000; Mouse; *Cell Signalling*, Cat. 3873S, Danvers, Massachusetts, USA). The secondary antibody was incubated for 2 hours (*Anti-rabbit*, *GE Healthcare*, 1:10000 or *Anti-mice*, *GE Healthcare*, 1:10000, Chicago, Illinois, USA). The bands were detected using chemiluminescence methods (ECLPrime®, Amersham, Little Chalfont, United Kingdom) and visualized using the ChemiDoc Imaging Systems (GE ImageQuant LAS, MA- United States). Intensities of specific bands were quantified using the Image Studio Lite (LI-COR, Nebraska, United States) and normalized to anti-α-tubulin protein levels.

Golgi analysis

The Golgi impregnation method was performed using the FD Rapid GolgiStain Kit (FD NeuroTechnologies, INC) according to the manufacturer's instructions. After the euthanasia, the mouse brains were removed and immersed in the potassium dichromate solution at room temperature for ten days. The tissues were then transferred to the washing solution and stored at room temperature for at least 72 hours before being sliced in a cryostat in 100μm sections placed on gelatinized slides. The slices were maintained hydrated until processing, which consisted of completing the reaction with mercury chloride and subsequent dehydration process in successive ethanol and xylene stages. The analysis was performed on pyramidal neurons of the mCPF layers II/III, selecting 10μm of the initial portion of tertiary dendrites. We quantified and classified the dendritic spines after scanning in the reflection mode of a confocal microscope (Leica TCS SPE) (Spiga et al., 2011).

Case series

We followed the clinical evolution of three patients that sought out our outpatient psychiatric unit due to TRD. The patients were clinically evaluated for eligibility by a general

medical exam (that included laboratory tests to assess serum electrolytes and hepatic, thyroid, and hematological parameters). The diagnosis of major depression disorder (MDD) experiencing current moderate to severe episodes without psychotic features and no comorbid conditions was confirmed independently by two psychiatrists (JAC and JECH) well-versed in using the Brazilian-Portuguese version of the Structured Clinical Interview for DSM5 (Osório et al., 2019). The neurological examination of both patients was unremarkable. All patients consented to the CBD add-on treatment and were included in the institutional review board-approved registry. Two of the patients had been under weekly psychoanalytical therapy for more than three years, which continued during the study period. The investigated outcomes included symptoms of depression, and anxiety assessed by the Brazilian-Portuguese versions of the Montgomery-Åsberg Depression Rating Scale (MADRS) (Dratcu et al., 1987), Patient Health Questionnaire-9 (PHQ-9) (De Lima Osório et al., 2009), Generalized Anxiety Disorder-7 (GAD-7) (Spitzer et al., 2006) scale, and Clinical Global Impression, severity subscale (Guy, 1976). The CARES, a scale adapted from a combination of the UKU Side Effect Rating Scale (UKU) (Lingjærde et al., 1987) and the Common Terminology Criteria for Adverse Events (CTCAE v5.0) items, severity levels, and anchor points, was used to assess CBD's adverse effects and tolerability. Any adverse event that could conceivably be attributable to the treatment was included.

We considered a patient to be a CBD responder if they had at least a 50% decrease from the baseline in their depressive symptoms, as assessed by their MADRS scores. Remission was defined as a score lower than 10 (Bryant et al., 2019). To determine the severity of the depression episode we used the following cutoff points: 0 to 6 (normal/absent); 7 to 19 (mild); 20 to 34 (moderate); >34 (severe) (Herrmann et al., 1998).

Patients received a daily oral dose of 200 mg CBD (99.6% purity with no other cannabinoids; commercially available, *Prati-Donaduzzi*®, Toledo, Brazil) divided into two

daily doses (100 mg/0.5mL) for one week. This dose was increased to 400mg (200 mg/1mL, twice a day) the following week and maintained on this dose for the duration of the trial. CBD was administered as add-on to the SSRs.. Patients completed the assessment instruments at baseline and after 1, 2, 4, 8, and 12 weeks (Table 1). We selected CBD dose based on previous studies that indicates that at 300-400 mg/day promotes anxiolytic effects with good safety and tolerability (Crippa et al., 2018). We used the CARE (CAse REports Guidelines) Statement and Checklist to prepare the case reports (Gagnier et al., 2013).

Statistical analysis

Data obtained were tested for normality using the Kolmorov-Smirnov test and for homogeneity of variances using the Levene test. In the experiments involving the comparison of behavioral effects of CBD and escitalopram, we used the Two-way ANOVA (factor 1: stress; factor 2: treatment). In the experiments analyzing the effects of the combination of cannabinoids and escitalopram, the stress factor effect was assessed using the t-test for independent samples (comparing the vehicle-treated non-stressed with the vehicle-treated stressed group). Then we used Two-way ANOVA to evaluate the genotype effect, treatment 1 (vehicle, escitalopram), and treatment 2 (vehicle, CBD, or URB597) factors followed by Duncan's post-hoc test. P values equal to or less than 0.05 were considered significant. Data are represented as mean ± standard error of the mean (SEM).

Results

CBD, but not escitalopram, produces fast-onset anti-stress action in stressed animals

To verify the time required for escitalopram or CBD to induce behavioral effects, we submitted C57Bl6 mice to either non-stress, seven or 14 days of CUS. All groups received vehicle, escitalopram (20mg/kg) or CBD (30 mg/kg). On the 7th or 14th day of the protocol, all groups were food-deprived for 20h and then submitted to the NSF. In this protocol,

escitalopram reduced the latency to eat in the novelty-induced hypophagia in the control, but not in the stressed group ($F_{2,52}$=6.442, p=0.003). On the other hand, CBD, but not escitalopram, diminished the time for the stressed animals to start eating in the novel context after seven days of treatment ($F_{5,52}$=4.596, p=0.002; One-way ANOVA followed by Duncan, Figure 1A). There was no stress or treatment effect on food consumption at the home-cage (Figure 1B).

On the 14-day stress protocol, CBD behavioral effects in stressed mice were maintained. Escitalopram also reduced eating latency in the CUS group after 14 days of treatment. Neither treatment affected the non-stressed animals (F2,50=4.778, p=0.013, Figure 1C). Therefore, CBD showed an anti-stress effect with a shorter time to onset than escitalopram. No difference in food consumption in the home-cage as detected at 14 days (Figure 1D).

Since alterations in adult hippocampal neurogenesis have been observed after repeated treatment with CBD (Campos et al., 2013) and SSRIs (Santarelli et al., 2003), we measured the number of cells positive for the neuroblast marker doublecortin (DCX) in the dentate gyrus (Figure S1). No association (Supplemental Table 1) or correlations (data not shown) were found between the anti-stress effects and the number of DCX-positive cells.

The combination of escitalopram with sub-effective doses of CBD prevents stress-induced anxiety-like behaviors.

Next, we investigated the effects of combining lower doses of escitalopram and CBD in a 10 days protocol of chronic stress (in which mice received daily treatment for 7 days , starting at day 4). One of the potential advantages of this approach is reducing the doses and, consequently, the occurrence of side effects of these compounds. Therefore, we decreased the CBD dose to 7.5mg/kg and reduced the escitalopram dose by half (10mg/kg) (based on unpublished data from our laboratory). Stressed mice treated with the combination of

escitalopram and CBD presented a significantly lower latency to feed in the new environment ($F_{5,60}$=2.437, p=0.045, Figure 2A). No differences were seen in the home-cage food consumption (Figure 2B). Increased anandamide levels have been associated with the behavioral effects of CBD in the CUS model (Campos et al., 2013; Fogaça et al., 2018). Therefore, we also sought to verify if URB597 (0.1mg/kg), an inhibitor of the fatty acid amide hydrolase (FAAH) that degrades AEA, in combination with escitalopram, would produce a similar effect as CBD. However, we did not observe any effect (Figure 2A).

We also evaluated the effects of the treatments on stressed mice in the tail suspension test (TST). CUS enhanced passive coping strategies in the TST, decreasing the latency to the first immobility episode (Student's t-test; t_{21}=2.652, p=0.015) (Figure S2A). Immobility time along the 6 minutes of the test increased in stressed animals ($F_{5,105}$=4.215, p=0.002). Mice submitted to CUS and treated with vehicle presented an increase in the overall immobility during the TST test ($F_{1,21}$=7.863, p=0.011) (Figure S2B). There were no significant differences between the stressed groups that received different treatment combinations (repeated measures ANOVA; Treatment 1: $F_{1,54}$=0.076, p=0.784; Treatment 2: $F_{2,54}$=0.492, p=0.614; Interaction: $F_{2,54}$=0.159, p=0.854).

Next, we tested whether the Escitalopram + CBD or Escitalopram + URB could be effective in a homotypic stress model, the social defeated stress. The stress and the treatment were conducted in the same schedule as in the CUS model. Like the CUS protocol, the combination of escitalopram and CBD, but not URB, significantly decreased the latency to feed in the new environment (Figure 2C; $F_{5,50}$=2,471, p=0.045, One-way ANOVA followed by Duncan). Furthermore, the stressed animals treated with the low dose of URB597 also had a reduced latency. Moreover, the stressed vehicle-treated group consumed more food in the home-cage than non-stressed mice (Student's t-test; t_{19}=2.821, p=0.011). Still, there was an interaction between the treatments ($F_{2,49}$=3.283, p=0.046) (Figure 2C). The SDS group treated

with escitalopram alone presented a home-cage consumption significantly diminished compared to the SDS group that received only vehicle (One-way ANOVA followed by Duncan) (Figure 2D).

An independent group of mice was submitted to SDS and tested in the TST. Stressed mice treated with vehicle presented a decrease in the first immobility episode (Figure S2C; Student's t-test; t_{15}=2.787, p=0.014). The SDS group treated with Escitalopram + CBD showed a significantly higher latency for the first immobility episode ($F_{5,44}$=1.690, p=0.157; One-way ANOVA followed by Duncan). This response is predictive of an antidepressant-like effect of the drug combination. Repeated measures ANOVA indicated an interaction between stress and time in the TST, indicating that the immobility response along the 6 minutes of the test varied depending on whether the mouse was or was not submitted to SDS ($F_{5,75}$=3.018, p=0.015). However, there was no significant effect of stress exposure ($F_{1,15}$=3.468, p=0.082). The treatment combinations did not significantly alter the immobility response in the TST (Figure S2D; Repeated measures ANOVA; Treatment 1: $F_{1,42}$=1.899, p=0.176; Treatment 2: $F_{2,42}$=0.599, p=0.554; Interaction: $F_{2,42}$=0.067, p=0.935) (Figure S2C-D).

Therefore, our results indicated that combining escitalopram with a sub-effective dose of CBD induces an anti-stress effect after only seven days of treatment in mice submitted to repeated stressors of different natures.

Escitalopram and CBD combination alter pre-synaptic protein markers' expression, but not the number of postsynaptic terminals in the PFC.

Next, we sought to evaluate whether the observed behavioral effects were associated with changes in markers of synaptic plasticity. In the PFC, no difference was observed in the presynaptic marker synaptophysin in mice submitted to CUS (Figure 3A). However, in the Escitalopram + CBD group, we observed increased relative expression of synaptophysin in the

SDS model in the PFC (Figure 3B, $F_{2,18}$=7.437, p=0.004). However, stress or treatments did not change synaptophysin expression were detected in the hippocampus (CUS model, Figure S3A). The combination of escitalopram and URB597 increased hippocampal synaptophysin in the SDS model (Figure S3B, $F_{2,17}$=8.203, p=0.003).

Synaptotagmin expression, a protein associated with presynaptic vesicles release, was not altered by the CUS protocol or treatments in the PFC (Figure 3C). Still, it was found increased after escitalopram treatment in the hippocampus ($F_{2,17}$=4.242, p=0.032, Figure S3C). In the SDS model, the combination of escitalopram and CBD increased synaptotagmin expression in the PFC (F2,18-=7.437, p=0.004, Figure 3D) and the hippocampus (F2,18=3.866, p=0.04, Figura S3D)

The changes observed in synaptophysin and synaptotagmin expression suggested a potential effect of the combination Escitalopram + CBD on presynaptic plasticity. Therefore, we used the Golgi staining technique to stain, visualize, and quantify the number and type of dendritic spines in pyramidal neurons of the layers II/III of the medial PFC (Figure 3E) and granule cells of the dentate gyrus of the hippocampus (Figure S3E) as a measure of post-synaptic structural plasticity. Based on their morphology, the dendritic spines were classified as: mushroom, thin, filopodia, or stubby (Figure 3E-F; Figure S3-F). The percentage of each subtype was quantified relative to the total number of dendritic spines (Figure 3F; Figure S3F). The percentage of spines with a thin morphology was augmented in the PFC of the CUS group compared to the non-stressed animals (Figure 3F; Student's t-test; t_8=2.097, p=0.020). CUS also decreased the total number of dendritic spines in tertiary dendrites of pyramidal neurons in the mPFC ($t_{5.219}$=2.604, p=0.046, Figure 3G). Again, there was no treatment effect on spine morphology.

We also evaluated the expression of the postsynaptic protein postsynaptic density 95 (PSD95) relative expression in the PFC (SDS model). The homotypic stress protocol and the

drug treatments did not significantly alter the relative expression of PSD95 in this region PFC (Figure 3H). Also, we did not observe changes in the markers of post-synaptic plasticity in the hippocampus (Figure S3E-H).

Our data indicated, therefore, that our stress protocol contributed to the impaired in structural postsynaptic plasticity in the PFC. The combination of escitalopram and CBD might induce the expression of presynaptic proteins involved in synaptic transmission.

Knock-down of NAPE-PLD using CRISPR/Cas9 prevents the behavioral effects of the ESC+CBD in stressed mice.

Endocannabinoids can modulate synaptic function by, for example, decreasing neurotransmitter release (Scarante et al., 2017). As discussed before, they could also be involved in the behavioral effects of SSRI and CBD (Campos et al., 2013; Fogaça et al., 2018; Smaga et al., 2014). To investigate if the behavioral effects of the combined CBD and escitalopram treatment depends on the synthesis of anandamide or 2-AG, we specifically knocked-down in the PFC two enzymes closely involved in their synthesis, NAPE-PLD, and DAGLα, using a CRISPR-Cas9 system (Figure 4A-B).

Animals received a bilateral intra-PFC injection of viral vectors delivering either a construct that induces the CRISPR-Cas9-mediated deletion of one of the enzymes (for the knock-down (KD) groups) or a scrambled sequence (for the wild-type – WT – groups) (Figure 4B).

Confirming the results observed in Figure 2A, the combination of escitalopram and CBD decreased the latency to eat in a novel environment in WT animals. There was, however, a significant interaction between genotype and treatment ($F_{1,21}$=4.393, p=0.048) and the effect of Escitalopram+CBD disappeared in NAPE-PLD KD mice (Figure 4C). There was no difference in food consumption in the home cage (Figure 4D). DAGLα deletion, on the other

hand, did not prevent the anti-stress effects of Escitalopram + CBD ($F_{1,34}$=8.075, p=0.008, Figure 4E). No differences were observed in the food-consumption in the home-cage after DAGLα knockdown (Figure 4F).

In addition to the behavioral analysis, we measured content of the endocannabinoids, AEA and 2-AG, content in the prefrontal cortex (global, not only in the site of CRISPR-cas9 infusions). However, we did not find any changes (Supplementary Table 2). Together, the results suggest that the anti-stress effects of the combination of CBD plus escitalopram combination involve the NAPE-PLD, but not the DAGLα enzyme in the PFC.

Add-on therapy with CBD to treatment-resistant depressive patients

Our final question was whether CBD would be useful in treatment-resistant depressive patients as an add-on therapy. The summary of the psychiatric scale results before and during treatment are presented in the Table 1. Patient A was a 41-year-old married man who had an MDD diagnosis since he was 19 years old with three previous mild to severe depressive episodes, which had been successfully treated with sertraline 50 to 150mg/day. He had a significant family history of mood disorders. He sought the psychiatry outpatient unit with a complaint of five years of severely depressed mood, anhedonia, tearfulness, hopelessness, loss of pleasure in most everyday activities and hobbies. However, he was still capable of working if making extra efforts. He also complained of anxiety, insomnia, guilt feelings on past minor failures, and difficulty in concentrating and making decisions. Mr. A. did not present suicidal thoughts nor reduced libido. He attributed the latter to the use of testosterone topical gel. Over the years, the patient had been treated with various medications for MDD, including sertraline, duloxetine, escitalopram, and agomelatine, with or without adjuvant clonazepam, trazodone, or quetiapine. He presented with nausea (duloxetine) or sedation (clonazepam and quetiapine) during the treatment with these medications, which precluded adequate drug trials.

Agomelatine (50 mg/day for three months) and desvenlafaxine (200 mg/day for six months) did not reduce the symptoms. He was also using 50 mg/day of a topical testosterone skin gel for more than one year, although his plasma testosterone levels were below the normal range (about 350 ng/dL when he started and around 800 ng/dL after the hormonal treatment). When CBD was added to desvenlafaxine, he had a marked reduction of all depressive symptoms within four weeks and improved social and labor functioning after eight and 12 weeks. MADRS cutoff scores showed a decrease to moderate after 1 and 2 weeks, mild after 4 weeks, and normal/absent levels after 8 and 12 weeks. Full remission occurred after 8 weeks, thus confirming the above outcome (Table 1). A six-month follow-up showed no relapse of the symptoms, and no other side-effects were evident during the CBD's treatment period.

Ms. B., a 29-year-old single woman, sought the psychiatry outpatient unit with a history of several previous mild to moderate depressive episodes, the first occurring when she was 12-years-old. For these MDD episodes, she had been treated, with partial success, with a combination of various medications, including paroxetine, sertraline, clomipramine, venlafaxine, escitalopram, lamotrigine, lithium, clonazepam, lorazepam, and bromazepam. She could tolerate the optimal dosage and treatment period of all these antidepressants, but for clomipramine (cholinergic symptoms) and venlafaxine (constipation and dizziness). Her former responses were only partial, and she complained of residual cognitive, sleep difficulties, and mild depressive mood between the significant episodes. She also had a significant family history of mood disorders, particularly bipolar, from both parents' side. She sought the psychiatry outpatient unit with a complaint of two years of severe feelings of sadness, extreme anxiety, anhedonia, tearfulness, loss of interest in most of her normal activities, such as sex, studies, and sport (running). She quit from a very competitive internship selection program that she had enjoyed due to feelings of worthlessness. Ms. B also complained of tiredness, unrest in sleep, slowed thinking, and presented with occasional suicidal thoughts without

concrete plans. When CBD was added to escitalopram 30mg/day and lorazepam 2mg/day, she had a significant decrease in anxiety within one and two weeks and decreased in depressive symptoms within four weeks. Complete remission was achieved after eight and 12 weeks, as assessed by the clinical report and rating scales' scores. After three months, escitalopram was reduced to 20mg/day, and bromazepam was discontinued MADRS cutoff scores showed a severity reduction to moderate after 4 and 8 weeks and normal/absent levels after 12 weeks. Full remission occurred after 12 weeks, in agreement with the above outcome (Table 1). The six-month follow-up (still on CBD) showed no relapse of the symptoms, and she did not present with any CBD-related side-effect.

Mr. C. is a 31-year-old single man who presented to the psychiatry outpatient unit with a history of mild and two moderate previous depressive episodes, the first being when he was 19-year-old. He had been successfully treated in the past with up to 150mg/day of sertraline. He had a significant family history of MDD and anxiety disorders. The current episode started four years ago, and he sought the mood disorder outpatient unit with a complaint of severe emptiness, hopelessness, concentration difficulties, irritability, fatigue, sleep problems, loss of interest in sex, and fluctuating physical aches – none of which could be attributed to a medical condition or medication. Most of the time he wanted to stay at home rather than going out to socialize or work. During the present MDD episode, Mr. C. received various antidepressant medications, at the maximum recommended doses, including sertraline, duloxetine, and escitalopram, with or without adjuvant clonazepam, bupropion, lithium, and/or quetiapine. He presented with constipation, dry mouth, and orthostatic hypotension with nortriptyline 100mg/day, which prevented an adequate trial duration of this tricyclic. Desvenlafaxine (200 mg/day for two months) only mildly decreased the symptoms. He was then treated with three six-weeks blocks of 12, 9, and 8 sessions of subcutaneous esketamine infusions (0.5-1mg/kg), with no response. CBD was then added to 200mg/day of desvenlafaxine, quetiapine

100mg/day, and 2 mg/day of clonazepam. The cannabinoid adjuvant led to a marked decrease in anxiety within one week. The MDD episode decreased after eight weeks, leading to his return to social and work activities. MADRS cutoff points showed a reduction to moderate after 2 weeks and to mild levels after 12 weeks. The patient was considered a CBD responder as he had a 61% decrease from his baseline depressive symptoms, as assessed by the MADRS scores (Table 1). After three months, desvenlafaxine was reduced to 50mg/day, and clonazepam was occasionally used. No relapse of the symptoms occurred in the six-month follow-up, and he did not present any CBD-related side-effects throughout the trial.

Therefore, the results presented indicate that, in addition to optimizing the anti-stress effects of escitalopram in pre-clinical models, CBD might also be an useful add-on therapy to improve the response of TRD patients to antidepressants.

Discussion

The results presented here allowed us to answer the questions: 1. Will the combination of sub-effective doses of CBD and escitalopram induced an anti-stress behavioral effect?; 2. Are these effects associated with plastic changes in the PFC?; 3. Are the behavioral effects of Escitalopram + CBD was dependent on the expression/activity of NAPE-PLD in the vmPFC? ; and 4. Will CBD be effective as an add-on therapy to improve the symptoms of three patients with TRD?

Escitalopram and CBD: Onset of action and a promising drug combination to optimize antidepressant action and treat TRD.

CBD induced an anti-stress effect more quickly than the SSRI escitalopram during escitalopram repeated treatment. Previous work have proposed that CBD could be a fast-acting antidepressant-like agent using animal models such as the forced swimming stress and

olfactory bulbectomy animal models (Linge et al., 2016; Sales et al., 2019). However, this potential fast-acting property had been poorl o investigated in models involving chronic stress. Previous studies in these models had shown CBD anti-stress effects after 14 (Campos et al., 2013; Fogaça et al., 2018) or 28 days of concomitant treatment and stress (Gáll et al., 2020). In addition, Xu and colleagues (2019) demonstrated that 4 administrations of CBD (given weekly from the second week of CUS) induce antidepressant-like effect in the FST (Xu et al., 2019).

We showed that adding a quarter of the effective CBD dose revealed an anti-stress effect of a low dose of escitalopram (10mg/Kg) within seven days.. The effect of the concomitant administration of sub-effective doses of CBD and escitalopram was seen both in a heterotypic and a homotypic stress protocol.

Our study is the first to describe a potential benefit of the combination of escitalopram with the phytocannabinoid CBD after repeated treatment in models of chronic stress. The acceleration of onset of action of typical antidepressant drugs has also been observed with other cannabinoid-related drugs but in acute treatment regimens of and using the forced swimming test as a readout (Adamczyk et al., 2008; Rutkowska and Jachimczuk, 2004; Sales et al., 2018). However, it is important to stress that the FST, is a controversial model of depression and to detect antidepressant-like activity (especially in mice). The immobility/swimming times measured during the FST better reflect an adaptative behavior (passive/active coping in the presence of an acute stress) rather than a chronic state (Molendijk and de Kloet, 2015).

Our study, however, has an apparent limitation. Neither CUS nor SDS induced statistically significant differences between the non-stressed groups and the vehicle-treated stressed groups. The non-stressed group (SDS and CUS protocols) showed at least two populations of responders: a subgroup that responded within the first 400 seconds of the test (85% of the control group in the CUS protocol and 69% of the control group in the SDS protocol) and a subgroup that responded in the cut-off of the test (600 seconds). This variability

might have been caused by operational issues during the protocol (for instance, the daily two intraperitoneal injections might be a stressor for the control group), by environmental variables (for instance, housing conditions), or by intrinsic factors (individual differences in response to acute stressors). In the SDS protocol, the stressed group consumed significantly more food at the home-cage than the non-stressed controls, indicating a metabolic effect of stress exposure that might have interfered with the hyponeophagia response in the NSF test. However, the absence of a statistically significant effect of stress in the NSF test does not invalidate the importance of our data related to the combination of escitalopram and CBD. All mice (except for 2 in the SDS protocol) treated with the combination presented a latency lower than 300 seconds, indicating a robust anxiolytic-like effect of the treatment in stressed mice.

Clinical effects of CBD

Augmentation agents are frequently used in combination with SSRIs in treatment-resistant depression. However, the most commonly prescribed add-on therapies, such as lithium or atypical antipsychotics, cause significant side effects, limiting patient adherence to the treatment (Barowsky and Schwartz, 2006).

CBD can produce a broad spectrum of favorable effects in several neuropsychiatric disorders, with a quick-onset and sustained therapeutic response associated with reasonable safety and tolerability profile (see Crippa et al., 2018; Hosseini et al., 2020). Moreover, several preclinical studies (Linge et al., 2016; Sales et al., 2019; Zanelati et al., 2010) have suggested that CBD possesses antidepressant-like properties. This possibility, however, has not yet been extensively investigated in humans. Neuroimaging human studies show that CBD modulates the activity of limbic and paralimbic areas in MDD patients (Crippa et al., 2018). A case report showed that CBD (100–600 mg/day) was effective and well-tolerated for treating depression in a teenager with multiple substance abuse and social anxiety (Laczkovics et al., 2020). Another recent report (Berger et al., 2020) described the case of a young-man case with MDD

comorbid to severe social anxiety disorder, insomnia, and attenuated psychotic symptoms who was successfully treated with mirtazapine with adjunctive CBD (200-800 mg/day) for six months. However, the mood symptoms were probably secondary to comorbid conditions, drug misuse, and social anxiety. The same might have occurred in cases in which CBD was used in MDD associated with general medical conditions (Crippa et al., 2019; Hegazy and Platnick, 2019; Selvarajah et al., 2010). The present case series, therefore, shows, for the first time to the best of our knowledge, that CBD could be a useful add-on drug for treatment-resistant depression.

A potential benefit of combining SSRIs and CBD could also be a diminishment in SSRIs-induced side effects. For instance, some of the most common side effects of escitalopram include nausea and an initial anxiogenic effect. Rock and co-workers (2020) showed that both acute and repeated treatment with a dose de 5 mg/kg of CBD induced an anti-nausea effect in a drug-induced vomiting model in Suncus murinus (Rock et al., 2020). As shown here and in other studies, CBD exerts an anxiolytic-like effect in murine models (Campos et al., 2013; Guimarães et al., 1990). Zuardi and c-workers (1982) showed in healthy volunteers that CBD reduces the anxiety induced by delta 9-tetrahydrocannabinol (Zuardi et al., 1982). Whether CBD would also diminish nausea and anxiety associated with SSRI treatment remains to be investigated.

Chronic stress, treatments, and the prefrontal cortex.

The PFC modulates cognitive and affective function, goal-directed behaviors, and defensive responses (Arnsten et al., 2015). This region is susceptible to stress, and functional impairment has been described as a result of repeated exposure to stressors (Anderson et al., 2019; Shepard and Coutellier, 2018). The stress-induced decrease in the number of dendritic spines in cortical pyramidal neurons observed in our stressed mice corroborates this finding. Shepard and Coutellier (2018) described a hypofunction in glutamatergic activity located at the

PFC after two weeks of CUS as a result of an increased glutamatergic activity specifically at parvalbumin-positive interneurons, increasing local inhibitory control (Shepard and Coutellier, 2018). Hence, the diminished number of dendritic spines, which may indicate a lower number of excitatory synapses in pyramidal neurons, might be a result of an increase in local inhibition mediated by parvalbumin interneurons.

The PFC has also been implicated in SSRI effects. Repeated treatment with SSRI increased PFC activity, as measured through functional magnetic resonance imaging in depressed patients submitted to an emotion-interference task (Fales et al., 2009). In chronic stress models, the rapid antidepressant-like effects of ketamine are proposed to depend on synaptic plasticity modulation in this region (Hare et al., 2017). CBD has also been proposed to induce a rapid antidepressant-like effect via mTOR-induced protein synthesis and synaptogenesis in the mPFC (Sales et al., 2019). This mechanism could contribute to the increased levels of some presynaptic protein markers induced by our treatments in the present study.

Involvement of NAPE-PLD activity in the mPFC in the effects of Escitalopram combined with CBD

Both escitalopram and CBD have been shown to alter levels of NAEs (Bisogno et al., 2001; Campos et al., 2013; Smaga et al., 2014; Watanabe et al., 1996). In stressed mice, we compared the effects of combining escitalopram with CBD or URB597, an inhibitor of FAAH. This enzyme catalyzes the hydrolysis of active fatty acid amides like AEA and OEA.

AEA levels are decreased in the PFC of rats exposed to chronic mild stress (Hill et al., 2008a). Furthermore, McLaughlin and colleagues (2012) showed that the injection of URB597 into the PFC triggers an anti-stress behavioral response accompanied by an increase in the cortical serotonin levels. The depletion of serotonin abolished the anti-stress effects of intra-

PFC URB597, indicating an interaction between the serotonergic and AEA signaling in the PFC (McLaughlin et al., 2012).

However, we did not observe a significant effect of the SSRI plus URB597 combination. In other words, facilitation of anandamide neuromodulation of the PFC is not enough to enhance escitalopram effects after short-term systemic administration. This lack of effect may rely on our protocol's duration: a ten days (3-day treatment-free) CUS protocol is not as robust as, for instance, a shock-induced pain (Walker et al., 1999). The fact that systemic administration of a FAAH inhibitor failed, in our models, to accelerate escitalopram effects suggests that this effect might depend on the local and stimulus-related synthesis of NAEs. Corroborating our results, Leishman and colleagues (2018) showed that acute CBD treatment induced a remarkable augmentation in NAEs cortical levels. This effect was absent in NAPE-PLD KO mice (Leishman et al., 2018).

Endocannabinoids such as anandamide are usually synthesized 'on-demand' (Scarante et al., 2017). On the other hand, our behavioral results have clearly shown that only NAPE-PLD KO in the PFC interfered with Escitalopram + CBD effects. Previous studies have characterized the impact of systemically deleting NAPE-PLD. Leishman and co-workers (2016) have shown that NAPE-PLD KO mice present a broad and region-specific alteration in the brain lipidome profile. In the cortex, NAPE-PLD KO mice demonstrated a remarkable decrease in AEA, OEA, PEA, and other NEAs and N-acyl ethanolamines. However, this decrease was accompanied by an increase in N-acyl levels glycine, prostaglandin PGE2, and PGF2α (Leishman et al., 2016). Therefore, we should not discard that the increase in these non-NEAs mediators could contribute to our NAPE-PLD KD mice's (specifically in the vmPFC) behavioral outcomes. Similarly, DAGLα KO's effects have been investigated and shown to induce a region-dependent reduction in 2-AG, arachidonic acid, and derivatives and, in some regions, AEA (Schuele et al., 2020; Tanimura et al., 2010; Wilkerson et al., 2017). The

CRISPR-Cas9 approach used in our study has the advantage of providing high selectivity towards our target (NAPE-PLD or DAGL) in a brain specific region different from global KO strategies (which are complicated by neurodevelopment adaptations) or the conditioned KO models (with considerable time investments and costs of housing in animal facilities).

Besides the products of oxidative pathways that produce prostaglandins and leukotrienes, unsaturated fatty acid can form conjugates with other neurotransmitters resulting in the production of N-acyl amino acid/neurotransmitter (NAAN): NEA+ glycine (N-acyl glycine), NEA+GABA (N-arachidonoyl GABA), NEA+dopamine (N-acyl dopamine) and NEA +serotonin (n-acyl-serotonins, including arachidonoyl-serotonin -AA5-HT) (Connor et al., 2010; Keereetaweep and Chapman, 2016). Some NAANs can interact directly with GPCRs (such as CB1) or transient receptor potential channels (e.g., TRPV1 receptor) (Connor et al., 2010). Therefore, it is possible that chronic stress induces a disruption in the biosynthesis of NAANs, decreasing their modulatory role in the vmPFC circuitry leading to the hypofunction of this brain structure. Consequently, the combination of CBD and escitalopram would restore the production of NAANs, such as acyl-serotonin compounds via two synergistic mechanisms: 1) escitalopram: by increasing the serotonergic content in the synaptic cleft and 2) CBD, but not URB597, by the former increasing NAE production through enhanced NAPE-PLD activity, potentiating the conjugation of neurotransmitter with arachidonic acid (Connor et al., 2010). This latter mechanism would suggest that anti-stress actions and the effects of CBD on neuroplasticity would not rely simply on the inhibition of FAAH; instead, its action would involve alternative unsaturated fatty acid metabolic routes leading to the formation of NAEs (Leishman et al., 2016) and NAANs.

Regarding possible local (vmPFC) cellular mechanisms involving NAPE-PLD and the effects of Escitalopram + CBD, Rivera et al. (2014) suggested that in the hippocampus of Wistar rats, NAPE-PLD is expressed in GABAergic interneurons expressing parvalbumin,

calretinin, and calbindin (Rivera et al., 2014). Therefore, the combination of escitalopram and CBD could counteract the effects of stress by recruiting in the vmPFC the formation of NAEs via activation of NAPE-PLD in GABAergic interneurons, leading to a negative feedback to counteract the stress-induced PFC hypofunction (Figure 5).

However, this hypothesis remains to be tested.

Conclusion

Altogether, our pre-clinical and clinical data indicate that CBD could be a viable strategy as an add-on therapy to optimize the therapeutic response to antidepressants. We propose that CBD could accelerate escitalopram's anti-stress effects via the recruitment of NAE-mediated signaling in the prefrontal cortex. Whether CBD would enhance the therapeutic response to other SSRIs or other classes of antidepressants through the same mechanism remains to be tested.

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CRediT authorship contribution statement

Franciele Franco Scarante: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original draft, Writing – Review and editing, Visualization. **Vinícius Detoni Lopes:** Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original draft. **Eduardo J. Fusse:** Methodology, Investigation. **Maria Adrielle Vicente:** Methodology, Investigation, Writing – Original draft. **Melissa R. Araújo:** Methodology, Investigation, Writing – Original draft. **Davi S. Scomparin:** Methodology, Investigation, Writing – Original draft. **Rafael P. Aguiar:** Methodology, Investigation, Writing – Original draft. **Francisco S. Guimarães:** Conceptualization, Resources, Writing – Review and editing, Funding acquisition. **Viviani Nardini:** Methodology, Investigation. **Carlos Arterio Sorgi:** Methodology, Investigation. **Lucia H. Faccioli:** Methodology, Investigation, Writing – Review and editing. **Jaime E. C. Hallak:** Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Writing – Original draft, Writing – Review and editing, Supervision, Funding acquisition. **Samia Joca:** Conceptualization, Methodology, Investigation, Writing – Review and editing. **Kenneth Mackie:** Conceptualization, Writing – Original draft, Writing – Review and editing. **Antonio Waldo Zuardi:** Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Writing – Original draft, Writing – Review and editing, Supervision, Funding acquisition. **José Alexandre S. Crippa:** Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Writing – Original draft, Writing – Review and editing, Visualization, Supervision, Project administration, Funding acquisition. **Alline C. Campos:** Conceptualization, Methodology, Formal Analysis, Resources, Writing – Original draft, Writing – Review and editing, Visualization, Supervision, Project administration, Funding acquisition.

Conflict of interest

ACC, JAC, JEH, FSG, and AWZ are coinventors of the patent "Cannabinoid-containing oral pharmaceutical composition, method for preparing and using same," INPI on September 16, 2016 (BR 112018005423-2). JAC, JEH, FSG, AWZ are coinventors of the patent "Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023," Def. US number Reg. 62193296; July 29, 2015; INPI on August 19, 2015 (BR1120150164927; Mechoulam R, Zuardi AW, Kapczinski F, Hallak JEC, Guimarães FS, Crippa JAS, Breuer A). Universidade de São Paulo (USP) has licensed this patent to Phytecs Pharm (USP Resolution No. 15.1.130002.1.1) and has an agreement with Prati-Donaduzzi to "develop a pharmaceutical product containing synthetic CBD and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson's disease, and anxiety disorders." JAC is a member of the International Advisory Board of the Australian Centre for Cannabinoid Clinical and Research Excellence (ACRE) – National Health and Medical Research Council (NHMRC). JAC and JEH have received travel support to attend scientific meetings and personal consultation fees from BSPG-Pharm. The other authors declare that they have no conflicts of interest.

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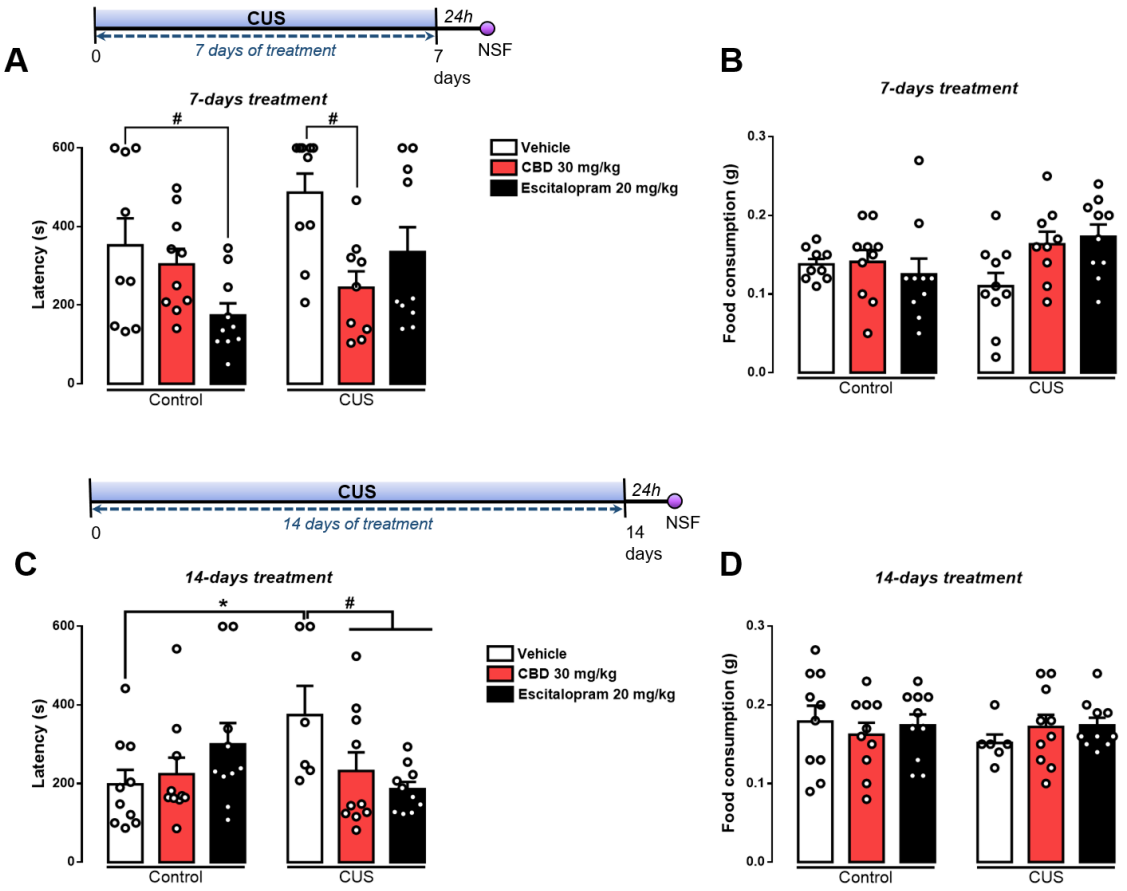


Figure 1. CBD induces an anti-stress effect more rapidly than escitalopram. Effect of 7 (A and B) and 14 days (C and D) of treatment with escitalopram (20mg/kg) or CBD (30mg/kg) on the response of mice undergoing the chronic unpredictable stress (CUS) model in the latency to start feeding in the novelty suppressed feeding (NSF) test (A and C) and on the home-cage food consumption (B and D). (*) indicates $p<0.05$ compared to the control group; (#) indicates $p<0.05$ compared to the stressed group treated with vehicle (N=6-10, Two-way ANOVA; One-way ANOVA followed by Duncan; t test for independent samples).

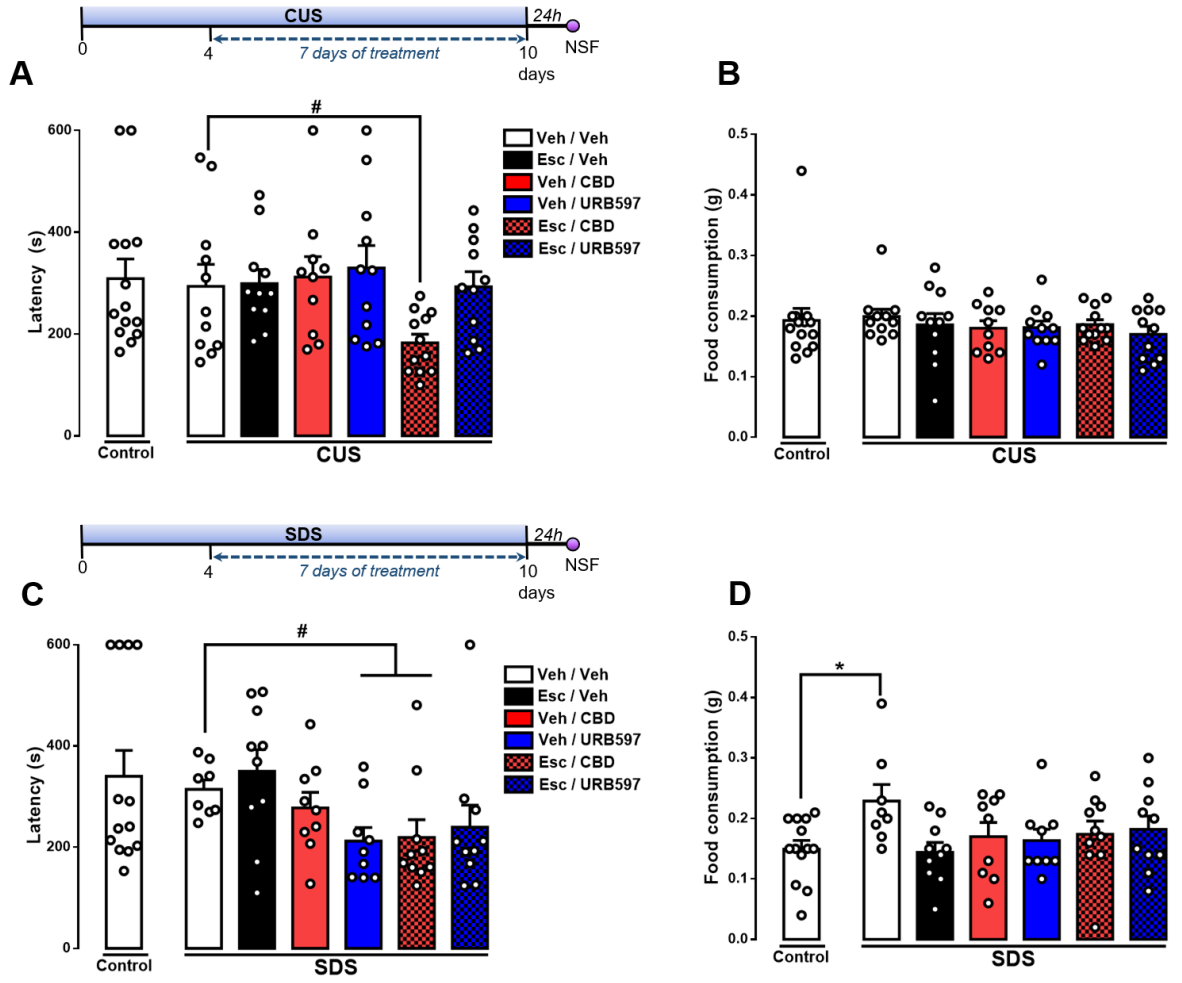


Figure 2. The combination of escitalopram and CBD accelerates the anxiolytic-like effect of the treatment in stressed animals. Effect of the 7-day treatment with the different combinations of vehicle or escitalopram (10mg/kg) and vehicle, CBD (7.5mg/kg) or URB597 (0.1mg/kg) on the latency to start feeding in the NSF test (A and C) and on the home-cage food consumption (B and D) in mice submitted to the CUS model (A and B) or to the social defeat model (SDS) (C and D). Data represented as Mean ± SEM (A-H); (*) indicates $p<0.05$ compared to the control group; (#) indicates $p<0.05$ compared to the stressed group treated with vehicle (N=8-14, Two-way ANOVA; One-way ANOVA followed by Duncan; t test for independent samples).

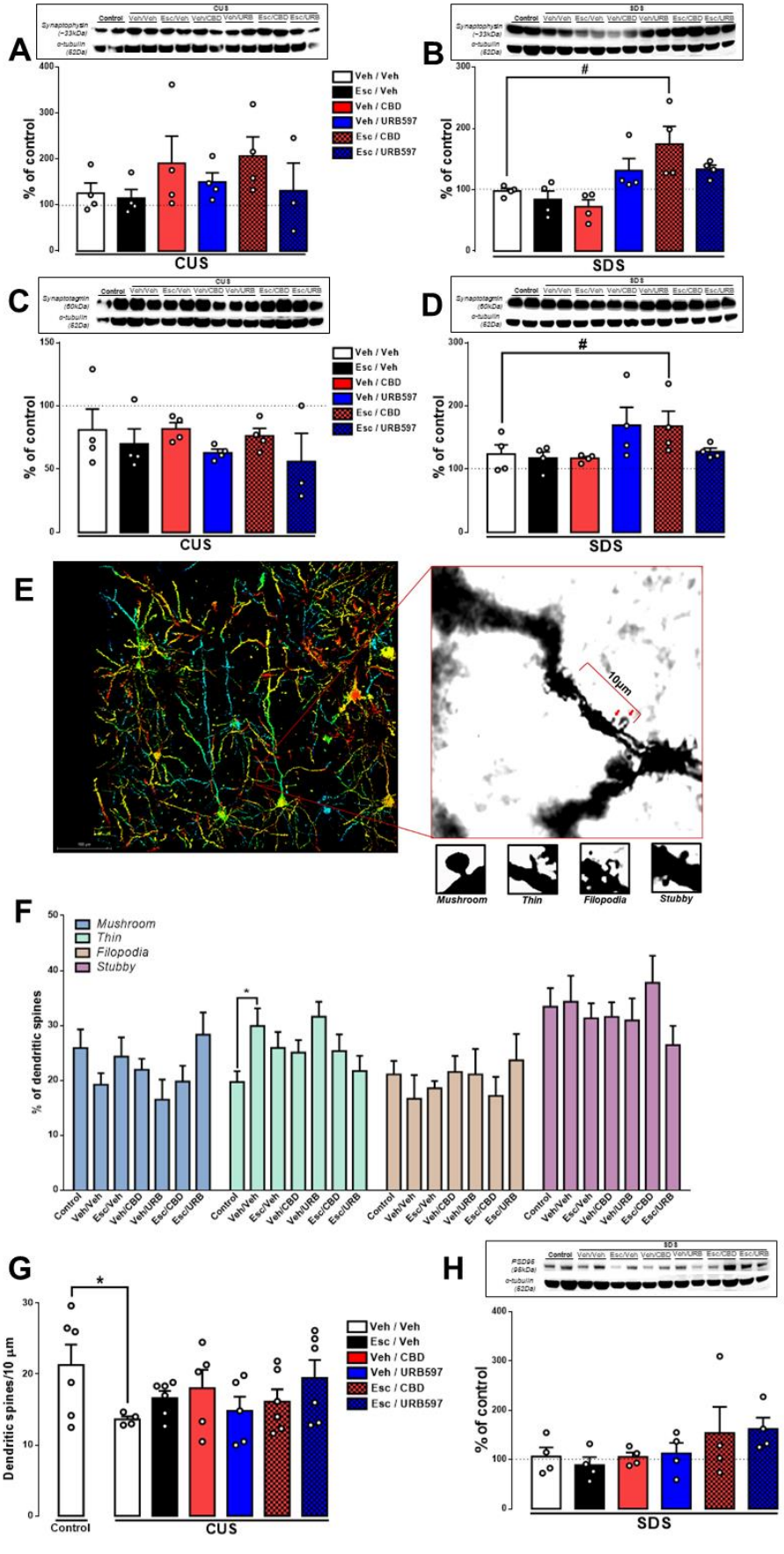


Figure 3. Stress causes a postsynaptic impairment and combined CBD and escitalopram increases the expression of presynaptic proteins in the SDS model. Effect of the 7-day treatment with the different combinations of vehicle or escitalopram (10mg/kg) and vehicle, CBD (7.5mg/kg) or URB597 (0.1mg/kg) on the relative expression of the presynaptic proteins synaptophysin (A and B) and synaptotagmin (C and D) in mice submitted to the CUS model (A and C) or to the social defeat model (SDS) (B and D). (E) Representative image of a pyramidal neuron in the PFC and explanation of the quantification and morphological analysis of dendritic spines; (F) Percentage of dendritic spines for each of the morphologies, relative to the total number of dendritic spines for each group; (G) Total number of dendritic spines in tertiary dendrites of mPFC pyramidal neurons in mice treated with the different drug combinations and submitted to the CUS protocol; (H) Relative expression of the postsynaptic protein PDS95 in mice submitter to the SDS protocol and treatment with the different treatment combinations. Data represented as the Mean percentage relative to control (non-stressed group) ± SEM (A-D and H) or as Mean ± SEM (F-G); (*) indicates $p<0.05$ compared to the control group; (#) indicates $p<0.05$ compared to the stressed group treated with vehicle (Two-way ANOVA; One-way ANOVA followed by Duncan; t test for independent samples).

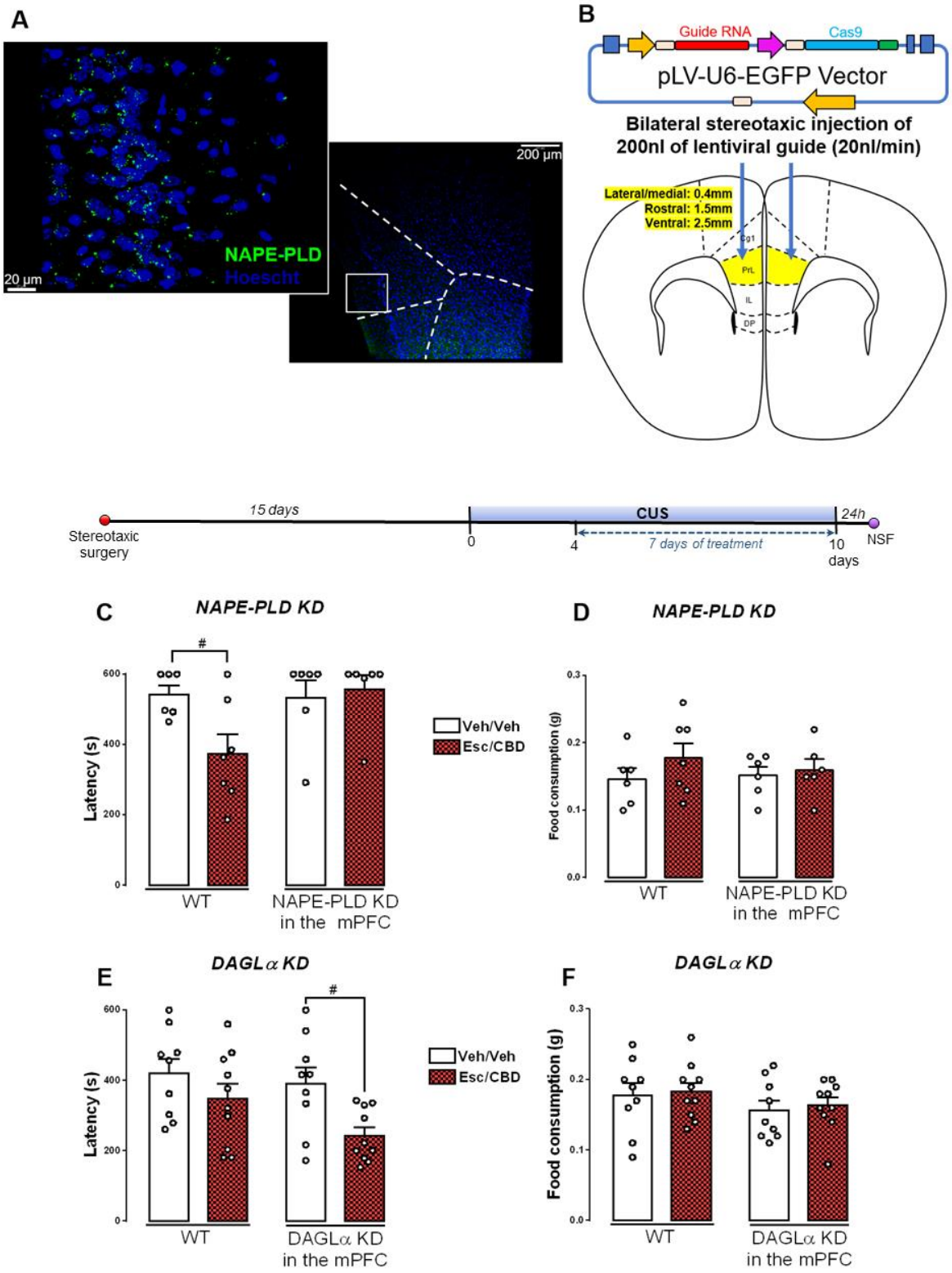


Figure 4. The anxiolytic-like effect of the combination of Escitalopram and CBD in stressed mice depends on NAPE-PLD, but not on DAGLα, activity in the PFC. ((A)

NAPE-PLD immunolabeling at the PFC; (B) Schematic representation of the viral vectors directing the CRISPR-Cas9-mediated deletion of NAPE-PLD or of DAGLα, detailing the stereotaxic coordinates, the volume and rate of the bilateral injection and the exon targeted by the guide RNAs for each enzyme; (C) Latency to feed in the novel environment and (D) food consumption in the home-cage of mice after 10 days of CUS and 7 days of treatment with Vehicle or with the combination of Escitalopram and CBD in which NAPE-PLD was knocked-down (KD) in the PFC; (E) Latency to feed in the novel environment and (F) food consumption in the home-cage of mice in which the knock-down of DAGLα was induced in the PFC after 10 days of CUS and 7 days of treatment with Vehicle or with the combination of Escitalopram and CBD. Data represented as Mean ± SEM (C-F); (#) indicates $p<0.05$ compared to the corresponding stressed group treated with vehicle (Two-way ANOVA; One-way ANOVA followed by Duncan).

Table 1. Assessment instruments scores completed at baseline and after 1, 2, 4, 8, and 12 weeks.

	Baseline	Week 1	Week 2	Week 4	Week 8	Week 12	%BL
Patient A.							
MADRS	40	31	22	10	03	01	97.5%
PHQ-9	19	14	10	05	01	00	100.0%
GAD-7	16	09	07	04	02	01	93.8%
CGI-S	06	05	04	03	01	01	83.3%
Patient B.							
MADRS	42	40	26	18	10	06	85.7%
PHQ-9	21	18	12	10	05	02	90.5%
GAD-7	17	14	09	07	03	02	88.2%
CGI-S	05	05	03	02	02	01	80.0%
Patient C.							
MADRS	44	42	31	25	20	17	61.4%
PHQ-9	22	20	17	14	11	10	54.5%
GAD-7	15	12	10	08	07	07	53.3%
CGI-S	06	06	05	04	03	03	50.0%

Abbreviations: MADRS; The Montgomery-Åsberg Depression Rating Scale; PHQ-9, 9-item Patient Health Questionnaire; GAD-7, 7-item Generalized Anxiety Disorder Scale; CGI-S, Clinical Global Index Severity, %BL percentage changes from Baseline.

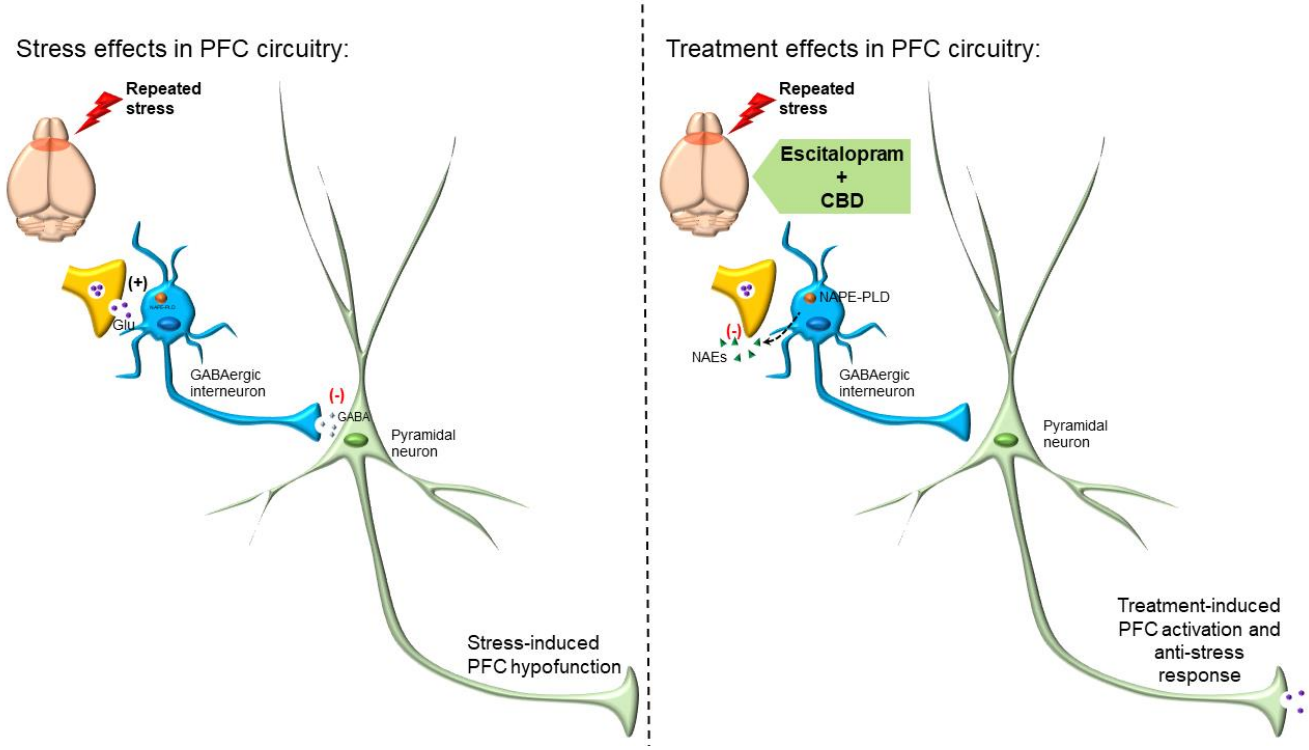


Figure 5. Hypothetical alterations in the PFC induced by stress and treatment. In stress conditions, there is an increase in the glutamatergic neurotransmission onto GABA interneurons, increasing the local inhibition over pyramidal cells and leading to a PFC hypofunction. Treatment with the combination of escitalopram and CBD recruits the activation of NAPE-PLD and the release of n-acylethanolamines, which act presynaptically to inhibit the activation of the GABAergic interneurons, allowing the pyramidal cells to be activated and generating the anxiolytic-like response in stressed mice.

Repeated stress

Reduced number of dendritic spines

Prefrontal cortex (PFC) hypofunction

Repeated stress

Treated with:

CBD (30 mg/kg)

1 week of treatment

Repeated stress

Treated with:

Escitalopram (20 mg/kg)

2 weeks of treatment

Repeated stress

Treated with:

Escitalopram (10 mg/kg) + CBD (7.5 mg/kg)

1 week of treatment

Anti-stress behavioral effect

NAPE-PLD gene

Intra-PFC injection of lentiviral vector directing the CRISPR-Cas9-mediated deletion of NAPE-PLD

Repeated stress

1 week of treatment

No antistress effect

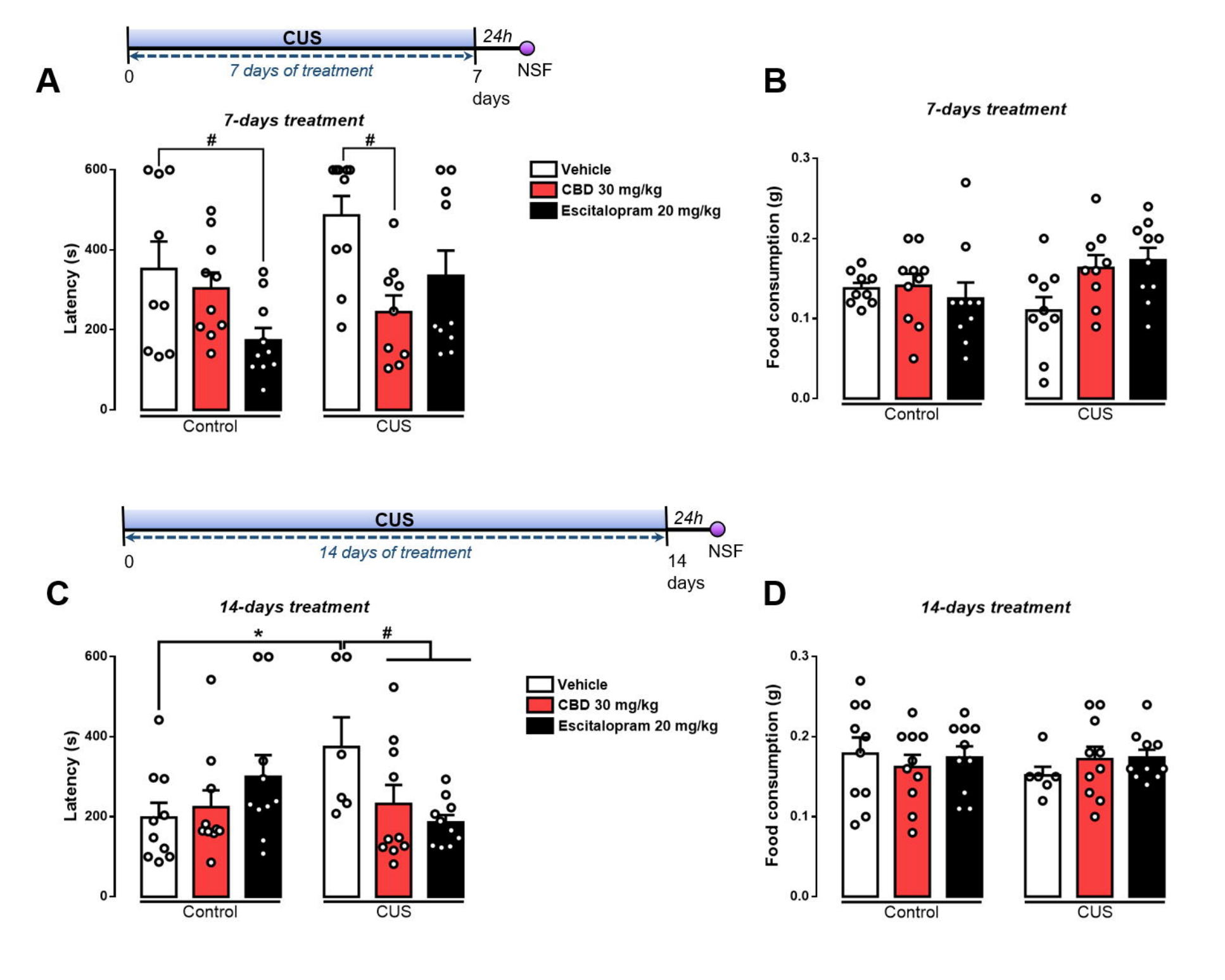
Patients with treatment-resistant depression

Adjunt therapy

CBD + Antidepressant

12 weeks

Total remission or reduction of depression and anxiety symptoms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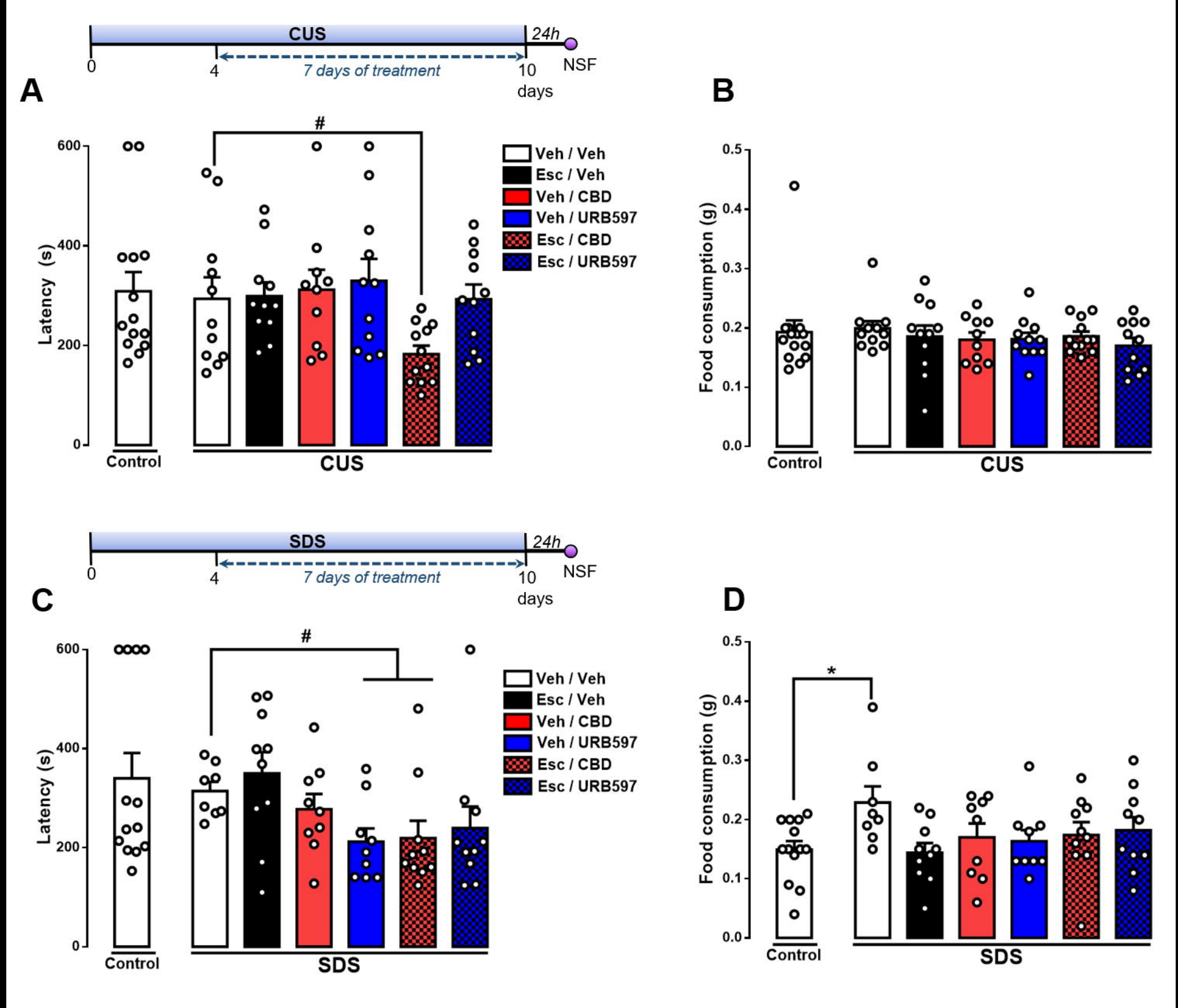
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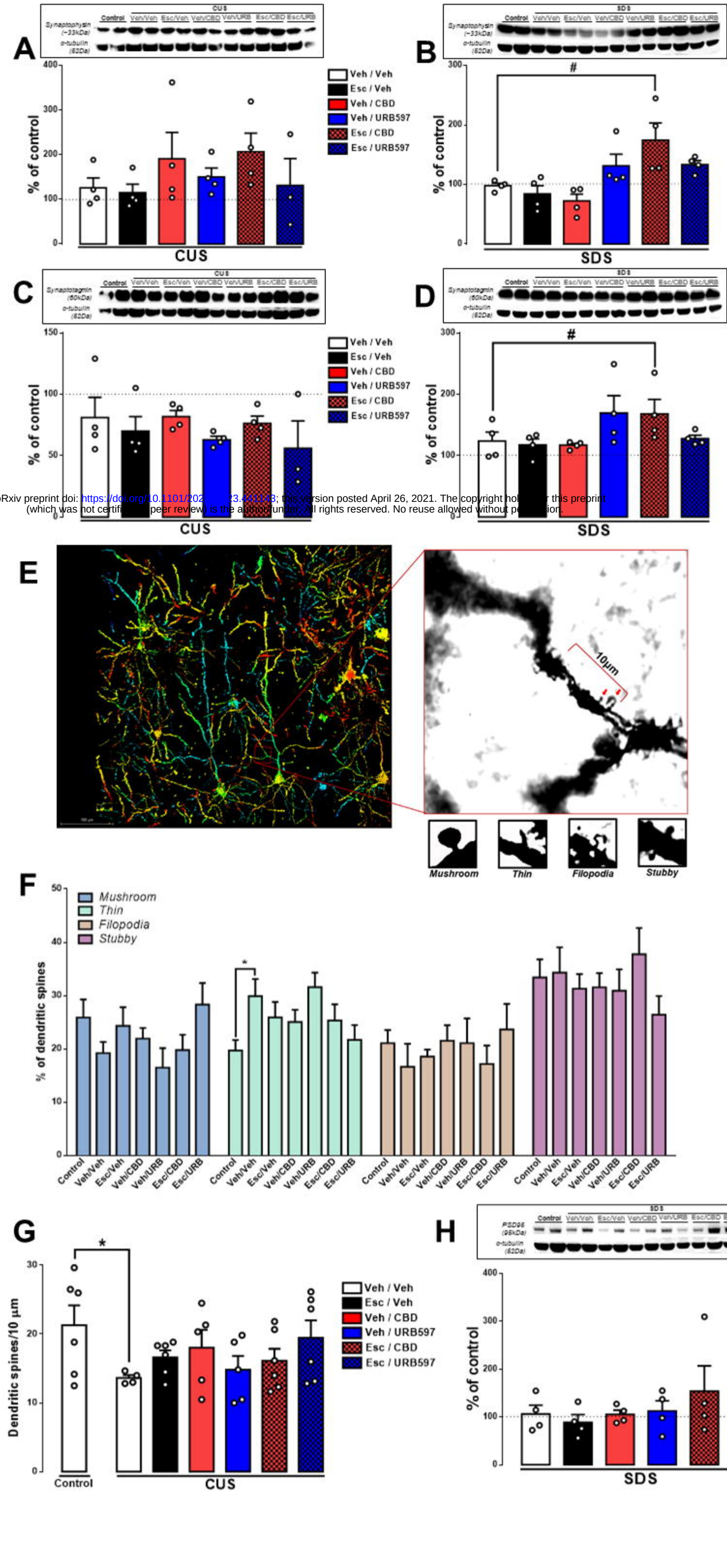
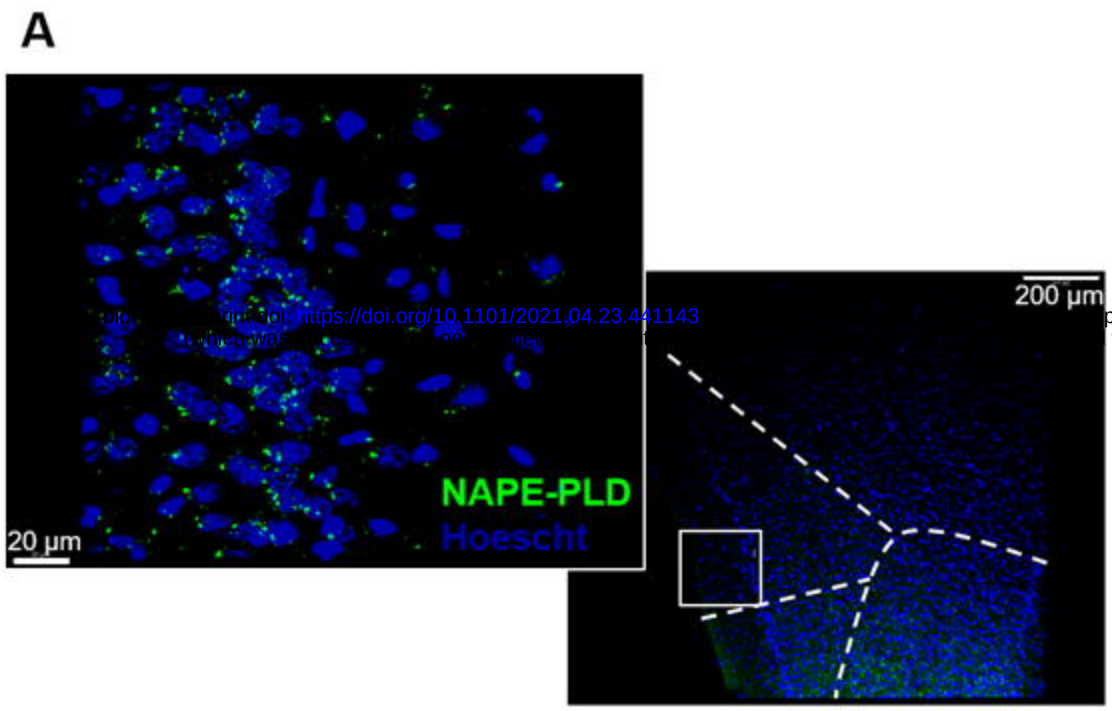
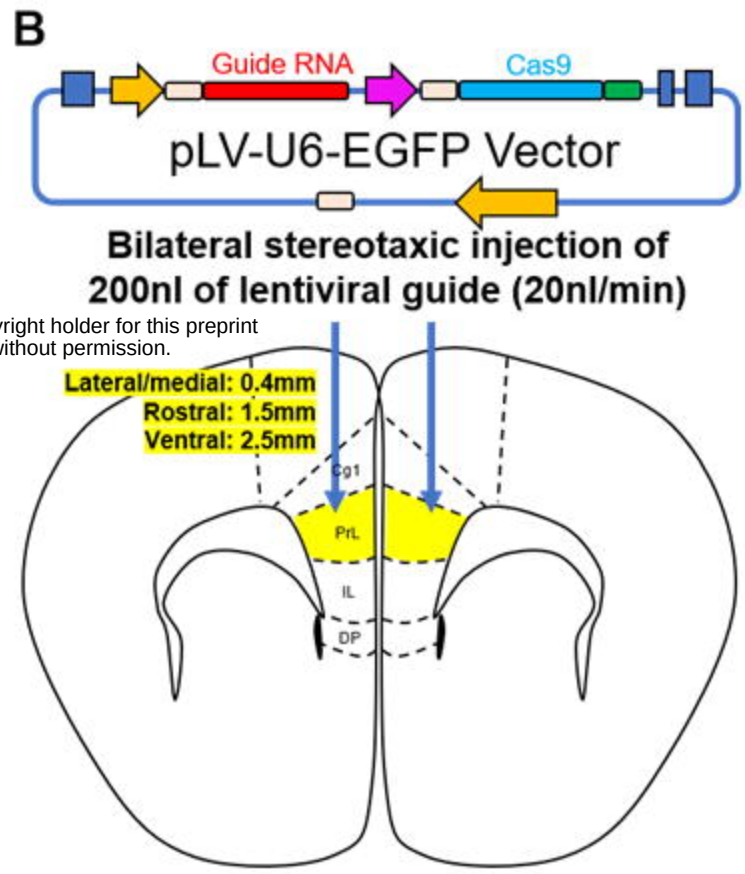
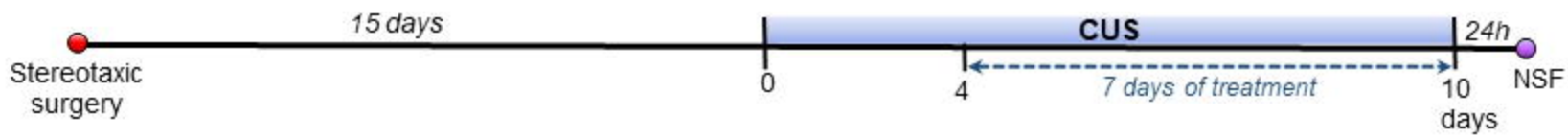
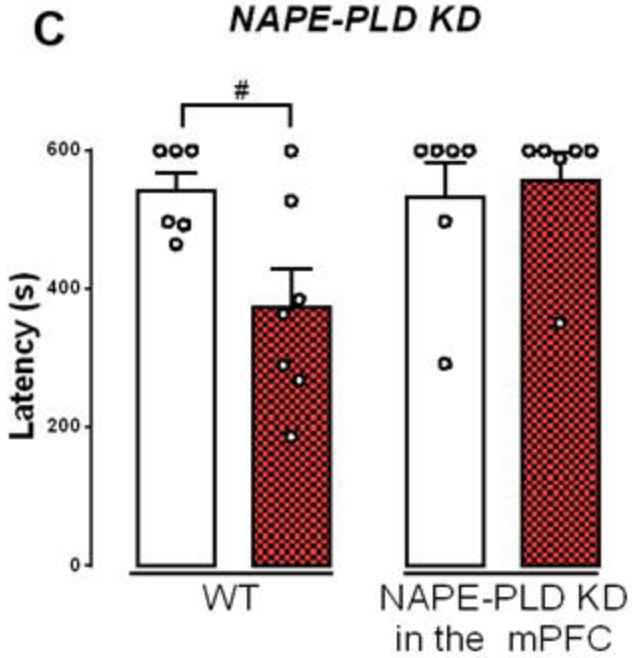
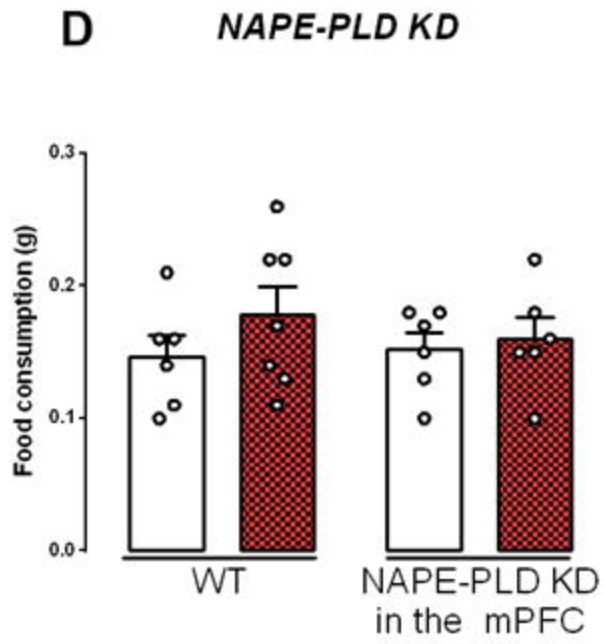


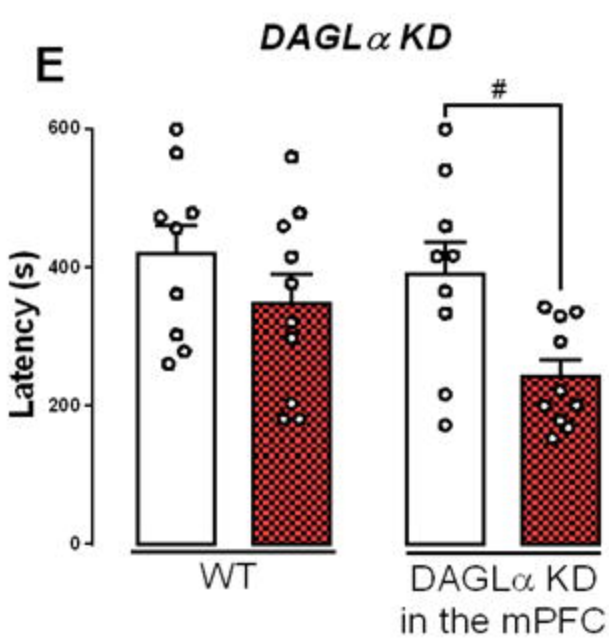


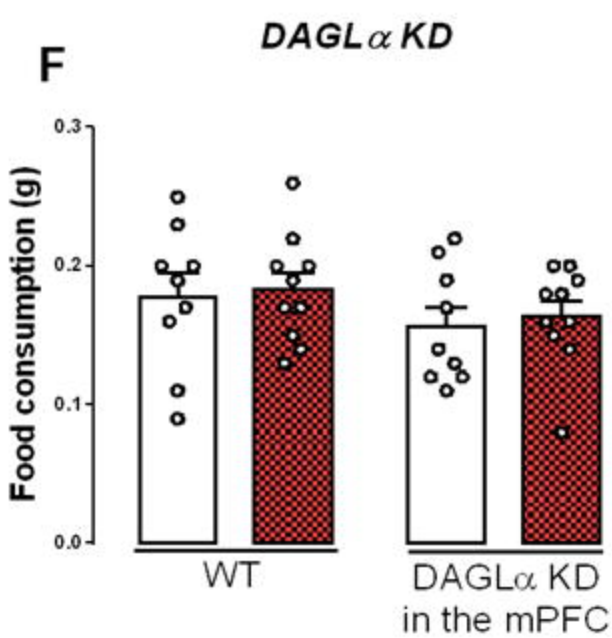




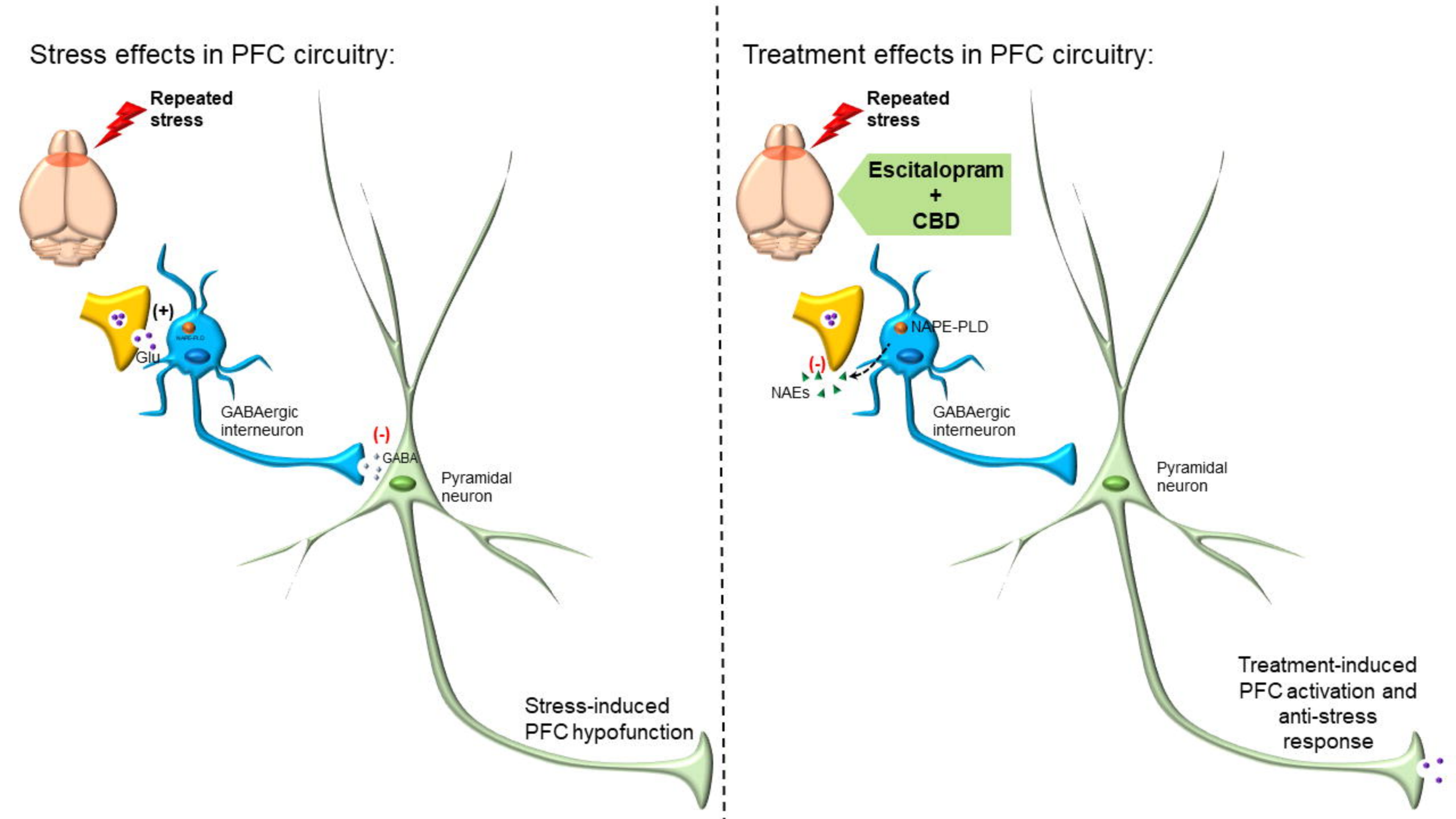


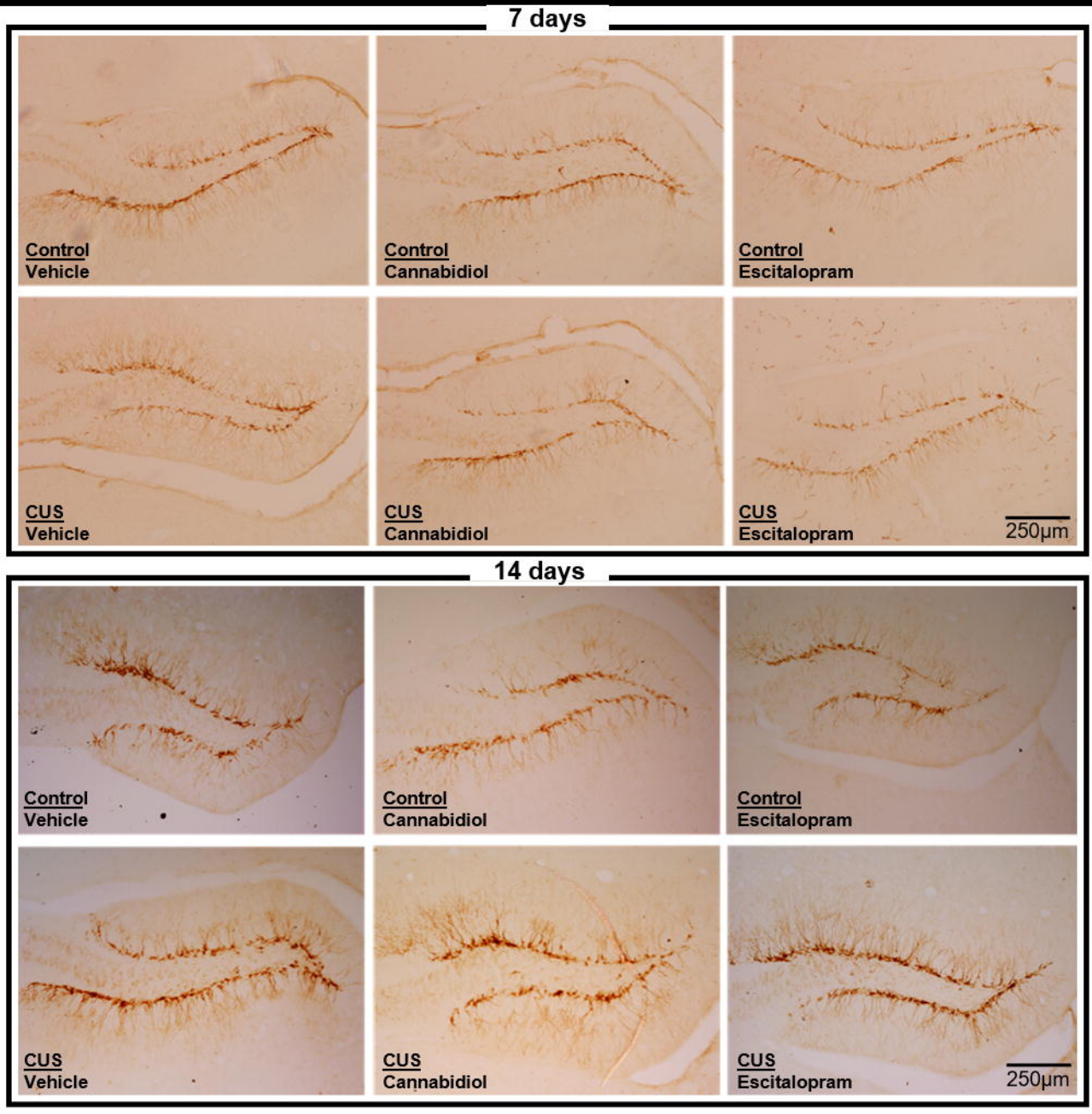


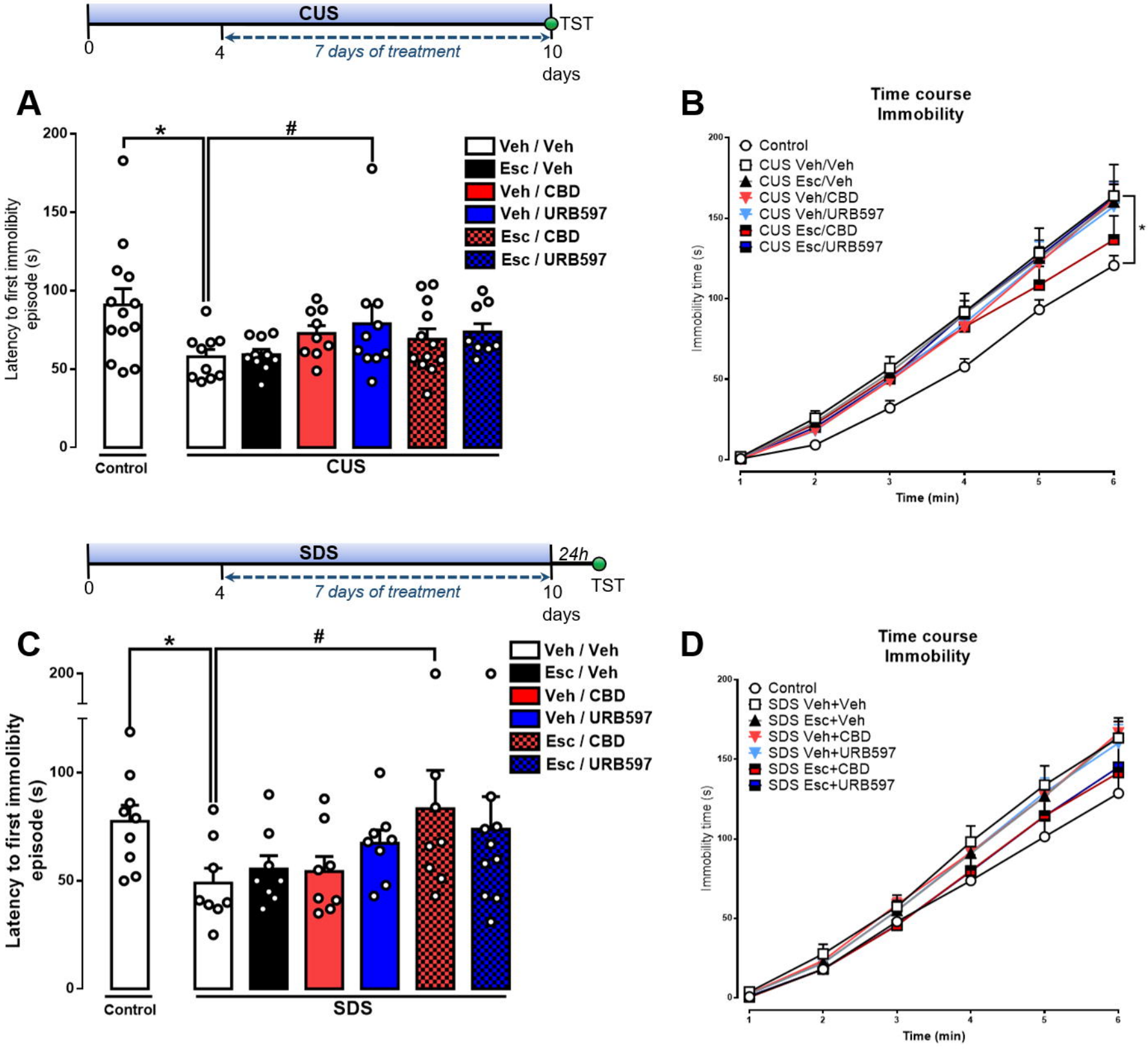




	Baseline	Week 1	Week 2	Week 4	Week 8	Week 12	%BL
Patient A.							
MADRS	40	31	22	10	03	01	97.5%
PHQ-9	19	14	10	05	01	00	100.0%
GAD-7	16	09	07	04	02	01	93.8%
CGI-S	06	05	04	03	01	01	83.3%
Patient B.							
MADRS	42	40	26	18	10	06	85.7%
PHQ-9	21	18	12	10	05	02	90.5%
GAD-7	17	14	09	07	03	02	88.2%
CGI-S	05	05	03	02	02	01	80.0%
Patient C.							
MADRS	44	42	31	25	20	17	61.4%
PHQ-9	22	20	17	14	11	10	54.5%
GAD-7	15	12	10	08	07	07	53.3%
CGI-S	06	06	05	04	03	03	50.0%


Stress effects in PFC circuitry:
Repeated stress
(+)
Glu
GABAergic interneuron
(-)
GABA
Pyramidal neuron
Stress-induced PFC hypofunction
Treatment effects in PFC circuitry:
Repeated stress
Escitalopram + CBD
NAPE-PLD
(-)
NAEs
GABAergic interneuron
Pyramidal neuron
Treatment-induced PFC activation and anti-stress response


7 days
Control
Vehicle
Control
Cannabidiol
Control
Escitalopram
CUS
Vehicle
CUS
Cannabidiol
CUS
Escitalopram
250µm
14 days
Control
Vehicle
Control
Cannabidiol
Control
Escitalopram
CUS
Vehicle
CUS
Cannabidiol
CUS
Escitalopram
250µm


CUS
TST
0
4
7 days of treatment
10
days
A
Latency to first immolibity episode (s)
200
150
100
50
0
*
#
Veh / Veh
Esc / Veh
Veh / CBD
Veh / URB597
Esc / CBD
Esc / URB597
Control
CUS
B
Time course
Immobility
Control
CUS Veh/Veh
CUS Esc/Veh
CUS Veh/CBD
CUS Veh/URB597
CUS Esc/CBD
CUS Esc/URB597
Immobility time (s)
Time (min)
SDS
24h
TST
0
4
7 days of treatment
10
days
C
Latency to first immolibity episode (s)
200
100
50
0
*
#
Veh / Veh
Esc / Veh
Veh / CBD
Veh / URB597
Esc / CBD
Esc / URB597
Control
SDS
D
Time course
Immobility
Control
SDS Veh+Veh
SDS Esc+Veh
SDS Veh+CBD
SDS Veh+URB597
SDS Esc+CBD
SDS Esc+URB597
Immobility time (s)
Time (min)