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Cannabidiol reverses memory impairments and activates components of the Akt/GSK3β pathway in an experimental model of estrogen depletion

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ABSTRACT

Clinical and preclinical evidence has indicated that estrogen depletion leads to memory impairments and increases the susceptibility to neural damage. Here, we have sought to investigate the effects of Cannabidiol (CBD) a non-psychotomimetic compound from *Cannabis sativa*, on memory deficits induced by estrogen depletion in rats, and its underlying mechanisms. Adult rats were subjected to bilateral ovariectomy, an established estrogen depletion model in rodents, or sham surgery and allowed to recover for three weeks. After that, they received daily injections of CBD (10 mg/kg) for fourteen days. Rats were tested in the inhibitory avoidance task, a type of emotionally-motivated memory. After behavioral testing they were euthanized, and their hippocampi were isolated for analysis of components of the Akt/GSK3β survival pathway and the antiapoptotic protein Bcl2. Results revealed that ovariectomy impaired avoidance memory, and CBD was able to completely reverse estrogen depletion-induced memory impairment. Ovariectomy also reduced Akt/GSK3β pathway's activation by decreasing the phosphorylation levels of Akt and GSK3β and Bcl2 levels, which were ameliorated by CBD.

The present results indicate that CBD leads to a functional recovery accompanied by the Akt/GSK3β survival pathway's activation, supporting its potential as a treatment for estrogen decline-induced deterioration of neural functioning and maintenance.

1. Introduction

Over time, our comprehension of estrogens' role crossed the field of reproduction and sexual differentiation and reached the central nervous system and its functioning [1]. It is the essential female hormone, and among the endogenous estrogens, estradiol is the most potent and prevalent [2,3]. In the brain, estradiol acts through its nuclear (alpha and beta) and membrane (G-coupled protein estrogen receptor - GPER) receptors where it plays crucial roles in memory, cognition, and behavior [4–6].

The function of estradiol in the brain has been understood from clinical and pre-clinical observations. In women, the period after menopause is associated with cognitive decline and an increased risk for Alzheimer's disease [7]. During the perimenopausal period, women experience a progressive decay on estradiol levels, reaching approximately 10 % of their premenopausal levels [8]. A suitable and widely used phenotypical animal model for studying the effects of estrogen depletion in cognition is the bilateral ovariectomy in rodents [9,10].

The integrity of hippocampal formation is required for episodic and contextual memory; moreover, the hippocampus encodes the context in fear and aversive conditioning tasks in humans and animals [4,11–13]. The density of apical dendritic spines and expression of synaptophysin in the CA1 region [reviewed in 4] and neurogenesis [14] in the dentate gyrus are physiologic substrates for memory formation and consolidation, and are

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positively influenced by estradiol [4]. Moreover, the relationship between hippocampal performance and estradiol levels can be expressed by an inverted U-shaped curve [3]. In rodents, ovariectomy leading to estrogen depletion is associated with impairment in memory tasks (e. g. object recognition and object localization) and decrease in dendritic spines in the hippocampus and medial prefrontal cortex in rats [15].

Animal studies have demonstrated that estradiol treatment can reverse neuronal damage in the hippocampus through activation of ER alpha and one of its downstream pathways, namely the phosphatidylinositol-3 kinase/protein kinase B (PI3K/Akt) pathway [16,17]. Akt is a serine/threonine protein kinase regulated through phosphatidylinositol-mediating signaling. Glycogen synthase kinase 3 (GSK3), downstream of Akt, is a serine/threonine protein kinase highly expressed in the central nervous system. Akt/GSK3β signaling pathway regulates several cellular processes including transcription, cell proliferation, and apoptosis [18]. Evidence indicates that estrogen, by modulating the PI3K/Akt/GSK3β pathway, upregulates Bcl2, a member of the Bcl2 family of the apoptotic cascade, which exerts anti-apoptotic effects [19].

Cannabidiol (CBD) is one of the main non-psychotomimetic components of *Cannabis* (*sativa*, *indica* and *ruderalis*) [20]. Cannabinoid compounds act through Cannabinoid type 1 and type 2 receptors (CB1 and CB2). CB1 is a G protein-coupled receptor localized at the pre-synaptic terminal in hippocampus, medial prefrontal cortex, basolateral and lateral amygdala (critical areas for fear learning) [21–23]. The therapeutic effects of CBD are related to a multitude of pharmacological pathways: increasing endocannabinoid levels, mainly anandamide, activation of transient receptor potential vanilloid (TRPV-1), inhibition of adenosine reuptake, agonism of serotonin 5-HT1A receptors, intracellular calcium increase, agonism of intracellular PPARγ receptors, and anti-oxidative effects [24,25]. Previous studies also report that CBD displays neuroprotective properties related to its anti-apoptotic effects [26,27]. Also, *in vitro* and *in vivo* studies have shown that CBD modulates the PI3K/AKT/GSK3β pathway [28,29].

In the present study, we aimed to understand the possible neuroprotective effect of CBD against estrogen depletion-induced emotional memory deficits, using an animal model of ovariectomy-induced estrogen depletion. Once CBD and estradiol modulate a common pathway, we speculated whether CBD would be able to reverse the deleterious effect of estradiol decline observed in menopause.

2. Materials and methods

2.1. Animals

Adult female Wistar rats (CrlCembe:WI) were obtained from the Centro de Modelos Biológicos Experimentais (CeMBE), Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil, and kept in groups of three to five in individually ventilated cages (Tecniplast, Italy) with sawdust bedding, in a room with temperature of 21 ± 1 °C and a 12/12 h light/dark cycle (lights on at 7:00 a.m.). Throughout the experiments, animals were given standardized pellet food and tap water *ad libitum*.

All behavioral experiments were performed at light phase between 09:00 a.m and 11:00 a.m. All experimental procedures were performed following the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI, Brazil) and approved by the Ethics Committee for the Use of Animals of Pontifical Catholic University of Rio Grande do Sul (SIPESQ #7922). All efforts were made to minimize the number of animals and their suffering.

2.2. Surgery

Adult female Wistar rats (3 months-old) were anesthetized with xylazine (7.5 mg/kg) and ketamine (75 mg/kg) intraperitoneally (i.p.). After anesthesia was confirmed, two small bilateral dorsolateral incisions in the skin and peritoneum were performed. The ovaries and tips of uterine horns were ligated and excised, leaving the ligatured uterine horns intact (OVX, n = 28). The muscle was then sutured and the skin stapled. The false-operated (sham, n = 25) groups were submitted to the same type of surgical incision, but with the ovaries' preservation. After the experiments, the success of ovariectomy (OVX) was confirmed by the development of uterine atrophy [9,10,30].

2.3. Cannabidiol

Three weeks after recovery from surgery, female rats (mean body weight at the beginning of treatments 440 g) received a daily intraperitoneal injection of vehicle (Tween 80 – saline solution 1:16 v/v; Sham = 10 and OVX = 14) or CBD (10 mg/kg, Sham = 15 and OVX = 14; approximately 99.9 % pure; kindly supplied by BSPG-Pharm, Sandwich, UK) for 14 consecutive days, at the same time of the day, starting at 9 a.m. Drug solutions were freshly prepared immediately prior to administration [26,31–33].

2.4. Inhibitory avoidance

We used the single-trial, step-down Inhibitory avoidance (IA) conditioning as an established model of fear-motivated memory. In IA training, animals learn to associate a location/context in the training apparatus with an aversive stimulus (footshock). The IA training and retention test procedures were described in previous reports [32,34,35]. The IA apparatus was a 50 × 25 × 25-cm^3 acrylic box (Albarsch, Porto Alegre, Brazil) whose floor consisted of parallel stainless steel bars (1-mm diameter) spaced 1 cm apart. A 7-cm wide, 2.5-cm high platform was placed on the floor of the box against the left wall. On the training trial, performed in the 13th day of pharmacological treatment, rats were placed on the platform and their latency to step-down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, rats received a 2-sec mild footshock (0.4 mA) and were removed from the apparatus immediately afterward. In the training day, CBD or vehicle were administered 6 h after the training session to prevent a possible acute effect during task acquisition. A retention test trial was carried out 24 h after the training trial. The retention test trial was procedurally identical to training, except that no footshock was presented. Step-down latencies (in seconds) on the retention test trial (maximum 180 s) were used as a measure of IA retention. Retention test was performed 4 h before the injections in the 14th day, in order to avoid any possible acute drug effect on retrieval.

Rats were euthanized by decapitation at 24 h after IA retention test. In the day of tissue collection, rats did not receive CBD injection. Brains were quickly dissected and dorsal hippocampi were isolated and stored at -80 °C for subsequent Western blot analyses.

2.5. Western blot analysis

Randomly selected hippocampi obtained from female rats euthanized by decapitation 24 h hours after IA retention test were homogenized in ice-cold lysis buffer containing 10 mM Tris−HCl pH 8.0, 1 mM EDTA pH 8.0, 100 mM NaCl, 1 mM Na_3VO_4, protease inhibitor cocktail, Triton X-100 0.5 %, and SDS 0.1 %. After 30 min in ice, samples were centrifuged at 13,500 rpm for 10 min [26,30,31,34]. The supernatant was collected and the protein content was determined using Bradford assay [36]. Aliquots were stored at −20 °C.

Thirty μg of protein was separated on a 10 % (or 12 % for Bcl2 analysis) SDS polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. Membranes were blocked with 5% albumin in TBS containing 0.05 % Tween 20 and were incubated overnight with one of the following antibodies: anti-pan-AKT (1:500; AB8805, Abcam), anti-phospho-AKT (THR308) (1:1,000; CELL-13038S, Cell Signaling Technology), anti-GSK3β (1: 1,000; AB32391, Abcam), anti-phospho-

GSK3β (Y216) (1: 500; AB75745, Abcam), Bcl2 (1: 500; AB196495, Abcam), anti-GAPDH (loading control; 1: 2000; AB37168, Abcam), which were dissolved in TBS-T with 5% bovine serum albumin. Membranes were then washed 3 times with TBS-T and incubated for 120 min at room temperature in TBS-T with 1% skimmed milk containing anti-rabbit IgG H&L (HPR) (Abcam, Cambridge, UK) secondary antibody, detected using ECL Western Blotting Substrate Kit (Abcam, Cambridge, UK). Pre-stained molecular weight protein marker (SuperSignal Molecular Weight Protein Ladder, THER-84785, Thermo Scientific, Rockford, USA) was used to determine the detected bands' molecular weight. The image acquisition for densitometric analysis was performed using L-Pix Chemi Express (Loccus Biotechnology, São Paulo, Brazil), followed by quantification using ImageJ software (http://rsb.info.nih.gov/ij/), by an experimenter blind to samples experimental condition [26,30,31,34]. Bcl-2 levels were quantified by normalizing the optical density of its band to a loading control band (GAPDH). In relation to Akt and GSK3β, our goal was to determine whether ovariectomy or CBD would affect the pathway activation, estimated by the phosphorylation levels of those key proteins. Results are expressed as a ratio of phosphorylated protein divided by total protein, as arbitrary units (AU), from each sample, in the same run, after stripping the membranes. Stripping procedure consisted of washing membranes twice with a mild stripping buffer (containing 15 g glycine, 1 g SDS, 10 mL Tween20, pH 2.2, final volume of 1, 0 L) for 30 min and twice with PBS for 10 min at room temperature, before incubating with antibodies.

2.6. Statistical analysis

Data from behavioral testing, i.e. latencies to step-down, and data from western blot experiments are expressed as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using SPSS software version 16.0. The comparisons were performed by means of a two-way analysis of variance (2-way ANOVA), using surgery (sham or ovariectomy) and pharmacological treatment (vehicle or CBD) as fixed factors. When significant interactions occurred, we have performed simple effect analyses. In 2-way ANOVA comparisons, *p* values of less than 0.05 were considered to indicate statistical significance, while for simple main effect analyses, *p* values of less than 0.025 were considered to indicate statistical significance.

3. Results

Our first goal was to determine if CBD treatment for 14 days after ovariectomy, would be able to ameliorate estrogen depletion-induced aversive memory impairments. Results from inhibitory avoidance task, a form of aversively-motivated learning are shown in Fig. 1. Statistical comparisons of latencies to step down in the retention test using two-way ANOVA indicated a significant main effect of treatment ($F_{(1, 49)} = 6.21$, $p = 0.016$) and a significant interaction ($F_{(1, 49)} = 4.37$, $p = 0.042$). Simple effect analyses indicated that OVX rats treated with CBD had a higher latency to step-down than OVX rats that received vehicle ($F_{(1, 49)} = 11.38$, $p = 0.001$), suggesting that ovariectomy induced memory deficits that were reversed by CBD (Fig. 1). When comparing retention test latencies of Sham groups, no significant simple effect of treatment was found ($p = 0.785$). No significant main effects of surgery ($F_{(1, 49)} = 0.99$, $p = 0.325$), treatment ($F_{(1, 49)} = 0.062$, $p = 0.805$) nor interactions ($F_{(1, 49)} = 0.053$, $p = 0.819$) were found when comparing training latencies.

To better understand the possible molecular mechanisms that could mediate the effects of CBD on ovariectomy-induced memory deficits, we decided to investigate components of the PI3K/Akt/GSK3β signaling pathway and the antiapoptotic potein Bcl2 in the hippocampus.

Two-way ANOVA comparisons of the pAKT/AKT ratio in the hippocampus indicated a significant main effect of OVX, which reduced pAKT/AKT ratio ($F_{(1, 11)} = 8.37$, $p = 0.015$; Fig. 2), and a significant interaction of OVX and CBD treatment ($F_{(1, 11)} = 6.50$, $p = 0.027$; Fig. 2). Simple effect analyses of CBD treatment on pAKT/AKT ratio fell short of significance ($F_{(1, 11)} = 4.41$, $p = 0.060$; Fig. 2) in ovariectomized rats, while in the Sham group no significant simple effect of CBD was observed ($p = 0.166$). Those results suggest that CBD tends to increase pAKT/AKT ratio found in ovariectomized rats.

Subsequently, we analyzed the pGS3Kβ/GS3Kβ ratio in the hippocampus (Fig. 3). Two-way ANOVA comparison indicated a significant main effect of CBD treatment ($F_{(1, 14)} = 4.77$, $p = 0.046$) and a significant interaction OVX-CBD ($F_{(1, 14)} = 4.66$, $p = 0.049$; Fig. 3). Further analyses for simple effects indicated that CBD was able to increase the pGS3Kβ/GS3Kβ ratio in the ovariectomized group in comparison to ovariectomized rats treated with vehicle ($F_{(1, 14)} = 9.43$, $p = 0.008$; Fig. 3), while no simple effects were observed in Sham groups ($p = 0.985$), suggesting that ovariecomy reduced the pGS3Kβ/GS3Kβ ratio and CBD treatment abolished this effect.

We also examined the effects of ovariectomy and CBD treatment on

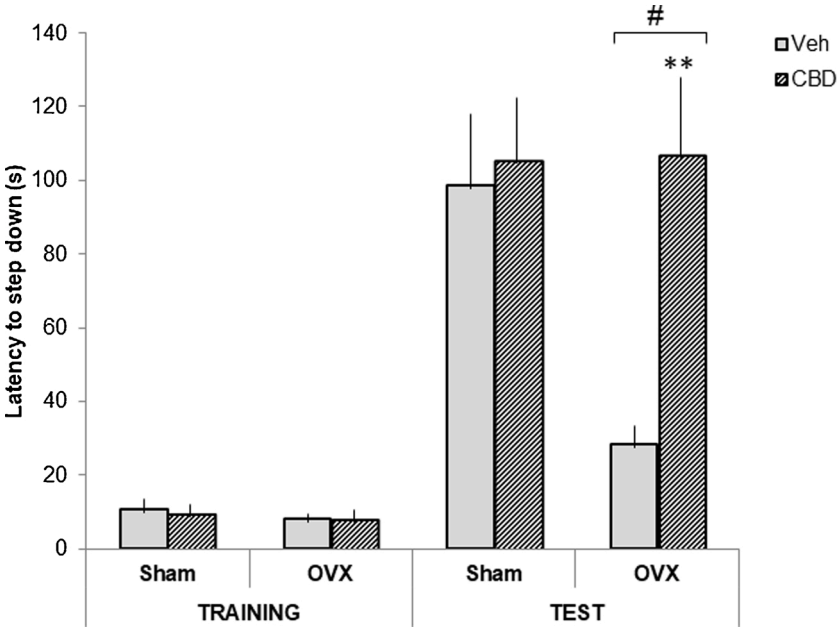

Fig. 1. Effects of cannabidiol in ovariectomized rats on inhibitory avoidance memory.
Female rats were submitted to sham surgery (sham) or ovariectomy (OVX) at 3-months of age. After three weeks recovering from surgeries, the animals received daily an i.p. injection of cannabidiol (CBD, 10 mg/kg) or vehicle (veh) for 14 days. On the 13th day of treatments, animals were trained in inhibitory avoidance (IA) and tested 24 h later. Statistical analysis was performed using two-way ANOVA, followed by simple effect analyses, when significant interactions occurred. Data expressed as mean ± S.E.M. (Sham-Veh N = 10; Sham-CBD N = 15; OVX-Veh N = 14; OVX-CBD N = 14). Significant interaction is indicated as # $p < 0.05$; significant simple effect of CBD treatment is indicated as ** $p < 0.01$.

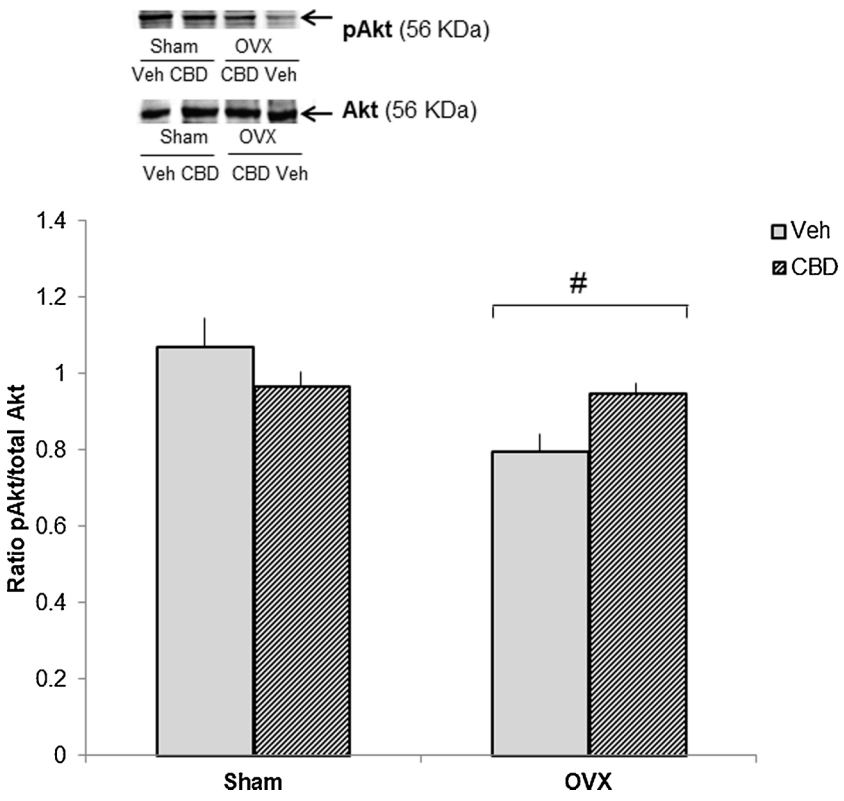

Fig. 2. Effects of cannabidiol in ovariectomized rats on pAKT/AKT ratio.
Western Blot of pAKT and total AKT in the hippocampus of adult female rats treated with vehicle (Veh) or cannabidiol (CBD) for 14 days. Thirty μg of protein were separated on SDS-PAGE and probed with specific antibodies. Representative Western Blots for pAKT and total AKT in the hippocampus are shown at the top panel. Statistical analysis was performed using two-way ANOVA, followed by simple effect analyses, when significant interactions occurred. Data expressed as mean ± S.E.M. pAKT/AKT ratio (Sham-Veh N = 4; Sham-CBD N = 4; OVX-Veh N = 3; OVX-CBD N = 4). Significant interaction OVX-CBD is indicated as # $p < 0.05$.

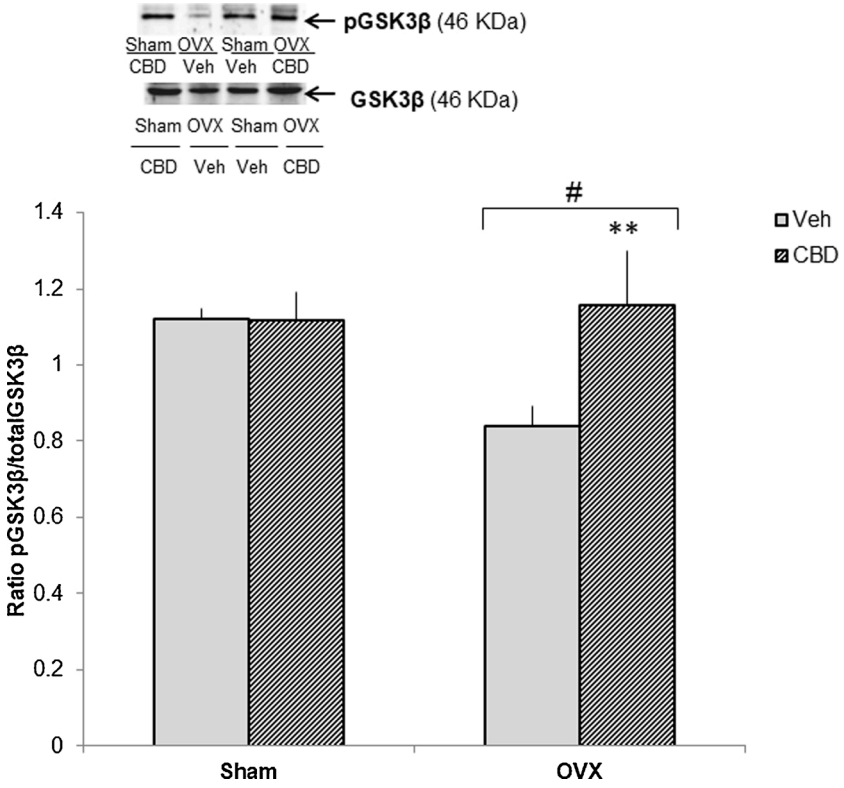

Fig. 3. Effects of cannabidiol in ovariectomized rats on pGSK3β/ pGSK3β ratio.
Western Blot of pGSK3β and total GSK3β in the hippocampus of adult female rats treated with vehicle (Veh) or cannabidiol (CBD) for 14 days. Thirty μg of protein were separated on SDS-PAGE and probed with specific antibodies. Representative Western Blots for pGSK3β and total GSK3β in the hippocampus are shown at the top panel. Statistical analysis was performed using two-way ANOVA, followed by simple effect analyses, when significant interactions occurred. Data expressed as mean ± S.E.M. pGSK3β/total GSK3β ratio (Sham-Veh N = 5; Sham-CBD N = 4; OVX-Veh N = 5; OVX-CBD N = 4). Significant interaction OVX-CBD is indicated as # $p < 0.05$; significant simple effect of CBD treatment is indicated as ** $p < 0.01$.

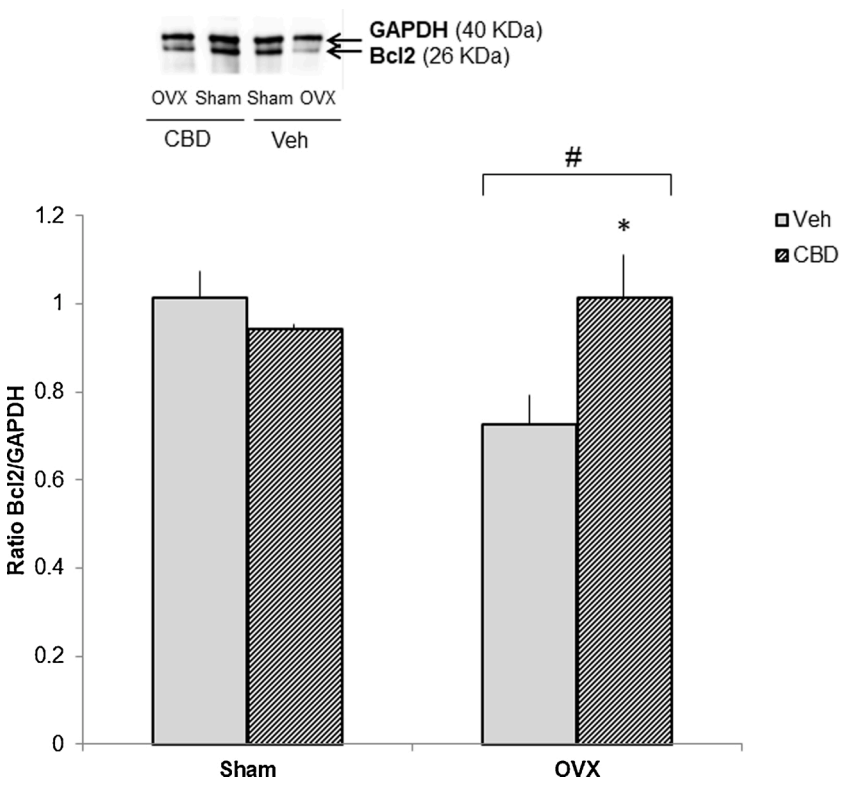

Fig. 4. Effects of cannabidiol in ovariectomized rats on Bcl2 levels.
Western Blot of Bcl2 in the hippocampus of adult female rats treated with vehicle (Veh) or cannabidiol (CBD) for 14 days. Forty μg of protein were separated on SDS-PAGE and probed with specific antibodies. Representative Western Blots for Bcl2 in the hippocampus are shown at the top panel. Statistical analysis was performed using two-way ANOVA, followed by simple effect analyses, when significant interactions occurred. Data expressed as mean ± S.E.M. Bcl2/GAPDH ratio (Sham-Veh N = 4; Sham-CBD N = 3; OVX-Veh N = 3; OVX-CBD N = 3). Significant interaction OVX-CBD is indicated as # $p < 0.05$; significant simple effect is indicated as * $p < 0.05$.

the antiapoptotic protein Bcl2. Although western blot results for Bcl2 followed the same pattern found for pAKT/AKT and pGS3Kβ/GS3K β ratios, no significant main effects of surgery ($F_{(1, 9)} = 2.05$, $p = 0.186$) or CBD treatment ($F_{(1, 9)} = 2.08$, $p = 0.183$) were found. Noteworthy, a significant interaction OVX-CBD was revealed ($F_{(1, 9)} = 5.58$, $p = 0.042$, Fig. 4). Simple effect analyses indicated that CBD increased Bcl2 levels in ovariectomized rats when compared to rats that received vehicle ($F_{(1, 9)} = 6.79$, $p = 0.029$, Fig. 4). In contrast, no simple effect was observed in Sham groups ($p = 0.518$), suggesting that ovariectomy reduced the antiapoptotic protein Bcl2 and CBD was able to restore Bcl2 levels in ovariectomized rats.

4. Discussion

Over the last decades, the investigation of estrogen depletion-related memory decline has received attention, particularly considering the controversial findings on memory decline in postmenopausal women and the observation that AD incidence increases after menopause in women compared to aged-matched men [37]. Using an established animal model, we have shown that estrogen depletion impairs emotionally-regulated memory in adult rats, corroborating previous results from our research group [30] and from others [38,39]. Previous studies also demonstrated that ovariectomized rats exhibit recognition memory deterioration in the object recognition task and its spatial version, object location task [30,40]. Memory deficits observed in ovariectomized adult rats are associated with reduced pyramidal neuron dendritic spine density in hippocampal CA1 and the medial prefrontal cortex [15]. The treatment of ovariectomized rats with CBD for fourteen days helped rescue memory impairments observed in the present study. Although the present results indicated that CBD has not affected training performance or memory retention in control sham-operated rats, we have not specifically addressed other possible factors that could be related to CBD's effects, such as reduction of anxiety and accompanying effects of stress-induced pain sensitivity, or motivation. Relatively few studies have examined the effects of CBD in memory-impaired rats. For instance, we have previously demonstrated that CBD recovered aversive and recognition memory deficits induced by iron overload [32]. CBD also ameliorated memory deficits induced by sepsis [41] and by pneumococcal meningitis [42] in male rats and improved memory, reducing hippocampal levels of inflammatory markers in middle-aged diabetic rats submitted to chronic cerebral hypoperfusion [43]. Recently, it was shown that CBD reversed object recognition deficits and delayed spatial learning in *APPxPS1* transgenic female mice [44]. To our knowledge, this is the first study that investigates the potential of CBD in attenuating estrogen depletion-related memory disruption.

Ovariectomy results in reduced levels of gonadal hormones, not only estrogens, but also progesterone. Although we cannot rule out the possibility that the effects found in the present study are related to estrogens and progesterone decline, previous evidence give support for a major role played by estrogen in functional deterioration related to menopause or surgical removal of ovaries. Moreover, the role of progesterone in memory and cognition, and possibly neuroprotection, is controversial. Singh and Su [45] reviewed the neuroprotection associated with progesterone and they summarized disease models where the treatment with progesterone seemed to be beneficial, although there was a great variation in the models used in different studies (from animal models to in vitro techniques). Briefly, progestogens have induced beneficial effects in animal/cellular models for traumatic brain injury, Alzheimer's disease, and ischemic stroke. For example, progesterone reduced oxidative injury resulting from glutamate and glucose deprivation induced-toxicity [46]. Nevertheless, some studies have found no effects or even deleterious effects of progestogens in the CNS. For instance, concomitant progesterone administration blocked the positive effects of estrogen administration on spatial memory, moreover, ovariectomized rats submitted to regimens of treatment with medroxyprogesterone performed poorer on spatial working memory tasks [47,48]. Thus, most of the studies using ovariectomy model of menopause focuses in investigating the beneficial effects of estrogen. It is well documented that estrogen plays a role in synaptic plasticity and synaptogenesis [49,50] and wield neuroprotective actions. Decreased estrogen levels resulting from ovariectomy reduce neural proliferation in the hippocampus [51] and may increase the risk of neuronal vulnerability against injury.

Mitochondrial dysfunction and neuroinflammation have also been related to estrogen levels decline [52].

Estrogen-mediated neuroprotection may involve different molecular mechanisms, including the activation of the PI3K/Akt/GSK3β survival pathway. Phosphorylated Akt (pAkt) is the active form of this kinase, which, in turn, phosphorylates a multitude of substrates, regulating cell survival, apoptosis, and energy metabolism. pAkt-mediated GSK3β phosphorylation inhibits GSK3β, protecting against neuroinflammation and apoptosis [53]. The present findings revealed that ovariectomy decreased the pAkt/Akt ratio, leading to a reduced pGSK3β/GSKβ ratio. A recent study has also found decreased Akt signaling that resulted in memory deficits and impaired autophagy in ovariectomized rats [54], while Spencer-Segal and coworkers [55] reported that exogenous estrogen administration was able to enhance Akt phosphorylation in the hippocampus of mice, effect not observed in estrogen receptor (ER) alpha and beta knockout mice.

A study evaluating pAkt levels in the hipppocampus of young and aged female rats found that pAkt-labeled synapses were significantly lessened in aged compared to young rats, and the treatment with estrogen significantly increased pAkt in pre- and post-synaptic sites [56]. Consistent with the present results, hippocampal GSKβ phosphorylation was also reduced in ovariectomized rats exhibiting spatial memory deficits [57]. Estradiol treatment rescued perinatal asphyxia-induced inactivation of PI3K/Akt/GSK3β pathway and recovered Bcl2 expression in male adult rats [16].

To further confirm estrogen depletion-induced inactivation of the survival signaling pathway in our study, we analyzed the anti-apoptotic protein Bcl2. Previous studies reported decreased Bcl2 levels in consequence of ovariectomy in several brain regions of young adult rats [58–60]. The present results showed a significant interaction between OVX and CBD treatment, indicating that CBD rescues OVX-induced alterations in Bcl2 levels.

Here we found that chronic CBD was able to functionally recover emotional memory in rats subjected to ovariectomy and reverse the effects of estrogen depletion on the Akt/GSk3β pathway. Although in the training day the animals were injected six hours after the training session, we cannot completely rule out the possibility that CBD could interfere with memory consolidation. For instance, Rossignoli and coworkers [61] found that CBD, injected in the prefrontal cortex five hours after fear conditioning, impaired fear-motivated memory when the animals were tested five days later.

Ovariectomy decreased the Akt/GSK3β survival pathway and CBD treatment returned pAkt/Akt and pGSK3β/GSk3β ratios and Bcl2 levels to values comparable to the control group (sham-vehicle). Inefficient activation of Akt/GSK-3β signaling may be relevant to neurodegeneration and correcting PI3K/Akt/GSK-3β signal deregulation in the CNS may be a potential therapeutic approach. In agreement with the present findings, it has been demonstrated that overexpression of the activated form of Akt inhibits neuronal death, caspase-3-like activation, and reverses decreased Bcl2 levels induced by NO in hippocampal neurons [62]. Activated Akt also phosphorylates, thereby inhibiting, proapoptotic proteins such as Bax and Bad, and regulating the binding of the Bcl-2/Bax complex [63]. Thus, it is possible that by enhancing Akt/GSK3β signaling pathway may inhibit apoptosis and contribute for neuroprotective effects of CBD. Some recent investigations have pursued to better understand the mechanisms underlying CBD's neuroprotective and prosurvival effects by acting on the PI3K/Akt/GSK3β pathway. For instance, experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis, induced downregulation of the PI3K/Akt/mTOR pathway, while CBD treatment activated PI3K/Akt/mTOR pathway by increasing the phosphorylation of PI3K, Akt, and mTOR [64]. CBD treatment also upregulated PI3K/Akt signaling and Bcl2 via cannabinoid receptor 1 and attenuated the neural apoptosis induced by hemorrhagic shock in rats [65]. In addition, it has been demonstrated that CBD facilitates endocannabinoid neural transmission through the inhibition of fatty acid amide hydrolase (FAAH), an enzyme responsible for the hydrolysis of the endogenous cannabinoid anandamide [66]. FAAH is also modulated by estrogen, and estrogen-induced reduction of FAAH activity has been implicated in its neuroprotective effects described in a focal brain ischemia rat model [67]. It has been shown that a cannabinoid receptor agonist, as well as a FAAH inhibitor, improve memory, while blocking PI3k/Akt abrogate this effect in rats submitted to chronic cerebral hypoperfusion [68]. Thus, further studies investigating the involvement of the endocannabinoid system in CBD-induced neuroprotection in an ovariectomy model are warranted.

In conclusion, the present results confirm that ovariectomy downregulates the Akt/GSK3β survival pathway, and support the use of CBD to oppose these effects. Akt/GSK3β pathway's activation, resulting in protection against apoptosis, may be one of the key mechanisms underlying the neuroprotective and memory-ameliorating effects of CBD. Considering that the menopausal hormone replacement therapy is controversial due to its association with several adverse outcomes, including increased risks of coronary heart disease, stroke, venous thromboembolism, and breast cancer, new onset asthma and development of erythematous systemic lupus, the search for therapeutic alternatives that counteract the effects of estrogen decline for menopausal women is crucial [69]. These observations, associated with a favorable safety and tolerability profile of this commercially available drug, added to existing evidence of neuroprotective, antioxidant, and anti-inflammatory properties of CBD, support our results immediately be translated to patient applications. Therefore, our findings can stimulate further controlled randomized clinical trials.

Authors' contributions

Márcio da Silveira Corrêa: Conceptualization and design of the work, Data acquisition and analysis.

Betânia Souza de Freitas: Data acquisition and analysis, Resources administration.

Gustavo Dalto Barroso Machado: Conceptualization and design of the work, drafting the work.

Vivian Naziaseno Pires: Data acquisition.

Elke Bromberg: Conceptualization and design of the work, review and editing the final version of the manuscript.

Jaime E. C. Hallak: Conceptualization and design of the work, Review and editing the final version of the manuscript, Funding acquisition.

Antônio Waldo Zuardi: Review and editing the final version of the manuscript.

José Alexandre S. Crippa: Conceptualization and design of the work, Review and editing the final version of the manuscript, Funding acquisition.

Nadja Schröder: Conceptualization and design of the work, Writing - Original Draft and Review and Editing the final version of the manuscript, Supervision, Project administration, Funding acquisition.

All authors read and approved the final manuscript.

Availability of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval

All experimental procedures were approved by the Institutional Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS/SIPESQ #7922).

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Declaration of Competing Interest

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All the other authors have no conflict of interest to disclose.

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