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Long-Term Cannabidiol Treatment Prevents the Development of Social Recognition Memory Deficits in Alzheimer's Disease Transgenic Mice

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Abstract. Impairments in cognitive ability and widespread pathophysiological changes caused by neurotoxicity, neuroinflammation, oxidative damage, and altered cholesterol homeostasis are associated with Alzheimer's disease (AD). Cannabidiol (CBD) has been shown to reverse cognitive deficits of AD transgenic mice and to exert neuroprotective, anti-oxidative, and anti-inflammatory properties *in vitro* and *in vivo*. Here we evaluate the preventative properties of long-term CBD treatment in male $A\beta PP_{Swe}/PS1\Delta E9$ (AβPP × PS1) mice, a transgenic model of AD. Control and AD transgenic mice were treated orally from 2.5 months of age with CBD (20 mg/kg) daily for 8 months. Mice were then assessed in the social preference test, elevated plus maze, and fear conditioning paradigms, before cortical and hippocampal tissues were analyzed for amyloid load, oxidative damage, cholesterol, phytosterols, and inflammation. We found that AβPP × PS1 mice developed a social recognition deficit, which was prevented by CBD treatment. CBD had no impact on anxiety or associative learning. The prevention of the social recognition deficit was not associated with any changes in amyloid load or oxidative damage. However, the study revealed a subtle impact of CBD on neuroinflammation, cholesterol, and dietary phytosterol retention, which deserves further investigation. This study is the first to demonstrate CBD's ability to prevent the development of a social recognition deficit in AD transgenic mice. Our findings provide the first evidence that CBD may have potential as a preventative treatment for AD with a particular relevance for symptoms of social withdrawal and facial recognition.

Keywords: Alzheimer's disease, amyloid load, behavior, cannabidiol, cholesterol, neuroinflammation, oxidative stress, phytosterol, social recognition memory, transgenic $A\beta PP_{Swe}/PS1\Delta E9$ mice

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease, which is associated with progressive memory loss. Other behavioral and cognitive symptoms include social withdrawal, poor facial recognition ability, increased motor agitation, and likelihood of wandering [1, 2]. AD is characterized by two main postmortem pathological hallmarks; amyloid-β (Aβ) protein aggregation forming plaque deposits and tau protein hyperphosphorylation resulting in neurofibrillary tangles. Microglia, the resident immune cells

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of the central nervous system, are activated for the phagocytosis of Aβ [3–5], but impaired clearance or reuptake of Aβ results in the release of inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and chemokines, that cause neuroinflammation. Brain tissue damage is further exacerbated by the release of glutamate and reactive oxygen and nitrogen species, resulting in neurotoxicity and oxidative damage, respectively [6]. Increased oxidative stress may be an early indication of AD risk [7, 8]. Disturbances in brain cholesterol metabolism are associated with the major pathological features of AD (including Aβ and tau pathology). In particular, decreased cholesterol synthesis correlates with the severity of neurodegeneration and dementia [9, 10], while late-stage AD patients also show decreased cholesterol circulation [11, 12]. Interestingly, dietary phytosterols (or plant sterols) found naturally in many foods (such as vegetable oils, nuts, grains, and grain-derived products) [13] can either interfere with critical functional processes in AD or decrease amyloidogenic processing [14]. Some phytosterols may even be relevant additional biomarkers for AD [15].

Current treatments available to AD patients do not slow the progression of the disease and only offer limited benefits for the cognitive abilities of patients (reviewed in [16]). Thus, it is important to explore novel alternative treatment strategies. The phytocannbinoid cannabidiol (CBD) may be a potential new candidate for AD therapy (for review, see [17]). CBD is derived from the *cannabis sativa* plant and is devoid of psychoactive properties. It has neuroprotective, anti-inflammatory, and anti-oxidative properties [18–22], thereby countering a number of AD-relevant pathological symptoms. *In vitro* studies have found that CBD prevents Aβ-induced tau protein hyperphosphorylation [23], neurotoxicity [23, 24], attenuates cell death, and promotes neurogenesis in mouse hippocampal cells [25, 26]. These biological functions of CBD promise therapeutic value for the neurodegenerative and neurotoxic components of AD. Indeed, *in vivo* studies reported that CBD reduced Aβ-induced neuroinflammation in rats and mice [25, 27] and rescued learning deficits in the Morris water maze in a pharmacological mouse model of AD [28]. The memory restoring properties of CBD were linked to a reduction in microglial activation and pro-inflammatory cytokines (i.e., decreased IL-6) [28].

Current research suggests existing interventions may be administered too late in the disease process when the damage caused by AD pathology is already too severe [17, 29, 30]. Thus, in the current study, we evaluated for the very first time the effectiveness of long-term oral CBD treatment to prevent the development of cognitive deficits and AD-relevant brain pathophysiology in an established transgenic mouse model of familial AD [31]. The double transgenic $A\beta PP_{Swe}/PS1\Delta E9$ (AβPP × PS1) mouse model co-expresses mutant amyloid-β protein precursor (AβPP) and presenilin 1 (PS1) genes [31–34]. Amyloid plaques are found as early as at 4 months of age in these AD transgenic mice [35]. Our past research established that male AβPP × PS1 mice demonstrate social recognition deficits, increased anxiety, and task-specific hyperlocomotion whereas sensorimotor gating and spatial memory were intact at 10–12 months of age [36]. Importantly, we also demonstrated recently that 3-weeks of CBD treatment effectively reversed the social and object recognition memory deficits of AβPP × PS1 males [37]. In the present study male AβPP × PS1 mice were treated with CBD (20 mg/kg) or vehicle using a daily voluntary oral administration scheme for 8 months beginning at 2.5 months of age when AD-like pathophysiology is still sparse (i.e., no Aβ burden reported for 4 months old AβPP × PS1 mice: [35]). Following this, mice were assessed in social recognition memory, associative memory (i.e., fear conditioning), and anxiety, before brain samples were analyzed for amyloid load, oxidative damage (i.e., markers of cerebral lipid oxidation), cholesterol levels as well as dietary phytosterols, and neuroinflammation markers. We selected the cytokines TNF-α and IL-1β as both have been most strongly implicated in the promotion of AD pathology in humans and in AD transgenic mouse models [38, 39]. Furthermore, inflammation driven by these cytokines is attenuated by CBD [27, 28].

METHODS

Animals

Double transgenic mice expressing chimeric mouse/human AβPP (Mo/HuAβPP695swe/Swedish mutations K595N/M596L) and mutant human PS1 (PS1/ΔE9) mice were obtained from Jackson Laboratory [Bar Harbor, USA; strain name: B6C3-Tg($A\beta PP_{Swe}/PS1\Delta E9$)85Dbo/Mmjax; stock no. 004462] and maintained as hemizygotes on the congenic C57BL/6JxC3H/HeJ background as described previously [31–33, 40]. Male double transgenic mice (AβPP × PS1) and their non-transgenic littermates (WT) were bred and group-housed in independently ventilated cages (Airlaw, Smithfield, Australia) at

Animal BioResources (Moss Vale, Australia). Test mice were transported to Neuroscience Research Australia (NeuRA) at around 10 weeks of age, where they were group-housed in Polysulfone cages (1144B: Techniplast, Rydalmere, Australia) with corn cob bedding (PuraCob Premium: Able Scientific, Perth, Australia) and some tissues for nesting. Mice were kept under a 12 : 12 h light:dark schedule [light phase: white light (illumination: 210lx); lights on 0700–1900 h]. Environmental temperature was automatically regulated at 21 ± 1°C and relative humidity was 40–60%. Food (Gordon's Rat and Mouse Maintenance Pellets: Gordon's Specialty Stockfeeds, Yanderra, Australia) and water were provided *ad libitum*, except where specified. Adult, male A/J mice from Animal Resources Centre (Canning Vale, Australia) were placed in the animal enclosures as standard opponents for the social preference test. Research and animal care procedures were approved by the University of New South Wales Animal Care and Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Drug treatment

Powdered cannabidiol (CAS: 13956-29-1, THC Pharm GmbH, Frankfurt/Main, Germany) was used at a dose of 20 mg/kg body weight, based on previous studies evaluating the behavioral properties of different doses of CBD [41] and the effectiveness of 20 mg/kg CBD to reverse spatial memory deficits of a pharmacological mouse model of AD [42]. Although chronic administration of CBD appears to be well tolerated by transgenic mice [37, 43], the stress of chronic injections may impact behavioral, cognitive, and/or physiological results. Thus, the gel pellet preparation and the oral administration regime were adapted from Zhang and colleagues [44, 45]: CBD or vehicle were dissolved in a highly palatable sweetened and chocolate flavored gel pellet, and administered at a volume of 8 ml/kg body weight. Due to the insolubility of CBD in water, CBD was first dissolved in 100% ethanol and an equal amount of Tween 80 (Sigma-Aldrich Co., St Louis, USA), then vortexed vigorously. CBD was dissolved in gel pellets with a final composition of 2.0% ethanol, 2.0% Tween 80, 15.2% Splenda (Splenda Low Calorie Sweetener: Johnson & Johnson Pacific Pty, Broadway, Australia), 8.7% gelatine (Davis Gelatine: GELITA Australia Pty, Josephville, Australia), 20.1% chocolate flavoring (Queen Flavouring Essence Imitation Chocolate: Queen Fine Foods Pty, Alderley, Australia), and 52.0% water for irrigation. Vehicle gel pellets were identical but contained no CBD. Mice were initially habituated to vehicle gel pellets in their home cages for seven days. Following this, the mice were isolated within their home cages for the treatment by placing a plastic divider in the home cage. Then animals were given either a vehicle or a CBD gel pellet (treatments were quasi-randomized), which they consumed within 2–5 min. The plastic divider was removed once mice had consumed the gel pellets. Mice were treated daily, late in the afternoon, to avoid potential acute effects of CBD confounding test outcomes (Table 1).

Behavioral phenotyping

Starting at 10 months of age, mice were tested in a number of behavioral tests (Table 1), with an inter-test interval of at least 48 h as described earlier ($n = 8$–14 mice per genotype/treatment) [36, 46, 47]. All tests were conducted during the first 5 h of the light phase to minimize effects of circadian rhythm.

Social preference test (SPT)

The SPT was used to assess sociability and social recognition memory [48] and performed as described

Table 1
Age of $A\beta PP_{Swe}/PS1\Delta E9$ (AβPP × PS1) mice and their WT counterparts (in days ± SEM) at the start of treatment, throughout behavioral testing and at the end of treatment

Treatment	Vehicle		CBD	
Genotype	WT	AβPP × PS1	WT	AβPP × PS1
Age at start of treatment	91.5 ± 11.5	97.1 ± 18.3	89.0 ± 8.2	95.9 ± 12.5
Number of days treated prior to start of testing	228.1 ± 38.5	234.8 ± 31.3	226.1 ± 33.0	237.8 ± 35.5
Social Preference Test	319.6 ± 34.5	331.9 ± 41.5	315.1 ± 30.8	333.8 ± 38.6
Elevated plus maze	324.1 ± 34.8	336.0 ± 41.7	319.3 ± 31.3	338.1 ± 38.9
Y-Maze	326.9 ± 34.5	338.4 ± 41.5	321.8 ± 31.2	340.6 ± 38.6
Fear conditioning	329.9 ± 34.5	341.4 ± 41.5	324.8 ± 31.2	343.6 ± 38.6
Tissue collection	333.1 ± 34.8	345.0 ± 41.7	328.3 ± 31.3	347.1 ± 38.9
Total Days of treatment	241.6 ± 38.9	247.9 ± 31.6	239.3 ± 33.4	251.2 ± 35.8

previously [36, 37]. Test animals were isolated for an hour prior to the start of testing. During the habituation trial, mice were allowed to freely explore a three-chamber apparatus, consisting of a center chamber (length: 9 cm; width: 18 cm; depth: 20 cm) and two outer chambers (16 cm × 18 cm × 20 cm), freely for 5 min. For the sociability test, an unfamiliar standard opponent (male A/J mouse) was placed in one of two animal enclosures (i.e., opponent chamber) in a quasi-randomized fashion (mouse enclosures allowed nose contact between mice but prevented fighting). The test mouse was returned to the apparatus and allowed to explore all three chambers and the animal enclosures for 10 min. Finally, test animals were observed in a 10 min social recognition test. For this, a second, unfamiliar standard opponent was placed in the previously empty chamber so that the test mouse had the choice to explore either the familiar mouse (from the previous trial) or the novel, unfamiliar mouse. The inter-trial interval (ITI) was 5 min. The chambers and enclosures were cleaned with 30% ethanol in-between trials and fresh corn cob bedding was added to the chambers prior to each test trial. AnyMaze^{TM} (Stoelting, Wood Dale, USA) tracking software was used to determine the time spent in the different chambers, number of entries and distance travelled by the test mice in each trial. Two mice (1 WT-VEH and 1 WT-CBD) were excluded from the sociability test due to recording issues.

Elevated plus maze (EPM)

The EPM assesses the natural conflict between the tendency of mice to explore a novel environment and avoidance of a brightly lit, elevated, and open area [49, 50] and was employed to determine potential effects of chronic CBD treatment on anxiety behavior. The '+' apparatus consisted of two alternate open arms (35 cm × 6 cm; without side walls) and two alternate enclosed arms (35 cm × 6 cm; height of enclosing walls 28 cm) connected by a central platform (6 cm × 6 cm), elevated 70 cm above the floor. Mice were placed at the center of the '+' of the grey PVC plus maze (for further details of apparatus, see [51]) facing an enclosed arm and were allowed to explore the maze for 5 min. The time spent on open arms, the percentage of entries onto open arms over total arm entries (open arm entries) and the distance travelled on the open and enclosed arms were recorded using AnyMaze^{TM} tracking software. One mouse was excluded (AβPP × PS1-CBD group) for falling off the apparatus.

Fear conditioning (FC)

FC assesses hippocampus- and amygdala-dependent associative learning whereby a previously neutral stimulus elicits a fear response after it has been paired with an aversive stimulus. On conditioning day, mice were placed into the test chamber (Model H10-11R-TC, Coulbourn Instruments, USA) for 2 min. An 80 dB conditioned stimulus (CS) was presented twice for 30 s with a co-terminating 0.4 mA 2-s foot shock (unconditioned stimulus; US) with an inter-pairing interval of 2 min. The test concluded 2 min later. The next day (context test), mice were returned to the apparatus for 7 min. On day 3 (cue test), animals were placed in an altered context for 9 min. After 2 min (pre-CS/baseline), the CS was presented continuously for 5 min. The test concluded after another 2 min, absent the CS (for more details, see [52, 53]). Time spent *freezing* was measured on all three experimental days using Any-Maze^{TM} software.

Biochemical analyses

Mice were anaesthetized and blood was collected through cardiac puncture. Blood samples were centrifuged (5000 rpm, 5 min, 4°C) in a microcentrifuge (Model No. 5415R, Eppendorf, Hamburg, Germany), and the plasma fraction was collected and stored at −80°C. Euthanized mice were perfused with phosphate buffered saline (PBS) transcardially as described previously [54]. Brains were sagittally divided and the right hemisphere was snap frozen in liquid nitrogen before being stored at −80°C. Cortex and hippocampal samples were dissected and weighed on dry ice prior to biochemical analyses [Sample numbers for ELISA and GC-MS were: $n = 8$ for WT-vehicle, $n = 10$ for AβPP × PS1-vehicle, $n = 10$ for WT-CBD, and $n = 10$ for AβPP × PS1-CBD].

Aβ enzyme-linked immunosorbent assay (ELISA) for Aβ pathology

Frozen cortex (20–30 mg) and hippocampal samples (∼5 mg) were homogenized and prepared as TBS soluble, and guanidine HCl (gHCl) soluble (TBS insoluble) fractions and stored at −80°C as described previously [54]. Both TBS-soluble and gHCl-soluble fractions were used in enzyme-linked immunosorbent assay (ELISA) to investigate the effect of CBD on Aβ levels in transgenic mice. Protein was quantified using the bicinchoninic (BCA assay) method.

$A\beta_{40}$ and $A\beta_{42}$ protein in TBS-soluble and gHCl-soluble fractions of brain homogenates were quantified using Beta Amyloid x-40 and x-42 ELISA kits (Cat

No. SIG-38954 and SIG-38956 respectively, Covance, Emeryville, USA) as described previously [54, 55].

Gas chromatography-mass spectroscopy (GC-MS) for cholesterol, oxidative damage, and CBD plasma levels

An Agilent 7000B triple quadrupole mass selective detector interfaced with an Agilent 7890A GC system gas chromatograph, equipped with an automatic sampler and computer workstation (Agilent Technologies, Santa Clara, USA) was used to analyze markers of oxidative damage in the cortical samples and CBD presence in plasma samples. GC-MS triple quadrupole provided very high analytical sensitivity for all analytes measured. Limits of detection (LOD: 0.05 ng/ml) were significantly less (at least 10-fold) than the levels of each analyte measured in plasma and brain. 150 µl of plasma were used for the analysis of CBD. The concentration obtained from the GC-MS was therefore multiplied by a factor of 6.67 to give the total amount of CBD per ml of plasma as shown in the Results section. The injection port and GC-MS interface were kept at 270°C and separations were carried out on a fused silica capillary column (20 m × 0.18 mm i.d. × 0.18 m film thickness, Restek Rxi-5 ms). Helium was the carrier gas with a flow rate of 0.8 ml/min (average velocity = 59 cm/s).

F_2-isoprostanes, oxidised sterols (oxysterols), and cholesterol

Frozen cortex samples (10–20 mg) were homogenized and hydrolyzed overnight for GC-MS analysis as described previously [56]. Samples were loaded into solid phase extraction columns (UCT CUQAX223 3 ml; United Chemical Technologies, Bristol, USA). Sterols and oxysterols, arachidonic acid, DHA, and F_2-isoprostanes were eluted from the SPE column separately. The sterol/oxysterol fractions were derivatized in 20 µl acetonitrile and 20 µl Selectra-SIL BSTFA [N,O-bis(trimethylsilyl)trifluoroacetamide] containing 1% TMCS (trimethylchlorosilane; United Chemical Technologies, Bristol, USA) prior to GC-MS analysis. Quantification of cholesterol oxidation products (COP) was as previously described [57]. Cholesterol was quantified using lathosterol-d_6 heavy isotope standard in a separate (0.6 µl split ratio 25 : 1). Relative molar response factors of all analytes were calculated from calibration curves constructed from different concentrations in triplicate. The F_2-isoprostane and fatty acid fractions were prepared and analyzed by GC-MS as described previously [56]. Quantification of F_2-isoprostanes and fatty acids were calculated by comparison of specific SRM transitions with their corresponding heavy isotope internal standards.

Quantification of CBD in plasma

Concentration of cannabidiol in plasma was quantified using the GC-MS as previously described with slight modifications [58, 59]. Plasma samples (150 µl) were treated using sodium acetate buffer pH 4.0, with MTBE and Hexane (1 : 1 v/v), rotated for 30 min and centrifuged at 1500 rpm for 2 min at 4°C, dried down, derivatized in 20 µl BSTFA and 20 µl 1% TMCS, and incubated at 70°C for 30 min. Derivatized samples were dried down, reconstituted in 40 µl of toluene and analyzed using the GC-MS (1 µl splitless). MRM was performed using EI mode similar to sterol analysis. Column temperature was held for 1 min and increased 40°C/min to 210°C, then 20°C/min to 300°C and held for 4 min. Quantification of CBD was calculated by comparison with specific MRM transitions corresponding with its heavy isotope internal standard (CBD-d3, Lipomed, Arlesheim, Switzerland).

Inflammatory markers (quantitative polymerase chain reaction)

RNA extraction: Frozen cortex samples (10–20 mg) were homogenized in Tri-reagent (TRIzol Reagent, cat no. 15596-018, Life Technologies, Mulgrave, VIC, Australia) as described previously [57]. RNA levels were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Scoresby, Australia), and diluted in RNAse-free water to obtain a concentration of 0.5 µg/µl. cDNA was synthesized using a Tetro cDNA Synthesis kit (Bioline, Alexandria, Australia), according to manufacturer instructions. The SensiFAST SYBR No-ROX kit (Bioline, Alexandria, Australia) was used to determine levels of inflammatory markers. Template concentration was 100 ng (1 : 10 dilution; 1µl cDNA). Forward and reverse primers for interleukin-1β (IL-1β; forward, 5′-CAACCAACAAGTGATATTCTCCATG-3; reverse, 5′-GATCCACACTCTCCAGCTGCA-3′), and tumor necrosis factor-α (TNF-α; forward, 5′-CATCTTCTC-AAAATTCGAGTGACAA-3′; reverse: 5′-TGGGAG-TAGACAAGGTACAACCC-3′) were used as biomarkers for the quantification of inflammation in transgenic mice, with β-actin as a housekeeping gene. Polymerase chain reaction (PCR) assays were reacted (3-step cycling; IL-1β: 45 cycles; TNF-α: 50 cycles) and analyzed using Roche LightCycler 480 (Roche Diagnostics, Castle Hill, Australia). Three mice were excluded (1×WT-CBD, 1×AβPP × PS1-vehicle and

1 × AβPP × PS1-CBD) as outliers (2 standard deviations away from mean).

Statistical analyses

One-way ANOVA was used to analyze effect of 'treatment' on Aβ levels in AβPP × PS1 mice. Two-way analysis of variance (ANOVA) was used to analyze behavioral parameters and biochemical data obtained for oxidative damage, CBD levels and quantification of inflammation for main effects of 'genotype' and 'treatment' in all tests. Repeated measures (RM) ANOVA was used to evaluate the effects of 'chamber' (SPT) and '1 min block' (FC) as published previously [36, 47]. Performance in the SPT was also assessed using one sample *t*-tests to clarify whether the percentage of time spent in the opponent/novel chamber was greater than chance (50%). Differences were regarded as significant if $p < 0.05$. Data are shown as means ± standard error of means (SEM). F-values and degrees of freedom are presented for ANOVAs and significant 'genotype' and 'treatment' effects are shown in figures and tables as '*' ($^{*}p < 0.05$, $^{**}p < 0.01$) and '#' ($^{\#}p < 0.05$) respectively whereas RM-ANOVA results for social novelty preference are presented by '+' ($^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$). Analyses were conducted using SPSS 20.0 for Windows.

RESULTS

Behavior

Sociability and social recognition

RM ANOVA revealed an effect of 'chamber' [$F(1,39) = 197.9$, $p < 0.001$] (Fig. 1A). All mice spent more time investigating the social opponent over the empty chamber, indicating intact sociability for all mice regardless of genotype and treatment. *T*-tests for percentage of time spent with the novel mouse confirmed that all mice demonstrated significant levels of sociability above chance [WT-VEH: $t(6) = 7.4$, $p < 0.001$; AβPP × PS1-VEH: $t(13) = 6.2$, $p < 0.001$; WT-CBD: $t(8) = 12.3$, $p < 0.001$; AβPP × PS1-CBD: $t(12) = 8.6$, $p < 0.001$] (data not shown).

In the SPT, RM ANOVA revealed a significant effect of 'chamber' for time spent investigating the novel over the familiar mouse [$F(1,41) = 23.6$, $p < 0.001$]. Importantly, a significant interaction between 'genotype' and 'treatment' was found [$F(1,41) = 4.8$, $p < 0.05$], where only vehicle-treated AD transgenic mice did not develop a preference for the novel mouse (Fig. 1B). Two-way ANOVA also revealed a trend toward an effect of CBD treatment [$F(1,41) = 3.1$, $p = 0.09$]. Indeed, ANOVA split by 'genotype' revealed that CBD increased the time AD transgenic mice spent with the novel mouse [$F(1,25) = 5.0$, $p < 0.05$], with no such effect observed in WT mice [$F(1,16) = 0.2$, $p = 0.7$] (Fig. 1B) showing that CBD had a beneficial effect on social recognition memory. *T*-tests confirmed that all animals, except vehicle-treated AβPP × PS1 mice, spent a significantly greater percentage of time with the novel mouse than the familiar mouse [WT-VEH: $t(7) = 2.5$, $p < 0.05$; AβPP × PS1-VEH: $t(13) = 0.3$, $p = 0.8$; WT-CBD: $t(9) = 3.3$, $p < 0.01$; AβPP × PS1-CBD: $t(12) = 3.7$, $p < 0.01$] (data not shown).

Anxiety

AβPP × PS1 transgenic mice demonstrated WT-like locomotion and anxiety ($p > 0.05$ for total distance travelled, time spent on open arms and open arm entries). Chronic treatment with CBD had no effect on EPM behaviors (all $p > 0.05$; Table 2).

Associative learning

All mice responded to the electric foot shocks during conditioning (i.e., vocalization detected in all mice). Two-way ANOVA found transgenic mice demonstrated increased amounts of *freezing* at baseline (i.e., first 2 min pre-conditioning) regardless of treatment [$F(1,41) = 4.5$, $p < 0.05$]. However, freezing duration during the first 2 min of the context and cue trials was similar for all mice across test conditions (all $p > 0.05$; Table 3) and all mice exhibited intact context memory regardless of treatment [$p > 0.05$; Fig. 2A]. Furthermore, memory of the cue was intact as all animals showed increased *freezing* post cue presentation [RM ANOVA: $F(1,41) = 52.9$, $p < 0.001$], regardless of 'genotype' or 'treatment' (Fig. 2B and Table 3).

Brain pathophysiology

Amyloid load

One-way ANOVA revealed that CBD had no effect on soluble and insoluble $A\beta_{40}$ or $A\beta_{42}$ in the cortex of AβPP × PS1 mice, although insoluble $A\beta_{42}$ was slightly higher after CBD treatment ['treatment': Soluble $A\beta_{40}$: $F(1,18) = 0.3$, $p = 0.6$; Insoluble $A\beta_{40}$: $F(1,18) = 2.4$, $p = 0.1$; Soluble $A\beta_{42}$: $F(1,18) = 0.1$, $p = 0.7$; Insoluble $A\beta_{42}$: $F(1,18) = 3.5$, $p = 0.08$] (Table 4). Similarly, Aβ levels remained unchanged after CBD treatment in the hippocampus ['treatment': Soluble $A\beta_{40}$: $F(1,17) = 0.4$, $p = 0.6$; Insoluble $A\beta_{40}$: $F(1,18) = 1.1$, $p = 0.3$; Soluble $A\beta_{42}$:

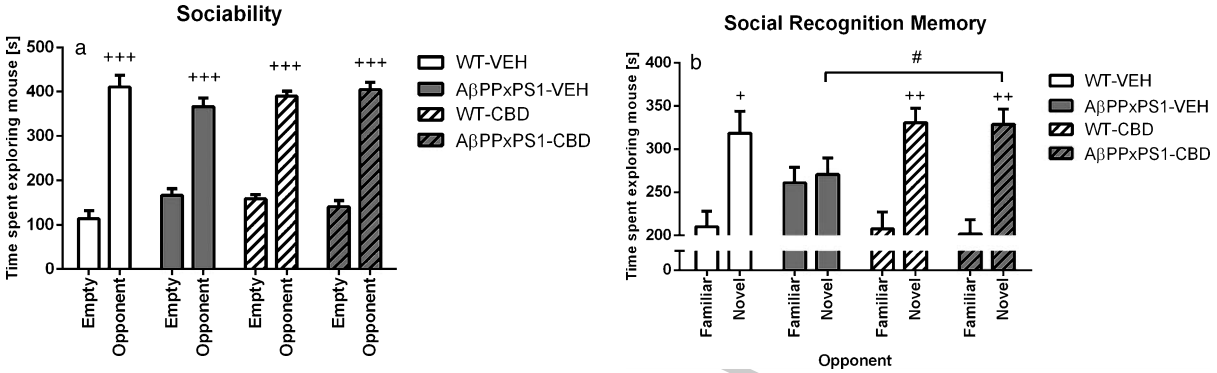

Fig. 1. Sociability and social recognition were measured using the social preference test. Graphs show total time spent [s] in test chambers by the test mice containing A) either an unfamiliar mouse (i.e., opponent) or an empty mouse enclosure (i.e., empty); or B) either a familiar or an unfamiliar (i.e., novel) mouse. Data for non-transgenic wild type-like control (WT) and double transgenic *AβPP$_{Swe}$/PS1ΔE9* (AβPP × PS1) male mice after vehicle or cannabidiol (CBD) treatment are shown as means + SEM. Significant 'treatment' effects are indicated with '#' (#p<0.01). RM ANOVA for novelty preference are presented by '$^{+}$' ($^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$).

Table 2
Anxiety-related behaviors (i.e., time spent on and entries onto open arms) and locomotion (total distance travelled) in the elevated plus maze (EPM). Parameters for wild type-like control mice (WT) and double transgenic *AβPP$_{Swe}$/PS1ΔE9* (AβPP × PS1) mice after vehicle or cannabidiol (CBD) treatment are shown as mean ± SEM

	Vehicle		CBD	
	WT	AβPP × PS1	WT	AβPP × PS1
Time spent on open arms [s]	7.2 ± 2.7	6.0 ± 2.3	6.7 ± 1.6	7.3 ± 2.7
Entries onto open arms [%]	13.3 ± 4.0	7.8 ± 3.1	11.6 ± 2.1	11.2 ± 2.7
Total distance travelled [m]	7.1 ± 0.9	8.0 ± 1.4	7.2 ± 0.9	7.9 ± 1.0

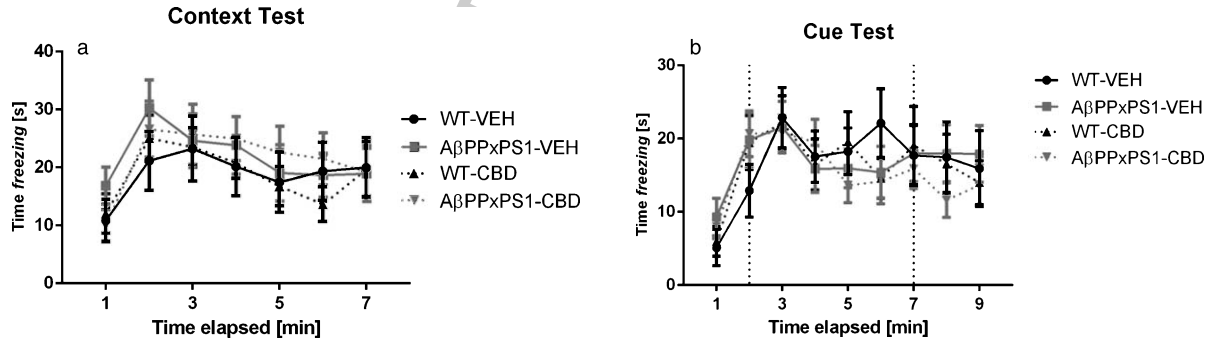

Fig. 2. Fear-associated learning was assessed in the fear conditioning test. Time spent *freezing* during A) the context test and B) the cue test for each is shown per '1 min bin'. Data for non-transgenic control wild type-like (WT) and double transgenic *AβPP$_{Swe}$/PS1ΔE9* (AβPP × PS1) mice after vehicle or cannabidiol (CBD) treatment are shown as means ± SEM.

F(1,15) = 0.3, p = 0.6; Insoluble Aβ$_{42}$: F(1,18) = 0.1, p = 0.7] (Table 4).

Oxidative damage

Total F$_2$-isoprostanes (free and esterified corrected for arachidonic acid; AA) were not significantly altered in AβPP × PS1 mice when compared to their WT littermates, regardless of 'treatment' (all $p>0.05$) (Table 5). We also measured the levels of oxysterols in the cortex. For enzymatically oxidized sterols, AβPP × PS1 mice demonstrated significantly decreased overall levels of 24-hydroxycholesterol compared to WT littermates ['genotype': F(1,34) = 4.9, $p<0.05$], whereas 'treatment' had no effect on sterols [F(1,34) = 0.07, p = 0.8] and no 'genotype' by 'treatment' interactions were found. No differences were found across all four groups for 27-hydroxycholesterol, and the reactive species oxidized sterols, 7β-hydroxycholesterol and 7-ketocholesterol (all $p>0.05$) (Table 5).

Table 3
Fear-associated memory in the fear conditioning paradigm. Freezing (i.e., time spent freezing [s]) at baseline, and during context test and cue test for non-transgenic wild type-like control (WT) and double transgenic *AβPP$_{Swe}$/PS1ΔE9* (AβPP × PS1) male mice after vehicle or cannabidiol (CBD) treatment are presented as mean ± SEM

	Vehicle		CBD	
	WT	AβPP × PS1	WT	AβPP × PS1
Baseline (first 2 min)				
Conditioning *freezing* [s]	2.3 ± 1.1	5.8 ± 1.7	4.7 ± 1.5	8.5 ± 1.9
Context *freezing* [s]	32.0 ± 7.8	47.1 ± 7.4	37.0 ± 6.0	36.7 ± 7.0
Context				
Total time spent *freezing* [s]	132.2 ± 29.6	152.3 ± 28.1	131.6 ± 15.4	150.7 ± 25.8
Cue				
Time spent *freezing* 2 min prior to cue onset [s]	18.0 ± 5.9	29.1 ± 5.7	25.5 ± 5.2	28.6 ± 4.4
Time spent *freezing* 2 min post cue onset [s]	40.4 ± 7.3^{+++}	37.4 ± 5.8^{+}	38.7 ± 6.7^{+}	40.4 ± 5.2^{+}

Significant effects of cue presentation on *freezing* response are indicated by '+' ($^{+}p < 0.05$ and $^{+++}p < 0.001$)

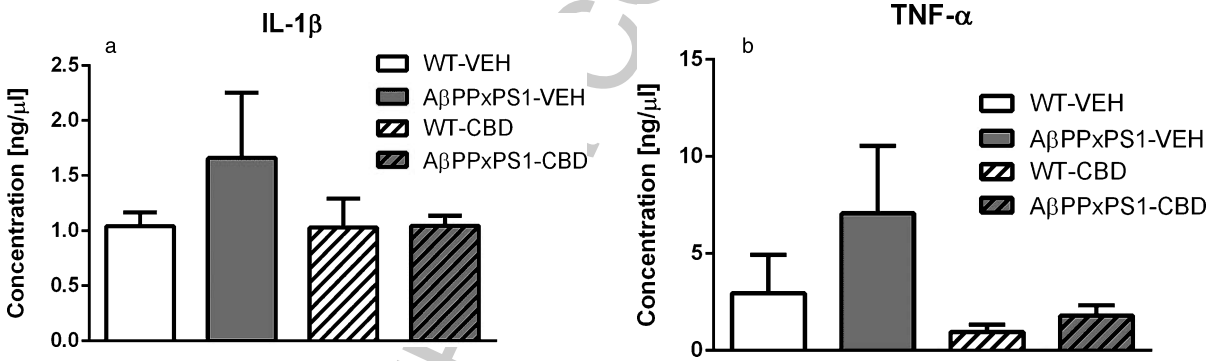

Fig. 3. Neuroinflammation markers in cortical tissue. Quantitative PCR was used to measure the concentration of A) interleukin-1β and B) TNF-α derived from the cortex of control (WT) and double transgenic *AβPP$_{Swe}$/PS1ΔE9* (AβPP × PS1) male mice after vehicle or cannabidiol (CBD) treatment. Concentrations [ng/μl] are presented as mean ± SEM.

Table 4
Amyloid-β Soluble and insoluble amyloid load in double transgenic *AβPP$_{Swe}$/PS1ΔE9* (AβPP × PS1) male mice after vehicle or cannabidiol (CBD) treatment are shown as means ± SEM

AβPP × PS1	Vehicle	CBD
Cortex		
Soluble $Aβ_{40}$ [pg/mg]	1033.2 ± 211.2	904.6 ± 118.8
Soluble $Aβ_{42}$ [pg/mg]	654.3 ± 102.6	613.4 ± 65.8
Insoluble $Aβ_{40}$ [pg/mg]	8184.6 ± 701.0	9758.3 ± 751.6
Insoluble $Aβ_{42}$ [pg/mg]	25601.4 ± 2138.6	30897.8 ± 1847.6
Hippocampus		
Soluble $Aβ_{40}$ [pg/mg]	464.4 ± 99.3	390.8 ± 69.6
Soluble $Aβ_{42}$ [pg/mg]	155.7 ± 196.8	196.8 ± 54.1
Insoluble $Aβ_{40}$ [pg/mg]	9854.5 ± 2217.7	12776.1 ± 1738.7
Insoluble $Aβ_{42}$ [pg/mg]	22295.1 ± 7937.3	26370.0 ± 8467.1

Cholesterol

Cholesterol was increased in cortical tissues of AβPP × PS1 mice compared to WT animals [$F(1,34) = 12.1$, $p < 0.01$] and CBD increased cholesterol levels [$F(1,34) = 11.0$, $p = 0.01$]. Further one-way ANOVA revealed that cholesterol was significantly higher in vehicle-treated AD transgenic mice [$F(1,16) = 7.7$, $p < 0.05$] compared to control mice, while CBD increased the cholesterol levels in WT mice [$F(1,16) = 25.1$, $p < 0.001$] but not AβPP × PS1 mice [$F(1,18) = 1.3$, $p = 0.3$] (Table 5).

Two-way ANOVA revealed a significant 'genotype' by 'treatment' interaction for the cortical levels of the dietary phytosterol, brassicasterol [$F(1,34) = 6.1$, $p < 0.05$], which was caused by CBD increasing brassicasterol levels in AβPP × PS1 mice only [WT: $F(1,16) = 0.5$, $p = 0.5$ AβPP × PS1: $F(1,18) = 6.9$, $p < 0.05$; Table 5]. Furthermore, the analysis detected a 'genotype' effect in CBD-treated mice [vehicle: $F(1,16) = 0.2$, $p = 0.7$, CBD: $F(1,18) = 9.9$, $p < 0.01$; Table 5]. The dietary phytosterol, campesterol, was also increased in AβPP × PS1 mice [$F(1,34) = 4.4$, $p < 0.05$]. More specifically, cortical campesterol was elevated in CBD-treated AβPP × PS1 mice [vehicle: $F(1,16) = 0.2$, $p = 0.6$, CBD: $F(1,18) = 9.0$, $p < 0.01$].

Table 5
Oxysterols, F_2-isoprostanes, cholesterol, and phytosterol levels. Oxidative damage and total cholesterol in the cortex of non-transgenic wild type-like control (WT) and double transgenic *AβPP$_{Swe}$/PS1ΔE9* (AβPP × PS1) male mice after vehicle or cannabidiol (CBD) treatment. Concentrations (in pg) are presented as mean ± SEM

	Vehicle		CBD	
	WT	AβPP × PS1	WT	AβPP × PS1
Oxidized sterols				
Reactive species oxidized				
7β-hydroxycholesterol [ng/mg]	0.29 ± 0.02	0.31 ± 0.05	0.30 ± 0.02	0.32 ± 0.02
7-ketocholesterol [ng/mg]	0.53 ± 0.04	0.50 ± 0.04	0.47 ± 0.03	0.57 ± 0.05
Enzymatically oxidized				
24-hydroxycholesterol [ng/mg]	38.5 ± 2.8	34.2 ± 3.0	39.4 ± 2.7	31.9 ± 1.8*
27-hydroxycholesterol [pg/mg]	48.3 ± 5.3	38.9 ± 5.7	46.1 ± 2.5	43.4 ± 4.6
F_2-isoprostanes (normalized for arachidonic acid)				
Total [pg/ng]	10.0 ± 1.0	8.1 ± 0.5	9.1 ± 0.4	8.3 ± 0.5
Cholesterol				
Total cholesterol [ng/mg]	20.5 ± 1.0	29.6 ± 2.8 *	29.3 ± 1.4$^{\#\#\#}$	33.1 ± 1.4
Dietary phytosterol				
Brassicasterol [pg/mg]	51.2 ± 3.8	48.3 ± 6.1	46.7 ± 4.9	70.4 ± 5.8$^{**\#}$
Campesterol [ng/mg]	15.1 ± 1.8	16.5 ± 2.3	15.0 ± 1.7	21.2 ± 1.1*

Significant effects of 'genotype' are indicated by '*' ($^{*}p < 0.05$ and $^{**}p < 0.01$) and effects of 'treatment' by '#' ($^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$)

There was also a trend for CBD to increase the cortical levels of this phytosterol in transgenic mice [WT: $F(1,16) = 0.0$, $p = 1.0$, AβPP × PS1: $F(1,18) = 3.4$, $p = 0.08$] (Table 5).

Inflammatory markers

Two-way ANOVA revealed no significant differences in the levels of mRNA for two inflammatory cytokine markers across test conditions ['genotype': IL-1β: $F(1,39) = 1.0$, $p = 0.3$ – 'TNF-α': $F(1,39) = 1.1$, $p = 0.3$] (Fig. 3A-B). There was no significant effect of 'treatment' on these cytokines [IL-1β: $F(1,39) = 1.3$, $p = 0.3$ – TNF-α: $F(1,39) = 2.5$, $p = 0.1$] either, although cytokine levels of CBD-treated AβPP × PS1 mice appeared closer to corresponding WT levels than levels of vehicle-treated AD transgenic mice (Fig. 3A-B).

CBD plasma levels

Two-way ANOVA revealed that all mice treated with CBD demonstrated significantly increased levels of plasma CBD (ng/ml) [WT-CBD: 2.1 ± 0.6; AβPP × PS1-CBD: 1.9 ± 0.4 – 'treatment': $F(1,30) = 21.3$, $p < 0.001$]. No significant 'genotype' differences or interactions were found (all $p > 0.05$). CBD could not be detected in mice that were treated with vehicle (values < 0.05 ng/ml).

DISCUSSION

Our study demonstrates for the first time the effects of long-term oral CBD treatment on the social recognition memory and pathophysiology of a double transgenic AβPP × PS1 mouse model for AD. We provide first evidence of a possible impact of CBD on dietary phytosterols, which can exert beneficial effects on cognition. We also suggest that the therapeutic effect of CBD may be linked to neuroinflammatory processes or changes in cholesterol but further research using additional CBD doses will be necessary to clarify this.

The SPT determined that vehicle-treated AβPP × PS1 mice exhibit a social recognition memory deficit, confirming our earlier findings [36, 37]. Importantly, long-term CBD treatment prevented this social recognition deficit from occurring in AβPP × PS1 mice. We previously found that intraperitoneal administration of CBD for three weeks reversed this cognitive deficit in the same AD mouse model [37]. Other recent studies also report social recognition deficits in AD transgenic mouse models, providing evidence for the increasing relevance of social recognition memory testing for AD research [60, 61]. Anxiety can confound the performance of mice in cognitive tests [62] and acute CBD has been found to modify anxiety-related behaviors [41, 63–67]. However, the AβPP × PS1 transgene did not influence anxiety parameters nor did CBD treatment.

The beneficial effect of CBD on social recognition memory was not associated with a direct effect on Aβ levels. Insoluble and soluble levels of $Aβ_{40}$ and $Aβ_{42}$ were no different between vehicle and CBD-treated AβPP × PS1 mice in cortex and hippocampus. Similarly, another study described improvements in spatial memory in *AβPP$_{Swe}$/PS1ΔE9* mice on a C57BL/6J background, which was not accompanied by changes

in Aβ levels [68]. The same study also found that levels of insoluble $A\beta_{40}$ and $A\beta_{42}$ in the parietal cortex did not correlate with cognitive deficits [68]. Nonetheless, various *in vitro* studies show CBD can attenuate Aβ-induced processes [23–26], reverse Aβ-induced memory impairments in rodents [28] and reduce Aβ formation [69].

The *in vivo* formation of isoprostanes is a marker for cerebral lipid oxidation and directly correlated with an increase in oxidative stress [7, 70–72]. Patients with AD are also known to have increased concentrations of F_2-isoprostanes in CSF even prior to disease diagnosis [7, 8, 71]. Levels of oxidation were not significantly altered in AβPP × PS1 mice in comparison to their age-matched WT littermates, nor did we detect changes in the level of lipid oxidation in the cortex of CBD-treated animals, despite its known antioxidant properties [73, 74]. These findings may be due to age as nucleic acid oxidation is significantly higher in 3 and 5 months old AβPP × PS1 mice compared to age-matched control mice. Importantly, this phenomenon is not evident in 10 and 15 month old mice, which is the age when brain tissue was collected for the current study [75].

Cholesterol was increased in AβPP × PS1 mice compared to WT mice, while CBD treatment increased cholesterol levels in WT mice. Our finding of increased cholesterol in AβPP × PS1 mice could indicate either an impaired reuptake process, or a compensatory mechanism for protection against neurodegeneration in AD mice. Maintenance of sufficient cholesterol is important to help combat synapse loss and neurodegeneration [76] and such a response is consistent with the reduced levels of 24-OH cholesterol detected in the AβPP × PS1 mice compared to WT. Formation of 24-OH cholesterol is the major pathway of cholesterol removal from the brain [77]. Insufficient amounts of cholesterol may interrupt essential processes such as myelin formation, synaptic transmission and cognitive ability in mice [78, 79], while a reduction in oxysterols has been shown to correlate with the severity of dementia and brain atrophy [9, 10, 80]. Interestingly, 8-month old $A\beta PP_{Swe}/PS1\Delta E9$ mice on a pure C57BL/6J background did not demonstrate significantly different levels of cholesterol, while 15-month old transgenic mice had significantly lower cholesterol levels compared to control mice [81]. It is noteworthy that decreased levels of cholesterol in cerebral spinal fluid and plasma have been found in patients with AD [11, 12].

Phytosterols are present naturally in a variety of different foods, including grains (e.g., sorghum and bran) found in mouse food pellets. CBD increased the levels of brassicasterol and campesterol in AβPP × PS1 mice. The accumulation and long-term consumption of dietary phytosterols do not interfere with memory [82, 83]. On the other hand, dietary supplementation of a fish oil-rich diet with phytosterols reduced insoluble $A\beta_{42}$ in $A\beta PP_{Swe}/PS1\Delta E9$ mice on a C57BL/6J background [68], while the phytosterol stigmasterol attenuated scopolamine-induced spatial memory deficits of mice [84]. These findings suggest a potentially beneficial effect of increased phytosterol levels for cognitive symptoms in the AD brain. It is possible that CBD interacted with AD pathophysiology by increasing the retention of specific phytosterols. Further research needs to be conducted in order to understand the effect of increased phytosterol levels in AD brains and how CBD might be involved in these processes.

Daily long-term administration of 20 mg/kg CBD did not result in a statistically significant effect. However, AβPP × PS1 mice have previously been shown to exhibit elevated levels of neuroinflammation (increased nitric oxide species and TNF-α) in the hippocampus [85] and inflammatory changes have been linked to impaired spatial memory of AβPP × PS1 mice [86]. Furthermore, Martin-Moreno and colleagues have demonstrated that Aβ-induced neuroinflammation was decreased after CBD treatment [28]. Thus, we suggest that CBD might be able to combat increased inflammation in AβPP × PS1 mice thereby impacting on the cognitive performance of these mice. Future research should consider additional CBD doses to determine the effects of long-term CBD treatment on neuroinflammation in AD mouse models. Furthermore, an escalating CBD dosage regime could be used as the dosage of AD-approved treatments is often increased over time as the condition of patients deteriorates [87].

In conclusion, our study is the first to demonstrate that long-term CBD treatment can prevent the development of a social recognition deficit in AβPP × PS1 mice. The findings suggest the mechanism involved in this prevention may be linked to CBD-induced retention of dietary phytosterols or neuroinflammatory processes in the brain of AD mice. We provide the first evidence that CBD has potential to be used as a long-term preventative treatment option for AD and may be especially relevant for symptoms of social withdrawal and facial recognition. The behavioral inertness of CBD and the fact that CBD is well tolerated in humans [88, 89] suggests that preclinical research findings could easily be followed up in clinical trials.

Future studies using cytokine arrays or an 'omics' approach may reveal which biochemical/genetic pathways contribute to the beneficial effects of CBD. It will also be important to clarify what receptors mediate the therapeutic-like effects of CBD: the peroxisome proliferator-activated receptor-γ [25, 69], N-methyl-D-aspartate receptors [90, 91], and receptors of the endocannabinoid system [92] are promising targets.

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