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Title: Effect of cannabidiol on muscarinic neurotransmission in the pre-frontal cortex and hippocampus of the poly I:C rat model of schizophrenia

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ABSTRACT

Cognitive impairment is a core symptom of schizophrenia; however, current antipsychotic drugs have limited efficacy to treat these symptoms and can cause serious side-effects, highlighting a need for novel therapeutics. Cannabidiol (CBD) is a non-intoxicating phytocannabinoid that has demonstrated pro-cognitive effects in multiple disease states, including a maternal immune activation (poly I:C) model of schizophrenia, but the mechanisms underlying the efficacy of CBD require investigation. Muscarinic neurotransmission is highly implicated in the cognitive impairments of schizophrenia; however, the effect of CBD on this system is unknown. We examined alterations in markers of muscarinic neurotransmission in the pre-frontal cortex (PFC) and hippocampus (HPC) following CBD treatment. Pregnant Sprague-Dawley rats (n = 16) were administered poly I:C (4 mg/kg) or saline. Adult offspring were treated (3-weeks) with CBD (10 mg/kg) or vehicle. Receptor autoradiography (using \[^{3}H\]pirenzepine) was used to examine changes in muscarinic M1/M4 receptor (M1/M4R) binding density. Levels of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) protein expression were examined using Western blot. M1/M4R binding density was downregulated in the PFC and CA1/CA2 and CA3 subregions in male poly I:C offspring. M1/M4R deficits were normalised after CBD treatment. ChAT protein expression was reduced in the HPC of male poly I:C offspring, while CBD treated poly I:C offspring exhibited control-like ChAT levels. AChE levels were unaltered in any of the groups. There were also no changes in muscarinic signalling in female offspring. These findings demonstrate that CBD can normalise muscarinic neurotransmission imbalances in male poly I:C offspring in regions of the brain implicated in cognition.

Keywords: cannabidiol; muscarinic; acetylcholine; maternal immune activation; schizophrenia; cognition.
1. INTRODUCTION

Cognitive impairment is experienced by 75-85% of patients with schizophrenia (Barch and Ceaser, 2012) and has been recognised as a core symptom domain from which the other symptoms may arise [1]. It often precedes the onset of psychotic symptoms, is evident in first episode psychosis [2] and can be a predictor of functional outcomes [3]. Current antipsychotic drugs (APDs) are limited in their ability to treat cognitive symptoms in schizophrenia and recent data suggests that high lifetime APD dosing potentiates cognitive deficits [4]. APDs can also cause negative side effects such as obesity and type 2 diabetes mellitus [5]. Therefore, there is a need to improve APD therapy to target cognitive dysfunction while avoiding adverse side effects.

Cannabidiol (CBD) is a major phytocannabinoid of Cannabis sativa L., produced as a metabolic by-product of the plant through decarboxylation of cannabidiolic acid [6]. It is psychoactive, i.e. alters brain function to affect behaviour [7], but there is currently no evidence that it causes the deleterious hallucinogenic, paranoia and anxiety-inducing effects that can occur with misuse of the delta-tetrahydrocannabinol (Δ-THC) type chemicals [8]. CBD has pro-cognitive effects in multiple disease states of cognitive impairment [see 9, 10 for review]. In addition, clinical studies have reported improved positive and negative symptoms in people with schizophrenia following CBD administration [11], with similar therapeutic efficacy to the APD amisulpride, a high tolerability and low negative side effects [12]. A limited number of clinical studies examined cognition in schizophrenia using CBD as an adjunct therapy to existing APDs showed only modest, if any, improvement in cognitive performance in patients with chronic stable schizophrenia [11, 13]. The effect of chronic CBD treatment alone on cognition in schizophrenia patients requires examination. We recently reported that chronic (3-weeks) CBD enhanced cognition and sociability in a maternal immune activation (MIA) rat model of schizophrenia [7]. In this model,
administration of polyinosinic-polycytidilic acid (poly I:C, a synthetic double-stranded RNA viral mimic) to pregnant rats during mid-late gestation increases pro-inflammatory cytokines that cross the placenta causing inflammation of the foetal brain and altering the neurodevelopmental trajectory of the offspring [14]. Poly I:C offspring display numerous behavioural, neurochemical and structural brain abnormalities observed in schizophrenia patients [14-16]. In particular, we found that male poly I:C offspring had deficits in recognition and working memory (assessed using the Novel Object Recognition (NOR) and T Maze tests, respectively), as well as reduced social interaction during the Social Interaction test, while CBD treated these deficits [7]. In female offspring, the poly I:C group exhibited deficits in recognition memory and social interaction that were treated using CBD, while female poly IC offspring did not exhibit impaired working memory [17]. However, the mechanisms by which CBD treats cognitive deficits in the poly I:C model are unknown.

The cholinergic system is composed of two major families of receptors: acetylcholine nicotinic and muscarinic receptors (mAChRs). Mice deficient in the muscarinic M1 receptor (M1R) subtype exhibit impairments in working memory and remote reference memory [18]. In addition, significant reductions in the expression of M1R and muscarinic M4 receptors (M4R) have been reported in post-mortem brains of schizophrenia patients in regions related to cognitive function, including the hippocampus and frontal cortex [reviewed in 19]. It has also been demonstrated that the expression of M1R protein, but not other mAChR subtypes, is decreased in the dorsolateral prefrontal cortex of people with schizophrenia [20]. Altogether, these findings suggest that alterations in the M1R may play a role in the cognitive dysfunction of schizophrenia. In cholinergic neurons, two main enzymes are essential to acetylcholine (ACh) neurotransmission: choline acetyltransferase (ChAT) for ACh synthesis in the pre-synaptic neuron, and acetylcholinesterase (AChE), which is located in the post-synaptic neuron and degrades ACh in the synaptic cleft following its release [21]. In previous
studies, decreased activity of ChAT has been correlated with poor cognitive functioning in schizophrenia patients [22], while AChE inhibitors have been shown to slow the cognitive decline in Alzheimer’s disease [23], demonstrating that ChAT and AChE activity impact cognitive functioning. Interestingly, acute CBD increases ACh in the basal forebrain within hours of systemic administration [24], unlike cannabinoid CB1 receptor (CB1R) agonists (Δ9-tetrahydrocannabinol, WIN 55,212-2 and CP 55,940 administered systemically) that inhibit ACh release in the rat pre-frontal cortex and hippocampus [25, 26]. Therefore, there is evidence of a link between CBD, the cholinergic muscarinic neurotransmission system, schizophrenia and cognitive function. The aim of this study was to examine the effect of CBD treatment on muscarinic neurotransmission (M1/M4R density, ChAT and AChE protein levels) in regions of the brain implicated in learning, memory and schizophrenia pathology in poly I:C offspring.

2. METHODS

2.1 Ethics Statement

Experimental procedures were approved by the Animal Ethics Committee of the University of Wollongong, Wollongong, NSW, Australia (AE15/05) and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council [27]. All efforts were made to minimise suffering and the number of animals used.

2.2 Animals and Treatment

The methods relating to the animal housing and treatment were previously reported by our laboratory [7] and in accordance with the Animal Research: Reporting of In Vivo
Experiments (ARRIVE) guidelines [28]. Briefly, sixteen pregnant Sprague-Dawley rats (Animal Resource Centre, Perth, WA, Australia) received an intravenous injection of either poly I:C (4mg/kg, Sigma-Aldrich, Sydney, NSW, Australia; \(n = 8\)) or saline solution (control; \(n = 8\)) at gestation day 15. Dose and timing of poly I:C administration was based on previous studies [15]. Male and female offspring received twice-daily intraperitoneal (i.p) injections of CBD (10 mg/kg; THC-Pharm GmbH, Frankfurt, Germany) dissolved in Tween 80 and saline (vehicle; 1:16 (v/v); Sigma-Aldrich), or vehicle alone (injection volume of 5ml/kg) (\(n = 12\) per group). Treatment began on postnatal day 56, which equates to late adolescence/early adulthood in humans [29]. After 2-weeks of treatment, rats were submitted to a series of behavioural tests, including the Novel object recognition (NOR) test, Rewarded T-maze alternation test and the Social interaction test to assess the effects of CBD on recognition memory, working memory and sociability, respectively [7, 17]. The behavioural data were published elsewhere [7, 17]. In order to examine the mechanisms by which CBD treated the cognitive deficits in poly I:C offspring, the brain tissue from our previous studies [7, 17] was analysed in the present study.

2.3 Histological Procedures
After 3 weeks of treatment, animals were euthanized via carbon dioxide asphyxiation followed by rapid decapitation, approximately 10-12 hours after the last drug treatment. Brains were removed, immediately frozen in liquid nitrogen and stored at -80ºC until processing. Rat brains (\(n = 8\) per group) were sectioned coronally into alternating 14 µM or 500 µM thick sections using a cryostat (Jung CM 3000, Leica Instruments GmbH, Nussloch, Germany). Sections included the prefrontal cortex (PFC, ie prelimbic and infralimbic cortices) and hippocampus (HPC, ie CA1/2 and CA3 subregions) (Bregma: 4.2 to 2.56 mm and -4.36 to -6.00mm, respectively) using a standard rat brain atlas [30] (Figure 1). Sections
collected for receptor autoradiography experiments (14 µM) were thaw-mounted on to Polysine\textsuperscript{TM} slides (Sigma-Aldrich, Castle Hill, NSW Australia) and stored at -20°C until further analysis. Sections collected for Western blot experiments (500 µM) were mounted on glass slides, microdissected using a micropuncture kit and stored at -80°C. The dissection methods enabled examination of receptor binding and protein expression from the same rat brain.

2.4 Receptor Autoradiography

2.4.1 M1/M4R binding

The methods used to detect M1/M4R binding density were conducted as previously described by our laboratory [31]. Briefly, slides containing two consecutive sections, per region for each rat, were air-dried and pre-incubated in 22 mM HEPES buffer (pH 7.5) for 15 min at room temperature. To examine total binding, sections were incubated in HEPES buffer containing 10 nM $[^3]$H)pirenzepine (72.8 Ci/mmol; PerkinElmer \textsuperscript{TM} Life and Analytical Sciences, Boston, USA) for 90 min at room temperature. Non-specific binding was determined by incubating an additional two consecutive sections in 10 nM $[^3]$H)pirenzepine in the presence of atropine (10 µM), a competitive antagonist of muscarinic receptors, in buffer (pH 7.5) for 90 min at room temperature. After incubation, sections were washed three times for 4 min in ice-cold HEPES buffer, then dipped in ice cold milliQ water and air-dried overnight.

2.4.2 Quantification

Slides were exposed to Amersham Hyperfilm ECL (GE Healthcare Life Sciences, Parramatta, NSW, Australia) for 7 weeks in X-ray film cassettes with a set of tritium
standards (Amersham, Buckinghamshire, United Kingdom). Autoradiographs were
developed using standard procedures, scanned using a GS-800 Imaging Densitometer (Bio-
Rad, Hercules, California, USA) and analysed with Image J software
(https://imagej.nih.gov/ij). Images were calibrated based on the Rodbard curve obtained from
the tritium standards to produce nCi/mg tissue equivalent (TE) values. Regions of interest
included the PFC and the CA1/2 and CA3 regions of the HPC. Specific binding densities
were calculated by subtracting non-specific binding from total binding values. Values were
converted to fmole $[^3H]$ ligand per mg TE, taking into account the specific activity of the
ligand, as we have described previously [32-34]. Anatomical structures of interest were
confirmed using a standard rat brain atlas [30].

2.5 Western Blot
Microdissected brain tissue samples (PFC, HPC with the CA1/2 and CA3 subregions
combined) were homogenised in homogenising buffer (0.1 M Tris-HCl, 2 mM EDTA, 10%
glycerol, 2% SDS, 0.5 mM PMSF, Protease Inhibitor Cocktail (P8340; Sigma-Aldrich, Castle
Hill, NSW, Australia) and Phosphatase Inhibitor Cocktail 2 (P5726; Sigma-Aldrich, Castle
Hill, NSW, Australia)). Total protein concentration was determined using a DC Protein
Assay kit (Bio-Rad, Gladesville, NSW, Australia). Proteins were loaded in equal amounts (10
ug, within the linear range for each antibody target) into TGX™ 4-20% Stain-free Precast
Gels (Bio-Rad, Australia) to undergo SDS-PAGE electrophoresis at 180V for 1 hr. Stain-free
gels were activated (GelDoc XR+ imaging system; Bio-Rad, Australia) [35, 36], proteins
were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Gladesville,
NSW Australia) at 100V for 1 hr, and imaged to capture total protein in each lane [35, 36].
PVDF membranes were washed in Tris Buffered Saline with Tween 20 (TBST) (3 x 5 min)
and blocked in non-fat milk for 1 hr at room temperature. Membranes were then incubated
overnight in anti-ChAT (1:10000, #ab181023, Abcam, Melbourne, VIC, Australia) or anti-AChE (1:5000, #ab183591, Abcam, Melbourne, VIC, Australia) primary antibodies. Membranes were washed in TBST (5 x 5 min) and incubated in goat anti-rabbit secondary antibody (1:5000, #AB307P, Merck Millipore, Bayswater, VIC, Australia) for 60 min at room temperature. Membranes were washed in TBST (3 x 5 min), incubated in ECL reagent (GE Healthcare, Parramatta, NSW, Australia) and exposed to a Gel Imager (600RB, Amersham, GE Healthcare, Parramatta, NSW, Australia). Band density was quantified using Image Lab software (ver 6, Bio-Rad Laboratories Inc, California, USA). Samples were examined in duplicate. The values for each signal were normalised to total protein in the respective lane to account for loading variability. Values were then normalised to an internal control (pooled sample) on each gel, to account for gel-to-gel variability and allow comparison of samples across gels.

2.6 Statistical Analysis

Data were analysed using SPSS (Version 21.0, IBM, Chicago, Illinois, USA). All data points included in analyses were within ±2SD of the mean. Shapiro-Wilk tests were used to test data for normality. A two-way Analysis of Variance (ANOVA) was used to test for an effect of PRENATAL INFECTION (POLY vs CONT) and OFFSPRING TREATMENT (CBD vs VEH) on receptor binding density and protein expression in the PFC and HPC. Where significant interactions were observed, pairwise comparisons (with Bonferroni’s adjustment) were made between groups. Male and female data were analysed separately due to sexual dimorphism in neuropsychiatric trajectory observed in the model [37] and the endocannabinoid system in rats [38]. Comparisons between vehicle-treated control and poly I:C offspring (CONT+VEH vs. POLY+VEH), vehicle and CBD-treated poly I:C offspring (POLY+VEH vs. POLY+CBD), as well as vehicle and CBD-treated control offspring
(CONT+VEH vs. CONT+CBD) were examined. Where applicable, comparisons between CBD-treated poly I:C offspring and vehicle-treated control offspring (POLY+CBD vs. CONT+VEH) were conducted using independent T tests to examine whether CBD treatment restored control-like levels in poly I:C offspring. Correlations between binding density, protein expression and behavioural parameters were examined using Pearson’s correlation tests for parametric data. Statistical significance was set at $p < 0.05$ and $p$ values between 0.05 and 0.1 were considered statistical trends. Data were presented as mean ± standard error of the mean (SEM).

3. RESULTS

We previously reported that male poly I:C offspring exhibit significantly reduced working memory (decreased percentage of correct entries in the T Maze), recognition memory (decreased Discrimination Ratio in the Novel Object Recognition Test) and social interaction (decreased Total Interaction Time in the Social Interaction test) compared to controls, whereas CBD treated these deficits [7]. We have also previously shown that the female poly I:C offspring had reductions in the Discrimination Ratio and Total Interaction Time that were treated using CBD, but unlike males, working memory performance in the T Maze test was not altered by poly I:C or CBD [17]. In the present study, we examined alterations to markers of cholinergic muscarinic neurotransmission in the PFC and HPC of the brain tissue from those rodents.

3.1 M1/M4R Binding Density in the PFC and HPC of offspring

Examples of $[^3]$H]Pirenzepine binding to M1/M4Rs are presented in Figure 1, with M1/M4R binding observed across the PFC, and CA1/CA2 and CA3 regions of the HPC. There were no significant main effects of PRENATAL INFECTION ($F_{(1, 19)} = 2.91, p = 0.10$) or
OFFSPRING TREATMENT \((F_{(1, 19)} = 1.40, \ p = 0.25)\) on M1/M4R binding density in the PFC of male offspring, but a significant interaction between the factors was observed \((F_{(1, 19)} = 5.34, \ p = 0.03)\). M1/M4R binding density was reduced in male poly I:C offspring compared to controls (-16.3%; POLY+VEH vs. CONT+VEH, \(p = 0.01\)) (Figure 2A); however, CBD treatment restored M1/M4R binding density levels in male poly I:C offspring that were no longer different to controls (POLY+CBD vs CONT+VEH, \(p = 0.18\)) (Figure 2A). In addition, CBD significantly reduced M1/M4R binding density in control offspring (-14.2%, CONT+CBD vs. CONT+VEH, \(p = 0.02\)) (Figure 2A).
Figure 1: Example autoradiographs to demonstrate $[^3]$H]Pirenzepine binding to M1/M4Rs in the offspring brain. Schematic diagrams (left panel) showing the approximate Bregma levels examined in the prefrontal cortex (PFC; 2.52 mm), which included the prelimbic and infralimbic cortices (shaded), and the hippocampus (HPC; -4.92 mm), which included the CA1/2 and CA3 regions. Example autoradiographs show total (row 1 and 3) and non-specific (row 2 and 4) binding in the PFC and HPC, respectively, in vehicle-treated male and female offspring from control dams. Schematic diagram modified from a standard rat atlas (Paxinos and Watson (2007). The Rat Brain in Stereotaxic Coordinates. 6th Ed. Elselvier Academic Press Inc, CA, USA).
Figure 2: The effect of cannabidiol (CBD) or vehicle (VEH) treatment on M1/M4R ([3H]Pirenzepine) binding density in the prefrontal cortex of (A) male and (B) female control (CONT) and poly I:C (POLY) offspring. Data expressed as mean ± SEM. n = 5-7 rats per group. *p<0.05 vs. CONT+VEH. TE = tissue equivalent.

Conversely, there was no significant main effect of PRENATAL INFECTION ($F_{(1, 19)} = 1.17, p = 0.29$) or OFFSPRING TREATMENT ($F_{(1, 19)} = 1.63, p = 0.22$) on M1/M4R binding density in the PFC of female offspring, and no interaction between the factors ($F_{(1, 19)} = 1.57, p = 0.23$) (Figure 2B).
In the HPC, there was a significant PRENATAL INFECTION x OFFSPRING TREATMENT interaction ($F_{(1, 18)} = 8.67, p = 0.01$) on M1/M4R binding density in male offspring. Pairwise comparisons revealed that maternal poly I:C exposure significantly reduced M1/M4R binding density in male offspring compared to controls (-25.93%; POLY+VEH vs. CONT+VEH, $p = 0.009$), but CBD-treated male offspring did not significantly differ to controls (POLY+CBD vs. CONT+VEH, $p = 0.51$) (Figure 3A). Similar to findings in the PFC, CBD administration significantly reduced M1/M4R binding density in the HPC of male control offspring (-22.59%; CONT+CBD vs. CONT+VEH, $p = 0.015$) (Figure 3A).

Given their functionally distinct roles in memory encoding and retrieval, further analysis was performed on the CA1/2 and CA3 subregions of the HPC. There was a significant interaction between the two factors ($F_{(1, 18)} = 8.41, p = 0.01$) for M1/M4R binding density in the hippocampal CA1/CA2 subregions of adult male offspring. Reflecting the changes observed in the initial HPC analysis, maternal poly I:C exposure reduced M1/M4R binding density (-24.5%, POLY+VEH vs CONT+VEH, $p = 0.01$), but CBD-treated rats did not differ to controls (POLY+CBD vs. CONT+VEH, $p = 0.79$). CBD administration significantly decreased M1/M4R binding density in control offspring (-22.1%, CONT+CBD vs CONT+VEH, $p = 0.02$) (Figure 3B). In the hippocampal CA3 subregion, there were also no significant main effects of PRENATAL INFECTION ($F_{(1, 17)} = 1.09, p = 0.31$) or OFFSPRING TREATMENT ($F_{(1, 17)} = 0.12, p = 0.73$), though a significant interaction between the two factors was observed ($F_{(1, 17)} = 5.09, p = 0.04$). Further analysis showed a significant reduction in M1/M4R binding density in the male poly I:C offspring compared to the controls (-23.3%, POLY+VEH vs CONT+VEH, $p = 0.04$) and a non-significant trend toward a decrease in M1/M4R binding density in the male control offspring administered
CBD treatment (-18.4%, CONT+CBD vs CONT+VEH, \( p = 0.09 \)) (Figure 3C). CBD treatment restored control-like levels of M1/M4R binding density in the CA3 region of poly I:C male offspring (POLY+CBD vs CONT+VEH groups \( p = 0.43 \)) (Figure 3C).

Conversely, maternal poly I:C exposure and CBD treatment did not affect M1/M4R binding in the HPC of adult female offspring (Figure 3D) (PRENATAL INFECTION: \( F(1, 15) = 0.11, \ p = 0.75 \); OFFSPRING TREATMENT: \( F(1, 15) = 0.38, \ p = 0.55 \); PRENATAL INFECTION x OFFSPRING TREATMENT: \( F(1, 15) = 0.26, \ p = 0.62 \). The same pattern was also observed when further analysis was conducted on the CA1/2 (Figure 3E) and CA3 (Figure 3F) subregions in adult female offspring (CA1/2: [PRENATAL INFECTION: \( F(1, 15) = 0.01, \ p = 0.92 \); OFFSPRING TREATMENT: \( F(1, 15) = 0.22, \ p = 0.65 \); PRENATAL INFECTION x OFFSPRING TREATMENT: \( F(1, 15) = 0.12, \ p = 0.73 \)], CA3: [PRENATAL INFECTION: \( F(1, 14) = 0.36, \ p = 0.56 \); OFFSPRING TREATMENT: \( F(1, 15) = 0.34, \ p = 0.57 \); PRENATAL INFECTION x OFFSPRING TREATMENT: \( F(1, 15) = 0.24, \ p = 0.63 \)].
Figure 3: The effect of cannabidiol (CBD) or vehicle (VEH) treatment on M1/M4R ([3H]Pirenzepine) binding density in the hippocampus (HPC), and CA1/2 and CA3 subregions in (A-C) male and (D-F) female control (CONT) and poly I:C (POLY) offspring. Data expressed as mean ± SEM. n = 4-6 rats per group, except female CONT+CBD group where n=3. *p<0.05, **p<0.01, t = 0.09 vs. CONT+VEH group. TE = tissue equivalent.
3.2 ChAT Protein Expression in the PFC and HPC of Offspring

In the PFC, there were no significant main effects of PRENATAL INFECTION ($F_{(1, 28)} = 2.55, p = 0.12$) or OFFSPRING TREATMENT ($F_{(1, 28)} = 0.50, p = 0.83$) and no significant interaction between these factors ($F_{(1, 28)} = 0.13, p = 0.72$) in male offspring (Figure 4A). Similarly in adult female offspring, no significant effects or interactions were observed on ChAT protein levels in the PFC (Figure 4B) (PRENATAL INFECTION: $F_{(1, 27)} = 2.30, p = 0.14$; OFFSPRING TREATMENT: $F_{(1, 27)} = 1.04, p = 0.32$; PRENATAL INFECTION x OFFSPRING TREATMENT: $F_{(1, 27)} = 1.22, p = 0.28$).

On the other hand, there was a significant main effect of PRENATAL INFECTION ($F_{(1, 27)} = 5.154, p = 0.03$) in the male HPC (CA1/2 and CA3 regions combined). Further analysis revealed a small yet significant decrease in ChAT protein expression in the HPC of male poly I:C offspring compared to the controls (-10.1%, POLY+VEH vs CONT+VEH, $p = 0.02$) (Figure 4C). However, there was no significant effect of OFFSPRING TREATMENT on ChAT protein expression in the HPC ($F_{(1, 27)} = 0.29, p = 0.60$) and no interaction between PRENATAL INFECTION and OFFSPRING TREATMENT ($F_{(1, 27)} = 1.36, p = 0.25$) in the male HPC. Hippocampal ChAT protein levels were unaltered in female offspring (PRENATAL INFECTION: $F_{(1, 28)} = 1.74, p = 0.20$; OFFSPRING TREATMENT: $F_{(1, 28)} = 0.05, p = 0.82$; PRENATAL INFECTION x OFFSPRING TREATMENT: $F_{(1, 28)} < 0.001, p = 0.99$) (Figure 4D).
Figure 4: The effect of cannabidiol (CBD) or vehicle (VEH) treatment on choline acetyltransferase (ChAT) protein levels in the (A, B) prefrontal cortex (PFC) and (C, D) hippocampus (HPC) in male and female control (CONT) and poly I:C (POLY) offspring. Representative immunoblots for ChAT are shown underneath each graph. Signal intensity was normalised to total protein levels in the respective lane and an internal control sample. *p<0.05 vs. CONT+VEH group. Data expressed as mean ± SEM. (n = 7-8 rats per group).
3.3 AChE Protein Expression in the PFC and HPC of Offspring

There was no significant main effect of PRENATAL INFECTION ($F_{(1, 27)} = 0.09, p = 0.76$), or OFFSPRING TREATMENT ($F_{(1, 27)} = 0.66, p = 0.42$) on AChE protein expression in the male PFC and no interaction between the two factors ($F_{(1, 27)} = 0.31, p = 0.59$) (Figure 5A). A similar pattern was also observed in the PFC of female offspring (Figure 5B) (PRENATAL INFECTION: $F_{(1, 28)} = 0.13, p = 0.72$; OFFSPRING TREATMENT: $F_{(1, 28)} = 1.65, p = 0.21$; PRENATAL INFECTION x OFFSPRING TREATMENT: $F_{(1, 28)} = 0.13, p = 0.72$).

Similar results were identified in the HPC, with no significant effects or interactions observed in male (Figure 5C) (PRENATAL INFECTION: $F_{(1, 28)} = 0.56, p = 0.46$; OFFSPRING TREATMENT: $F_{(1, 28)} = 0.71, p = 0.41$; PRENATAL INFECTION x OFFSPRING TREATMENT: $F_{(1, 28)} = 0.58, p = 0.46$) or female offspring (Figure 5D) (PRENATAL INFECTION: $F_{(1, 28)} = 0.09, p = 0.76$; OFFSPRING TREATMENT: $F_{(1, 28)} = <0.001, p = 0.99$; PRENATAL INFECTION x OFFSPRING TREATMENT: $F_{(1, 28)} = 0.39, p = 0.54$).
Figure 5: The effect of cannabidiol (CBD) or vehicle (VEH) treatment on acetylcholinesterase (AChE) protein levels in the (A, B) prefrontal cortex (PFC) and (C, D) hippocampus (HPC) in male and female control (CONT) and poly I:C (POLY) offspring. Representative immunoblots for AChE are shown underneath each graph. Signal intensity was normalised to total protein levels in the respective lane and an internal control sample. Data expressed as mean ± SEM. (n = 7-8 rats per group).
3.4 Correlations

Correlations were performed to assess the relationship between markers of muscarinic neurotransmission in male and female offspring. In the PFC, protein levels of ChAT and AChE were positively correlated in female (r = 0.57, p = 0.001) (Figure 6A) and male offspring (r = 0.45, p = 0.011) (Figure 6B). There was also a positive association between AChE protein levels and M1/M4R binding density levels in the HPC of female offspring (r = 0.64, p = 0.003) (Figure 6C), but this relationship was not apparent in males (r = -0.10, p = 0.67) (Figure 6D). We also examined correlations between markers of the muscarinic system in the HPC and PFC of male and female offspring and their previously reported cognitive behaviour [7, 17]. Performance in the T-maze test (percentage of correct entries) was positively correlated with M1/M4R binding density levels (r = 0.474, p = 0.04) (Figure 6E) and AChE protein levels (r = 0.503, p = 0.004) (Figure 6F) in the HPC of female offspring that showed comparable working memory across treatment groups. Conversely, in male offspring that did show treatment group differences in working memory performance, these correlations were not significant (M1/M4R: r = 0.15, p = 0.52; AChE: (r = 0.04, p = 0.83). Instead, T-maze performance of male offspring was positively correlated to hippocampal ChAT protein levels (r = 0.41, p = 0.03) (Figure 6G).
Figure 6: Correlations between muscarinic cholinergic markers in the (A, B) prefrontal cortex (PFC) and (C, D) hippocampus (HPC), and with performance in the T-maze test (percentage of correct entries) (E, F, G) in male and female offspring.
4. DISCUSSION

The mechanisms underlying the pro-cognitive efficacy of CBD are largely unknown. We investigated the effect of chronic CBD treatment on muscarinic neurotransmission in regions of the brain implicated in cognitive function in the poly I:C model. These experiments revealed a downregulation of M1/M4R binding density in the PFC and hippocampal CA1/CA2 and CA3 subregions in male poly I:C offspring that were not present in the brains of poly I:C offspring treated with CBD. Levels of ChAT protein expression were also reduced in the HPC of male poly I:C offspring compared to healthy controls, whereas CBD-treated poly I:C offspring had control-like ChAT levels, while AChE remained unaltered in any of the groups. CBD administration to healthy male control offspring reduced M1/M4R binding density in the PFC and hippocampal subregions examined. Muscarinic neurotransmitter signalling parameters were unaltered in the PFC or HPC and its subregions of female offspring.

Our finding of an M1/M4R downregulation in the PFC and HPC of male poly I:C offspring compared to offspring born to control dams is consistent with numerous post-mortem studies that have reported decreased levels of M1/M4R binding density in the schizophrenia brain, including the PFC [39-41], anterior and posterior cingulate cortices [42, 43], superior temporal gyrus [44], HPC [39, 45, 46] and caudate-putamen [47]. This result suggests that the poly I:C model mimics muscarinic dysfunction in a manner that is relevant to human schizophrenia; however, we also found that M1/M4R binding density was unaltered in the brains of female offspring. The existence of sex differences in the M1/M4R in the human schizophrenia brain is unclear. Reduced [\(^3\)H]pirenzepine binding to M1/M4R in the HPC and PFC has been reported in the post-mortem schizophrenia brain in studies using predominantly (70-80%) male tissue cohorts [39, 40]; however, decreased M1/M4R binding density has also
been reported in the anterior cingulate cortex of a predominantly female schizophrenia cohort [42]. A possible explanation for the difference between male and female M1/M4R binding density results is the potential influence of the estrous cycle, as ACh levels fluctuate depending on the stage of the cycle [48]. The estrous cycle was not measured in the present study and should be considered for future studies examining cholinergic systems in female rodents; however, the low data variability in our study suggests that the estrous cycle did not affect these results. The difference in M1/M4R binding density results between males and females could also be partly attributed to sexual dimorphism in the severity of cognitive symptoms. We have previously shown that female poly I:C offspring have impaired recognition memory but intact working memory [17], which differed to the male poly I:C offspring who showed impairments in both cognitive measures [7]. There is also clinical evidence that male patients can exhibit greater cognitive impairment than females [49]. Literature suggests that muscarinic dysfunction, especially M1/M4R signaling, in schizophrenia patients strongly contributes to psychotic and cognitive symptoms [reviewed in 19]. Indeed, CBD treated the cognitive impairment exhibited by poly I:C male offspring [7] and restored cholinergic and M1/M4R deficits to control-like levels the PFC and HPC, ie regions of the brain relevant to cognition, in the same cohort of rats. These findings suggest that impairments in the muscarinic system are partly responsible for the cognitive deficits observed in the poly I:C male offspring and suggest that normalization of the M1/M4R binding density by CBD may have contributed to the improvement in cognition in these rats.

M1/M4R binding density and AChE protein levels did not differ between female treatment groups and we observed a positive correlation between these markers in the HPC of female offspring. Furthermore, both markers were positively correlated with working performance in the T maze test, in which female treatment groups did not differ in performance [17]
Conversely, in male offspring that showed alterations in muscarinic neurotransmission, hippocampal M1/M4R binding density and AChE protein levels did not correlate with each other, or with T maze performance. Taken together, these findings could suggest that tight regulation of M1/M4R expression and ACh metabolism in the HPC is important for functional working memory. In other studies, decreased ChAT levels and M1R G protein-coupling were reported in the PFC of Alzheimer’s disease patients with severe cognitive impairment, with M1R levels correlating to the rate of cognitive decline [50]. Decreased muscarinic receptor G protein-coupled signalling in the PFC and HPC correlated to reduced spatial memory in aged rats [51] and decreased ChAT was correlated to age-related learning and memory deficits in mice [52]. Spatial working memory tasks that involve the HPC appear dependent on adequate cholinergic neurotransmission, particularly M1Rs that are expressed on excitatory pyramidal neurons [53]; therefore, hippocampal M1R downregulation may explain, in-part, the working memory deficits that we observed in male offspring.

Down-regulated M1/M4R binding density in the poly I:C model could represent reduced cholinergic neurotransmission or a compensatory decrease in receptor density due to hyper-cholinergic tone. However, while the mechanism underlying the CBD-induced alterations to muscarinic receptor density is unclear, one study recently reported that systemic administration of CBD to mice dose-dependently increased ACh levels in the forebrain within hours [24]. Indeed, we found that levels of the ACh synthesising enzyme, ChAT, were reduced in the HPC of poly I:C offspring, whereas CBD treatment resulted in control-like ChAT levels. On the other hand, the degrading enzyme, AChE, was unaltered in the HPC or PFC of any of the treatment groups. Therefore, the downregulated M1/M4R binding density in the poly I:C model is likely to have represented reduced cholinergic tone (ACh and
M1/M4R signalling), whereas CBD alters muscarinic receptor density by restoring normal levels of ACh synthesis, not preventing degradation. To that effect, we observed a positive correlation between ChAT and percentage of correct entries in the T Maze in male offspring, but no significant correlation between M1/M4R or AChE levels and cognitive performance in males. We also observed a significant positive correlation between ChAT and AChE protein levels in the PFC of both male and female offspring, suggesting that acetylcholine synthesis and metabolism is tightly regulated in this brain region. Our finding that CBD did not reduce AChE protein expression suggests that this molecule may not play a major role in CBD-induced cognitive performance; however, AChE enzymatic activity may be worth examining in future studies. The CB1R agonist Δ9-THC is a competitive inhibitor of AChE, *in-vitro* [54], but Δ9-THC effects on cognition can be either deleterious or beneficial. On the other hand, CBD may have a different mechanism of action as it did not alter AChE in the present study and was previously identified as a CB1R negative allosteric modulator, i.e., binds to an allosteric site on the receptor to reduce its activity, while traditional agonists/antagonists bind to the orthosteric site [55, 56]. Interestingly, cholinergic neurons projecting from the septum to the HPC release ACh, which increases production of post-synaptic endocannabinoids (via the M1R [57]) that regulate hippocampal excitation by controlling major inhibitory and excitatory neurotransmitters, GABA and glutamate, respectively [58]. Hippocampal excitation then affects signalling to higher brain regions, including the PFC, and is thought to contribute to the psychotic and cognitive symptoms of schizophrenia [59, 60]. Indeed, we previously reported imbalances in the level of glutamatergic NMDAR binding density in the PFC of female poly I.C offspring, while CBD treatment restored levels to that of the controls [17]. Taken together, CBD may alter signalling in the HPC and PFC through interactions with the cholinergic muscarinic, glutamatergic, GABAergic and endogenous cannabinoid...
systems to influence cognitive behaviour. However, further studies are required to confirm this mechanism.

Given that CBD can improve cognitive function in a range of disease states that manifest impairment [9], its pro-cognitive effects may be attributed to a number of mechanisms. For example, one study showed that CBD is a dopamine D2 receptor (D2R) partial agonist, similar to the antipsychotic drug, aripiprazole [61]. Differing to risperidone and olanzapine, which have been associated with impaired cognition in some schizophrenia patients [62], aripiprazole improves cognition, including processing speed, attention and memory [63]. D2R activation has also been shown to increase hippocampal ACh levels in rats [64]. Therefore, the D2R partial agonist activity of CBD may be a contributing mechanism in its pro-cognitive effects via cholinergic signalling in the poly I:C model. CBD is also anti-inflammatory, neuroprotective and can stimulate neurogenesis [reviewed in 9, 10]. In a mouse model of cerebral malaria, CBD improved cognitive performance that was associated with increased neurogenic markers, such as brain-derived neurotrophic factor (BDNF), and reduced pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, in the HPC and PFC [65]. Therefore, future studies may consider investigating the role of these markers in the mechanisms underlying the pro-cognitive effects of CBD in the poly I:C model. In addition, while this study focussed on brain regions relevant to cognitive function, the mechanisms underlying the improvements in social interaction in the poly I:C model of schizophrenia following CBD treatment remain unknown [7] and further investigations focussing on regions involved in social functioning, particularly glutamatergic and GABAergic signalling in the amygdala [66], are required.
Our study and others [67], have shown that CBD is psychoactive (i.e. alters brain signalling and changes behaviour), and is a promising novel therapeutic for a range of illnesses [9, 10]. However, the result that CBD treatment decreases M1/M4R binding density in the HPC and PFC of male control offspring in the present study has implications about its use in non-pathological states, particularly during adolescence/early adulthood which is a critical neurodevelopmental period. Further clinical studies are required to extrapolate these findings in humans.

5. Conclusion: This study has revealed reductions in M1/M4R binding density and ChAT protein expression in the HPC and PFC of cognitively impaired male poly I:C offspring. The cholinergic deficits were treated using CBD. Further studies are required to confirm whether alterations to cholinergic neurotransmission are a cause or effect of cognitive deficits in the poly I:C model, as our data indicate a potential correlation between these observations. For example, pre-treating poly I:C offspring with a cholinergic/ChAT protein upregulator, such as donepezil [68], could provide useful data towards answering this question. Overall, our study revealed for the first time that muscarinic signalling in regions of the rat brain implicated in cognitive function is altered by CBD treatment.

6. DECLARATIONS

6.1 Declaration of Interest: None

6.2 Author Contributions: KWG and ALO designed the study; CJN, ALO and KWG performed the experiments; CJN, ALO and KWG analysed the data; all authors have contributed to writing the manuscript and have approved the final manuscript for publication.
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From 2. Methods

2.1 Ethics Statement

Experimental procedures were approved by the Animal Ethics Committee of the University of Wollongong, Wollongong, NSW, Australia (AE15/05) and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council [28]. All efforts were made to minimise suffering and the number of animals used.
Highlights:

- Male poly I:C offspring have reduced M1/M4R density and ChAT in the PFC and hippocampus
- CBD treated deficits in cholinergic markers in male poly I:C offspring brain
- ChAT levels positively correlated to working memory performance in the T Maze
- Cholinergic markers were unaltered in female offspring
- This study revealed a role for cholinergic signaling in CBD’s pro-cognitive effects