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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.

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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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26 **ABSTRACT**

27 Cognitive impairment is a core symptom of schizophrenia; however, current antipsychotic
28 drugs have limited efficacy to treat these symptoms and can cause serious side-effects,
29 highlighting a need for novel therapeutics. Cannabidiol (CBD) is a non-intoxicating
30 phytocannabinoid that has demonstrated pro-cognitive effects in multiple disease states,
31 including a maternal immune activation (poly I:C) model of schizophrenia, but the mechanisms
32 underlying the efficacy of CBD require investigation. Muscarinic neurotransmission is highly
33 implicated in the cognitive impairments of schizophrenia; however, the effect of CBD on this
34 system is unknown. We examined alterations in markers of muscarinic neurotransmission in
35 the pre-frontal cortex (PFC) and hippocampus (HPC) following CBD treatment. Pregnant
36 Sprague-Dawley rats (n =16) were administered poly I:C (4 mg/kg) or saline. Adult offspring
37 were treated (3-weeks) with CBD (10 mg/kg) or vehicle. Receptor autoradiography (using
38 [^{3}H]pirenzepine) was used to examine changes in muscarinic M1/M4 receptor (M1/M4R)
39 binding density. Levels of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE)
40 protein expression were examined using Western blot. M1/M4R binding density was
41 downregulated in the PFC and CA1/CA2 and CA3 subregions in male poly I:C offspring.
42 M1/M4R deficits were normalised after CBD treatment. ChAT protein expression was reduced
43 in the HPC of male poly I:C offspring, while CBD treated poly I:C offspring exhibited control-
44 like ChAT levels. AChE levels were unaltered in any of the groups. There were also no changes
45 in muscarinic signalling in female offspring. These findings demonstrate that CBD can
46 normalise muscarinic neurotransmission imbalances in male poly I:C offspring in regions of
47 the brain implicated in cognition.

48

49 **Keywords:** cannabidiol; muscarinic; acetylcholine; maternal immune activation;
50 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

76 I:C, a synthetic double-stranded RNA viral mimic) to pregnant rats during mid-late gestation
77 increases pro-inflammatory cytokines that cross the placenta causing inflammation of the foetal
78 brain and altering the neurodevelopmental trajectory of the offspring [14]. Poly I:C offspring
79 display numerous behavioural, neurochemical and structural brain abnormalities observed in
80 schizophrenia patients [14-16]. In particular, we found that male poly I:C offspring had deficits
81 in recognition and working memory (assessed using the Novel Object Recognition (NOR) and
82 T Maze tests, respectively), as well as reduced social interaction during the Social Interaction
83 test, while CBD treated these deficits [7]. In female offspring, the poly I:C group exhibited
84 deficits in recognition memory and social interaction that were treated using CBD, while
85 female poly IC offspring did not exhibit impaired working memory [17]. However, the
86 mechanisms by which CBD treats cognitive deficits in the poly I:C model are unknown.

87 The cholinergic system is composed of two major families of receptors: acetylcholine nicotinic
88 and muscarinic receptors (mAChRs). Mice deficient in the muscarinic M1 receptor (M1R)
89 subtype exhibit impairments in working memory and remote reference memory [18]. In
90 addition, significant reductions in the expression of M1R and muscarinic M4 receptors (M4R)
91 have been reported in post-mortem brains of schizophrenia patients in regions related to
92 cognitive function, including the hippocampus and frontal cortex [reviewed in 19]. It has also
93 been demonstrated that the expression of M1R protein, but not other mAChR subtypes, is
94 decreased in the dorsolateral prefrontal cortex of people with schizophrenia [20]. Altogether,
95 these findings suggest that alterations in the M1R may play a role in the cognitive dysfunction
96 of schizophrenia. In cholinergic neurons, two main enzymes are essential to acetylcholine
97 (ACh) neurotransmission: choline acetyltransferase (ChAT) for ACh synthesis in the pre-
98 synaptic neuron, and acetylcholinesterase (AChE), which is located in the post-synaptic neuron
99 and degrades ACh in the synaptic cleft following its release [21]. In previous studies, decreased
100 activity of ChAT has been correlated with poor cognitive functioning in schizophrenia patients

101 [22], while AChE inhibitors have been shown to slow the cognitive decline in Alzheimer's
102 disease [23], demonstrating that ChAT and AChE activity impact cognitive functioning.
103 Interestingly, acute CBD increases ACh in the basal forebrain within hours of systemic
104 administration [24], unlike cannabinoid CB1 receptor (CB1R) agonists (Δ9-
105 tetrahydrocannabinol, WIN 55,212-2 and CP 55,940 administered systemically) that inhibit
106 ACh release in the rat pre-frontal cortex and hippocampus [25, 26]. Therefore, there is evidence
107 of a link between CBD, the cholinergic muscarinic neurotransmission system, schizophrenia
108 and cognitive function. The aim of this study was to examine the effect of CBD treatment on
109 muscarinic neurotransmission (M1/M4R density, ChAT and AChE protein levels) in regions
110 of the brain implicated in learning, memory and schizophrenia pathology in poly I:C offspring.

111

112 ## 2. METHODS

113

114 ### 2.1 Ethics Statement

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

120

121 ### 2.2 Animals and Treatment

122 The methods relating to the animal housing and treatment were previously reported by our
123 laboratory [7] and in accordance with the Animal Research: Reporting of *In Vivo* Experiments
124 (ARRIVE) guidelines [28]. Briefly, sixteen pregnant Sprague-Dawley rats (Animal Resource
125 Centre, Perth, WA, Australia) received an intravenous injection of either poly I:C (4mg/kg,

126 Sigma-Aldrich, Sydney, NSW, Australia; *n* = 8) or saline solution (control; *n* = 8) at gestation
127 day 15. Dose and timing of poly I:C administration was based on previous studies [15]. Male
128 and female offspring received twice-daily intraperitoneal (i.p) injections of CBD (10 mg/kg;
129 THC-Pharm GmbH, Frankfurt, Germany) dissolved in Tween 80 and saline (vehicle; 1:16
130 (*v/v)*; Sigma-Aldrich), or vehicle alone (injection volume of 5ml/kg) (n = 12 per group).
131 Treatment began on postnatal day 56, which equates to late adolescence/early adulthood in
132 humans [29]. After 2-weeks of treatment, rats were submitted to a series of behavioural tests,
133 including the Novel object recognition (NOR) test, Rewarded T-maze alternation test and the
134 Social interaction test to assess the effects of CBD on recognition memory, working memory
135 and sociability, respectively [7, 17]. The behavioural data were published elsewhere [7, 17]. In
136 order to examine the mechanisms by which CBD treated the cognitive deficits in poly I:C
137 offspring, the brain tissue from our previous studies [7, 17] was analysed in the present study.
138

139 **2.3 Histological Procedures**

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

151 using a micropuncture kit and stored at -80ºC. The dissection methods enabled examination of
152 receptor binding and protein expression from the same rat brain.
153
154 **2.4 Receptor Autoradiography**
155
156 *2.4.1 M1/M4R binding*
157 The methods used to detect M1/M4R binding density were conducted as previously described
158 by our laboratory [31]. Briefly, slides containing two consecutive sections, per region for each
159 rat, were air-dried and pre-incubated in 22 mM HEPES buffer (pH 7.5) for 15 min at room
160 temperature. To examine total binding, sections were incubated in HEPES buffer containing
161 10 nM [^{3}H]pirenzepine (72.8 Ci/mmol; PerkinElmer TM Life and Analytical Sciences, Boston,
162 USA) for 90 min at room temperature. Non-specific binding was determined by incubating an
163 additional two consecutive sections in 10 nM [^{3}H]pirenzepine in the presence of atropine (10
164 μM), a competitive antagonist of muscarinic receptors, in buffer (pH 7.5) for 90 min at room
165 temperature. After incubation, sections were washed three times for 4 min in ice-cold HEPES
166 buffer, then dipped in ice cold milliQ water and air-dried overnight.
167
168 *2.4.2 Quantification*
169 Slides were exposed to Amersham Hyperfilm ECL (GE Healthcare Life Sciences, Parramatta,
170 NSW, Australia) for 7 weeks in X-ray film cassettes with a set of tritium standards (Amersham,
171 Buckinghamshire, United Kingdom). Autoradiographs were developed using standard
172 procedures, scanned using a GS-800 Imaging Densitometer (Bio-Rad, Hercules, California,
173 USA) and analysed with Image J software (https://imagej.nih.gov/ij). Images were calibrated
174 based on the Rodbard curve obtained from the tritium standards to produce nCi/mg tissue
175 equivalent (TE) values. Regions of interest included the PFC and the CA1/2 and CA3 regions

176 of the HPC. Specific binding densities were calculated by subtracting non-specific binding
177 from total binding values. Values were converted to fmoles [^{3}H] ligand per mg TE, taking into
178 account the specific activity of the ligand, as we have described previously [32-34]. Anatomical
179 structures of interest were confirmed using a standard rat brain atlas [30].
180

181 **2.5 Western Blot**

182 Microdissected brain tissue samples (PFC, HPC with the CA1/2 and CA3 subregions
183 combined) were homogenised in homogenising buffer (0.1 M Tris-HCl, 2 mM EDTA, 10%
184 glycerol, 2% SDS, 0.5 mM PMSF, Protease Inhibitor Cocktail (P8340; Sigma-Aldrich, Castle
185 Hill, NSW, Australia) and Phosphatase Inhibitor Cocktail 2 (P5726; Sigma-Aldrich, Castle
186 Hill, NSW, Australia)). Total protein concentration was determined using a DC Protein Assay
187 kit (Bio-Rad, Gladesville, NSW, Australia). Proteins were loaded in equal amounts (10 ug,
188 within the linear range for each antibody target) into TGX^{TM} 4-20% Stain-free Precast Gels
189 (Bio-Rad, Australia) to undergo SDS-PAGE electrophoresis at 180V for 1 hr. Stain-free gels
190 were activated (GelDoc XR+ imaging system; Bio-Rad, Australia) [35, 36], proteins were
191 transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Gladesville, NSW
192 Australia) at 100V for 1 hr, and imaged to capture total protein in each lane [35, 36]. PVDF
193 membranes were washed in Tris Buffered Saline with Tween 20 (TBST) (3 x 5 min) and
194 blocked in non-fat milk for 1 hr at room temperature. Membranes were then incubated
195 overnight in anti-ChAT (1:10000, #ab181023, Abcam, Melbourne, VIC, Australia) or anti-
196 AChE (1:5000, #ab183591, Abcam, Melbourne, VIC, Australia) primary antibodies.
197 Membranes were washed in TBST (5 x 5 min) and incubated in goat anti-rabbit secondary
198 antibody (1:5000, #AB307P, Merck Millipore, Bayswater, VIC, Australia) for 60 min at room
199 temperature. Membranes were washed in TBST (3 x 5 min), incubated in ECL reagent (GE
200 Healthcare, Parramatta, NSW, Australia) and exposed to a Gel Imager (600RB, Amersham,

201 GE Healthcare, Parramatta, NSW, Australia). Band density was quantified using Image Lab
202 software (ver 6, Bio-Rad Laboratories Inc, California, USA). Samples were examined in
203 duplicate. The values for each signal were normalised to total protein in the respective lane to
204 account for loading variability. Values were then normalised to an internal control (pooled
205 sample) on each gel, to account for gel-to-gel variability and allow comparison of samples
206 across gels.

207

208 **2.6 Statistical Analysis**

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

226 statistical trends. Data were presented as mean ± standard error of the mean (SEM).

227

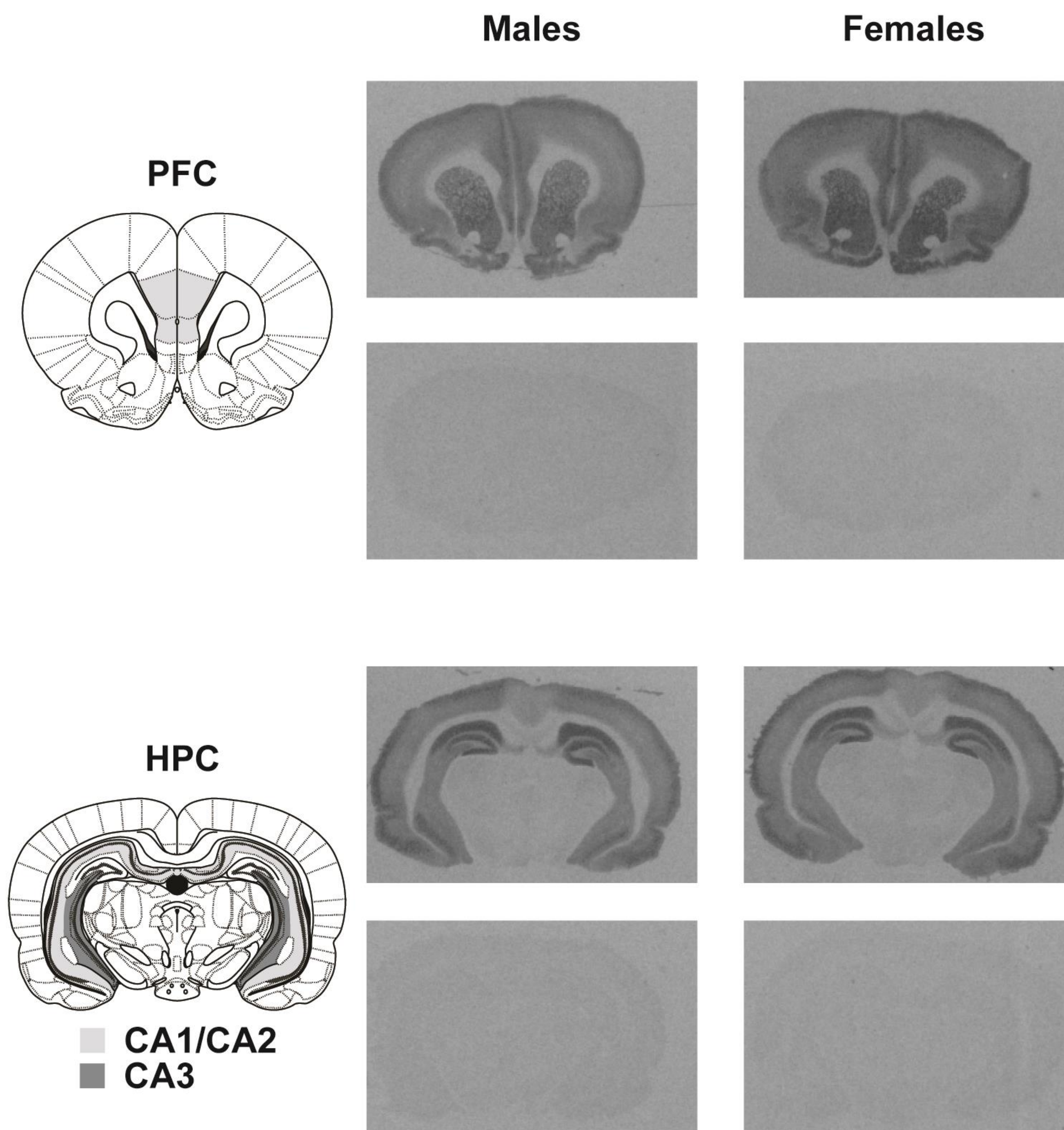
228 **3. RESULTS**

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

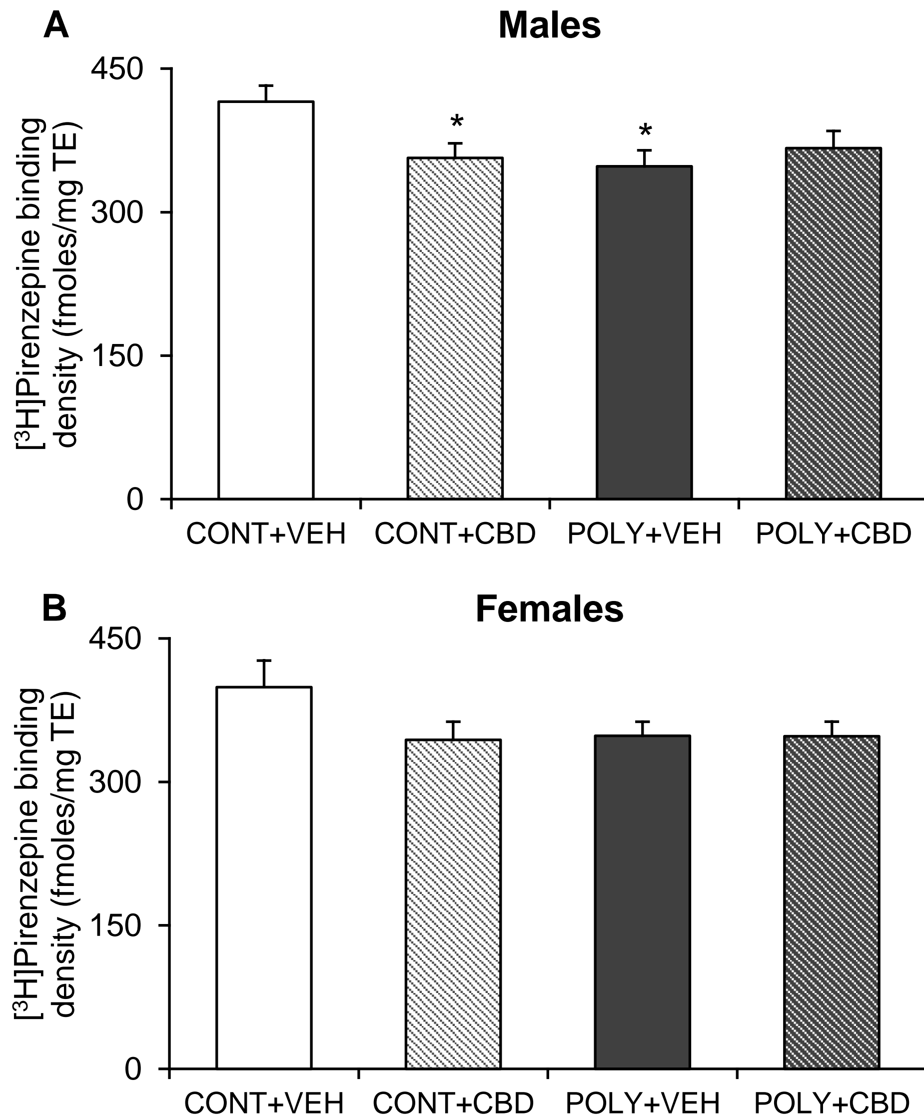
238

239 **3.1 M1/M4R Binding Density in the PFC and HPC of offspring**

240 Examples of [^{3}H]Pirenzepine binding to M1/M4Rs are presented in Figure 1, with M1/M4R
241 binding observed across the PFC, and CA1/CA2 and CA3 regions of the HPC. There were no
242 significant main effects of PRENATAL INFECTION ($F_{(1, 19)} = 2.91$, $p = 0.10$) or OFFSPRING
243 TREATMENT ($F_{(1, 19)} = 1.40$, $p = 0.25$) on M1/M4R binding density in the PFC of male
244 offspring, but a significant interaction between the factors was observed ($F_{(1, 19)} = 5.34$, $p =$
245 0.03). M1/M4R binding density was reduced in male poly I:C offspring compared to controls
246 (-16.3%; POLY+VEH vs. CONT+VEH, $p = 0.01$) (Figure 2A); however, CBD treatment
247 restored M1/M4R binding density levels in male poly I:C offspring that were no longer
248 different to controls (POLY+CBD vs CONT+VEH, $p = 0.18$) (Figure 2A). In addition, CBD
249 significantly reduced M1/M4R binding density in control offspring (-14.2%, CONT+CBD vs.
250 CONT+VEH, $p = 0.02$) (Figure 2A).



251
252 **Figure 1:** Example autoradiographs to demonstrate [^{3}H]Pirenzepine binding to M1/M4Rs in
253 the offspring brain. Schematic diagrams (left panel) showing the approximate Bregma levels
254 examined in the prefrontal cortex (PFC; 2.52 mm), which included the prelimbic and
255 infralimbic cortices (shaded), and the hippocampus (HPC; -4.92 mm), which included the
256 CA1/2 and CA3 regions. Example autoradiographs show total (row 1 and 3) and non-specific
257 (row 2 and 4) binding in the PFC and HPC, respectively, in vehicle-treated male and female
258 offspring from control dams. Schematic diagram modified from a standard rat atlas (Paxinos
259 and Watson (2007). The Rat Brain in Stereotaxic Coordinates. 6th Ed. Elselvier Academic Press
260 Inc, CA, USA).



261

262 **Figure 2**: The effect of cannabidiol (CBD) or vehicle (VEH) treatment on M1/M4R
263 ([^{3}H]Pirenzepine) binding density in the prefrontal cortex of (**A**) male and (**B**) female control
264 (CONT) and poly I:C (POLY) offspring. Data expressed as mean ± SEM. n = 5-7 rats per
265 group. *$p<0.05$ vs. CONT+VEH. TE = tissue equivalent.

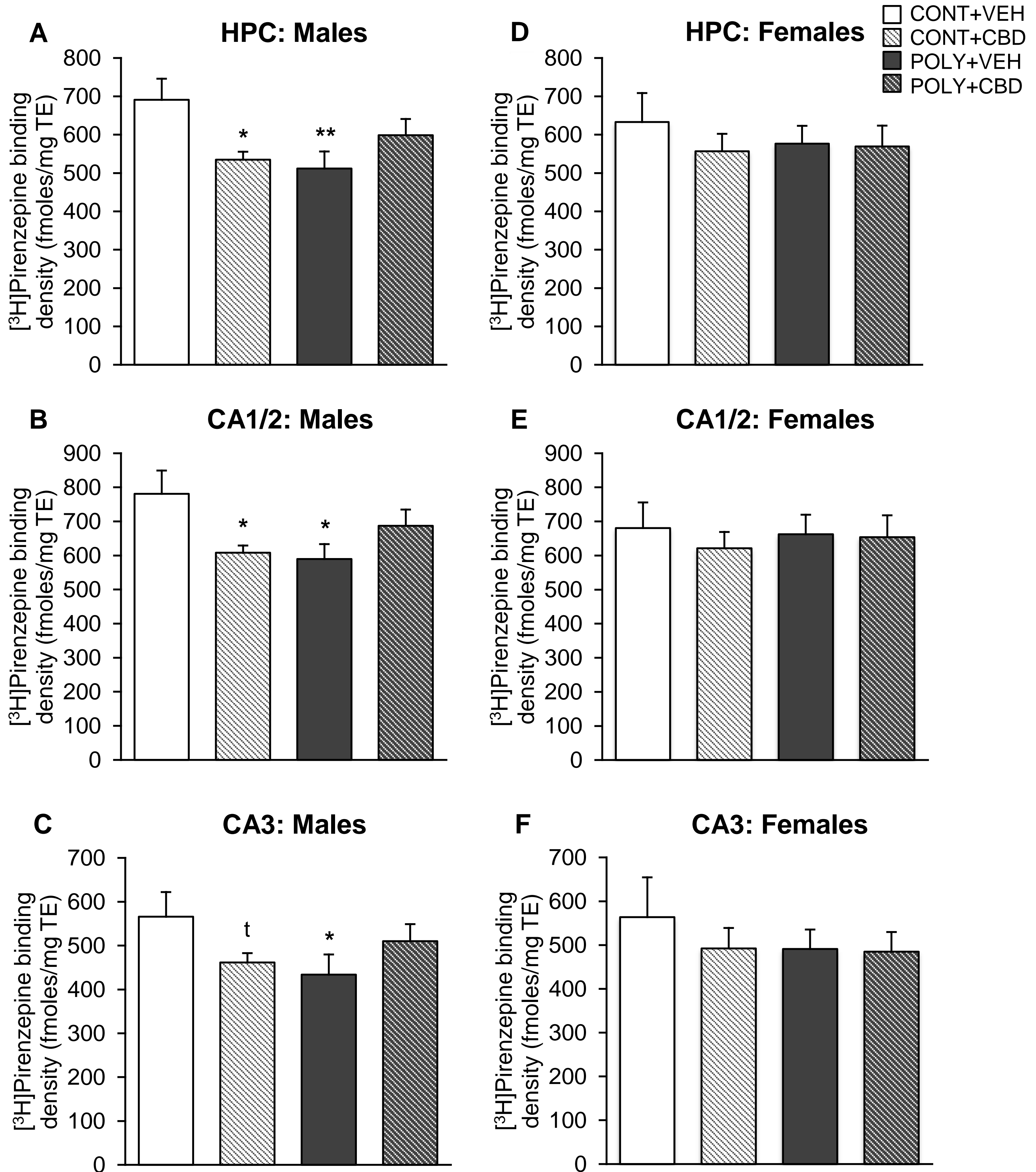
266

267 Conversely, there was no significant main effect of PRENATAL INFECTION ($F_{(1, 19)} = 1.17$,
268 $p = 0.29$) or OFFSPRING TREATMENT ($F_{(1, 19)} = 1.63$, $p = 0.22$) on M1/M4R binding density
269 in the PFC of female offspring, and no interaction between the factors ($F_{(1, 19)} = 1.57$, $p = 0.23$)
270 (Figure 2B).

271

272 In the HPC, there was a significant PRENATAL INFECTION x OFFSPRING TREATMENT
273 interaction ($F_{(1, 18)} = 8.67$, $p = 0.01$) on M1/M4R binding density in male offspring. Pairwise
274 comparisons revealed that maternal poly I:C exposure significantly reduced M1/M4R binding
275 density in male offspring compared to controls (-25.93%; POLY+VEH vs. CONT+VEH, $p =$
276 0.009), but CBD-treated male offspring did not significantly differ to controls (POLY+CBD
277 vs. CONT+VEH, $p = 0.51$) (Figure 3A). Similar to findings in the PFC, CBD administration
278 significantly reduced M1/M4R binding density in the HPC of male control offspring (-22.59%;
279 CONT+CBD vs. CONT+VEH, $p = 0.015$) (Figure 3A).
280
281 Given their functionally distinct roles in memory encoding and retrieval, further analysis was
282 performed on the CA1/2 and CA3 subregions of the HPC. There was a significant interaction
283 between the two factors ($F_{(1, 18)} = 8.41$, $p = 0.01$) for M1/M4R binding density in the
284 hippocampal CA1/CA2 subregions of adult male offspring. Reflecting the changes observed
285 in the initial HPC analysis, maternal poly I:C exposure reduced M1/M4R binding density
286 (-24.5%, POLY+VEH vs CONT+VEH, $p = 0.01$), but CBD-treated rats did not differ to
287 controls (Figure 3B) (POLY+CBD vs. CONT+VEH, $p = 0.79$). CBD administration
288 significantly decreased M1/M4R binding density in control offspring (-22.1%, CONT+CBD
289 vs CONT+VEH, $p = 0.02$) (Figure 3B). In the hippocampal CA3 subregion, there were also no
290 significant main effects of PRENATAL INFECTION ($F_{(1, 17)} = 1.09$, $p = 0.31$) or OFFSPRING
291 TREATMENT ($F_{(1, 17)} = 0.12$, $p = 0.73$), though a significant interaction between the two
292 factors was observed ($F_{(1, 17)} = 5.09$, $p = 0.04$). Further analysis showed a significant reduction
293 in M1/M4R binding density in the male poly I:C offspring compared to the controls (-23.3%,
294 POLY+VEH vs CONT+VEH, $p = 0.04$) and a non-significant trend toward a decrease in
295 M1/M4R binding density in the male control offspring administered CBD treatment (-18.4%,
296 CONT+CBD vs CONT+VEH, $p = 0.09$) (Figure 3C). CBD treatment restored control-like

297 levels of M1/M4R binding density in the CA3 region of poly I:C male offspring (POLY+CBD
298 vs CONT+VEH groups (p = 0.43) (Figure 3C).
299
300 Conversely, maternal poly I:C exposure and CBD treatment did not affect M1/M4R binding in
301 the HPC of adult female offspring (Figure 3D) (PRENATAL INFECTION: $F_{(1, 15)}$ = 0.11, p =
302 0.75; OFFSPRING TREATMENT: $F_{(1, 15)}$ = 0.38, p = 0.55; PRENATAL INFECTION x
303 OFFSPRING TREATMENT: $F_{(1, 15)}$ = 0.26, p = 0.62). The same pattern was also observed
304 when further analysis was conducted on the CA1/2 (Figure 3E) and CA3 (Figure 3F)
305 subregions in adult female offspring (CA1/2: [PRENATAL INFECTION: $F_{(1, 15)}$ = 0.01, p =
306 0.92; OFFSPRING TREATMENT: $F_{(1, 15)}$ = 0.22, p = 0.65; PRENATAL INFECTION x
307 OFFSPRING TREATMENT: $F_{(1, 15)}$ = 0.12, p = 0.73], CA3: [PRENATAL INFECTION: $F_{(1,}$
308 $_{14)}$ = 0.36, p = 0.56; OFFSPRING TREATMENT: $F_{(1, 15)}$ = 0.34, p = 0.57; PRENATAL
309 INFECTION x OFFSPRING TREATMENT: $F_{(1, 15)}$ = 0.24, p = 0.63]).



310 **Figure 3**: The effect of cannabidiol (CBD) or vehicle (VEH) treatment on M1/M4R
311 ([^{3}H]Pirenzepine) binding density in the hippocampus (HPC), and CA1/2 and CA3 subregions
312 in (**A-C**) male and (**D-F**) female control (CONT) and poly I:C (POLY) offspring. Data
313 expressed as mean ± SEM. n = 4-6 rats per group, except female CONT+CBD group where
314 n=3. *p<0.05, **p<0.01, t = 0.09 vs. CONT+VEH group. TE = tissue equivalent.

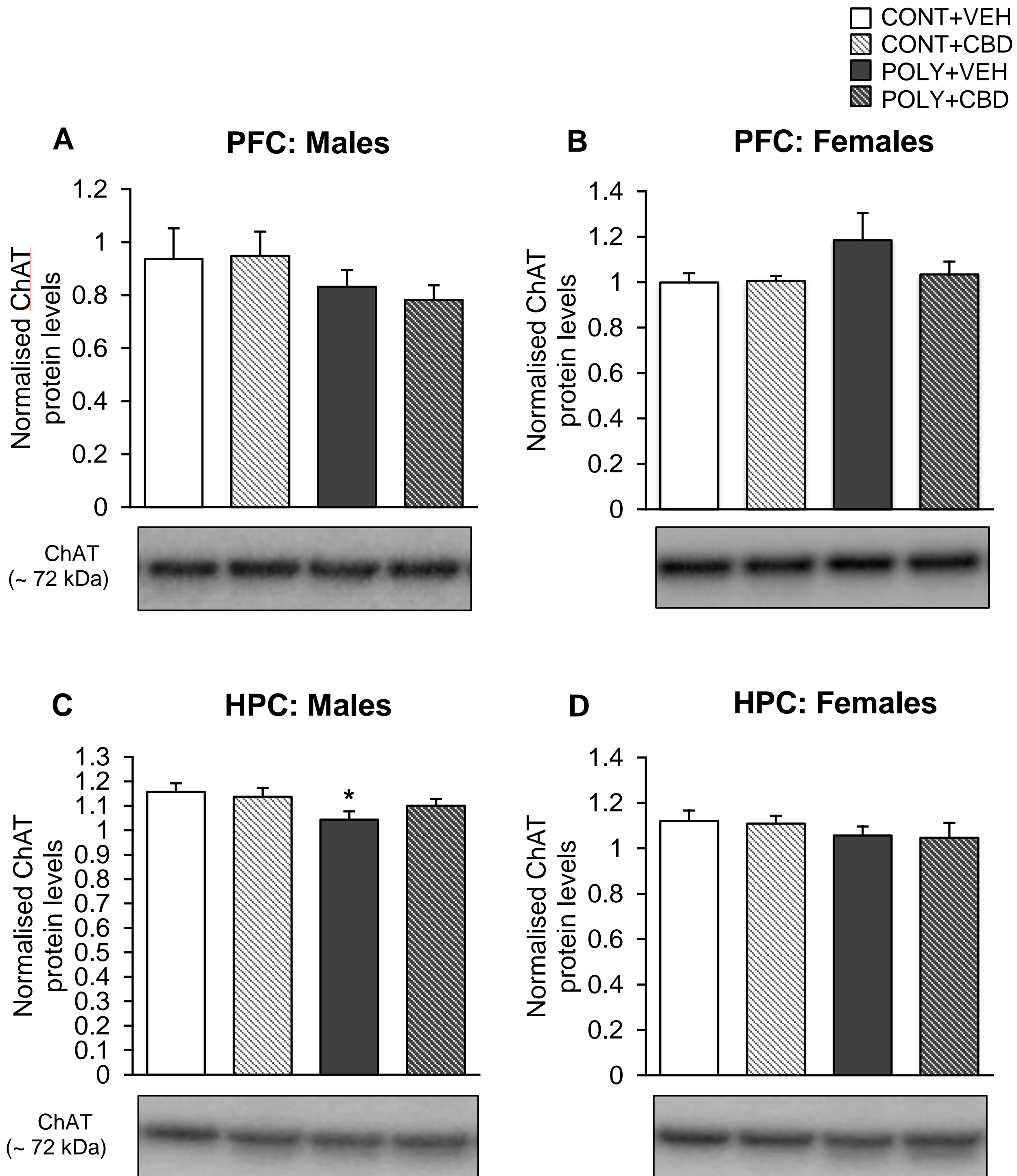
315 **3.2 ChAT Protein Expression in the PFC and HPC of Offspring**

316 In the PFC, there were no significant main effects of PRENATAL INFECTION ($F_{(1, 28)} = 2.55$,
317 $p = 0.12$) or OFFSPRING TREATMENT ($F_{(1, 28)} = 0.50$, $p = 0.83$) and no significant
318 interaction between these factors ($F_{(1, 28)} = 0.13$, $p = 0.72$) in male offspring (Figure 4A).
319 Similarly in adult female offspring, no significant effects or interactions were observed on
320 ChAT protein levels in the PFC (Figure 4B) (PRENATAL INFECTION: $F_{(1, 27)} = 2.30$, $p =$
321 0.14; OFFSPRING TREATMENT: $F_{(1, 27)} = 1.04$, $p = 0.32$; PRENATAL INFECTION x
322 OFFSPRING TREATMENT: $F_{(1, 27)} = 1.22$, $p = 0.28$).

323

324 On the other hand, there was a significant main effect of PRENATAL INFECTION ($F_{(1, 27)} =$
325 5.154, $p = 0.03$) in the male HPC (CA1/2 and CA3 regions combined). Further analysis
326 revealed a small yet significant decrease in ChAT protein expression in the HPC of male poly
327 I:C offspring compared to the controls (-10.1%, POLY+VEH vs CONT+VEH, $p = 0.02$)
328 (Figure 4C). However, there was no significant effect of OFFSPRING TREATMENT on
329 ChAT protein expression in the HPC ($F_{(1, 27)} = 0.29$, $p = 0.60$) and no interaction between
330 PRENATAL INFECTION and OFFSPRING TREATMENT ($F_{(1, 27)} = 1.36$, $p = 0.25$) in the
331 male HPC. Hippocampal ChAT protein levels were unaltered in female offspring
332 (PRENATAL INFECTION: $F_{(1, 28)} = 1.74$, $p = 0.20$; OFFSPRING TREATMENT: $F_{(1, 28)} =$
333 0.05, $p = 0.82$; PRENATAL INFECTION x OFFSPRING TREATMENT: $F_{(1, 28)} < 0.001$, $p =$
334 0.99) (Figure 4D).

335



336

337 **Figure 4**: The effect of cannabidiol (CBD) or vehicle (VEH) treatment on choline
338 acetyltransferase (ChAT) protein levels in the (**A**, **B**) prefrontal cortex (PFC) and (**C**, **D**)
339 hippocampus (HPC) in male and female control (CONT) and poly I:C (POLY) offspring.
340 Representative immunoblots for ChAT are shown underneath each graph. Signal intensity was
341 normalised to total protein levels in the respective lane and an internal control sample. **p*<0.05
342 vs. CONT+VEH group. Data expressed as mean ± SEM. (*n* = 7-8 rats per group).

343 **3.3 AChE Protein Expression in the PFC and HPC of Offspring**

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

350

351 Similar results were identified in the HPC, with no significant effects or interactions observed
352 in male (Figure 5C) (PRENATAL INFECTION: $F_{(1, 28)} = 0.56$, $p = 0.46$; OFFSPRING
353 TREATMENT: $F_{(1, 28)} = 0.71$, $p = 0.41$; PRENATAL INFECTION x OFFSPRING
354 TREATMENT: $F_{(1, 28)} = 0.58$, $p = 0.46$) or female offspring (Figure 5D) (PRENATAL
355 INFECTION: $F_{(1, 28)} = 0.09$, $p = 0.76$; OFFSPRING TREATMENT: $F_{(1, 28)} = <0.001$, $p =$
356 0.99; PRENATAL INFECTION x OFFSPRING TREATMENT: $F_{(1, 28)} = 0.39$, $p = 0.54$).

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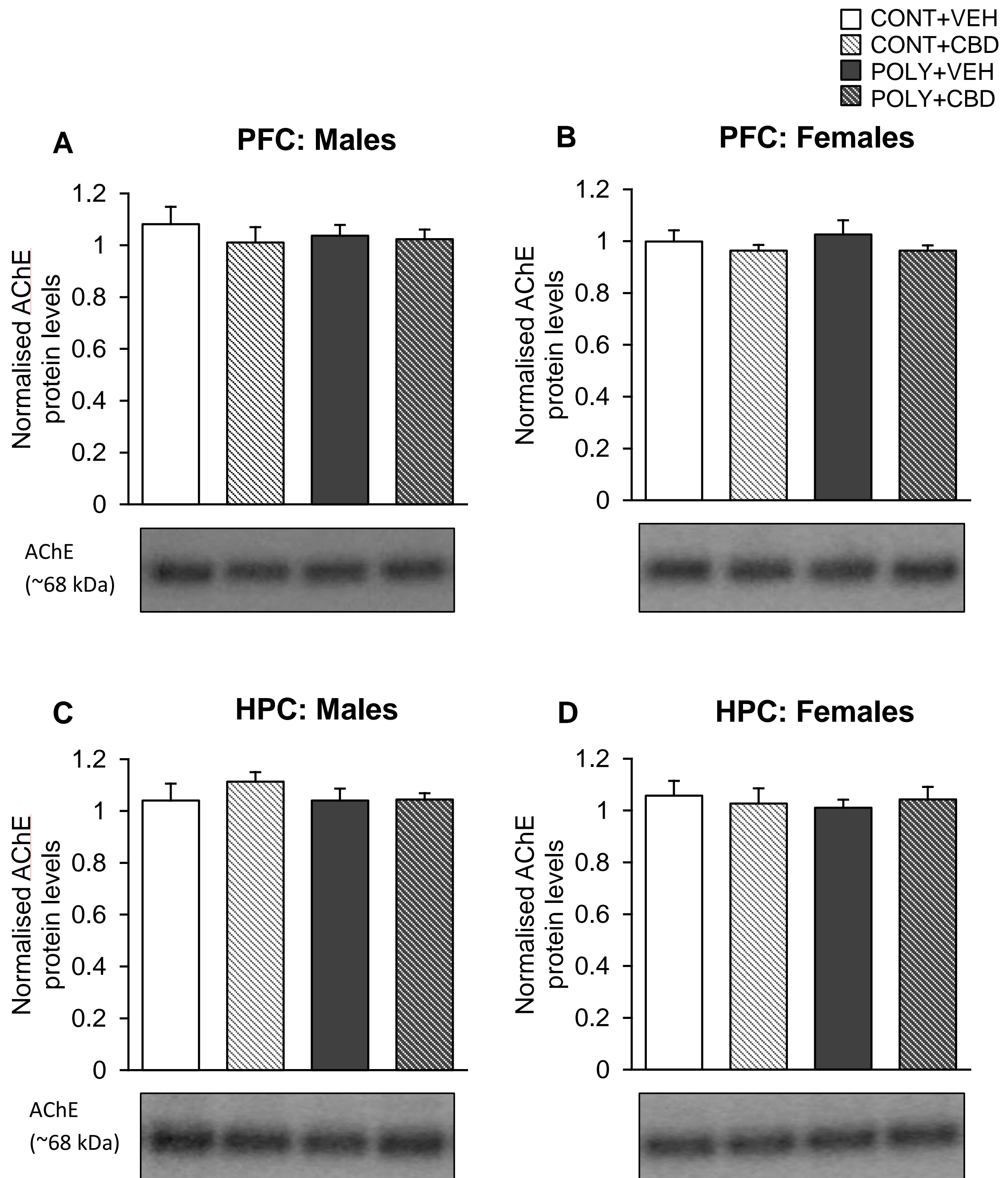

365

366 **Figure 5**: The effect of cannabidiol (CBD) or vehicle (VEH) treatment on acetylcholinesterase
367 (AChE) protein levels in the (**A**, **B**) prefrontal cortex (PFC) and (**C**, **D**) hippocampus (HPC) in
368 male and female control (CONT) and poly I:C (POLY) offspring. Representative immunoblots
369 for AChE are shown underneath each graph. Signal intensity was normalised to total protein
370 levels in the respective lane and an internal control sample. Data expressed as mean ± SEM. (*n*
371 = 7-8 rats per group).

372

373 **3.4 Correlations**

374 Correlations were performed to assess the relationship between markers of muscarinic
375 neurotransmission in male and female offspring. In the PFC, protein levels of ChAT and AChE
376 were positively correlated in female (r = 0.57, p = 0.001) (Figure 6A) and male offspring (r =
377 0.45, p = 0.011) (Figure 6B). There was also a positive association between AChE protein
378 levels and M1/M4R binding density levels in the HPC of female offspring (r = 0.64, p = 0.003)
379 (Figure 6C), but this relationship was not apparent in males (r = -0.10, p = 0.67) (Figure 6D).
380 We also examined correlations between markers of the muscarinic system in the HPC and PFC
381 of male and female offspring and their previously reported cognitive behaviour [7, 17].
382 Performance in the T-maze test (percentage of correct entries) was positively correlated with
383 M1/M4R binding density levels (r = 0.474, p = 0.04) (Figure 6E) and AChE protein levels (r =
384 0.503, p = 0.004) (Figure 6F) in the HPC of female offspring that showed comparable working
385 memory across treatment groups. Conversely, in male offspring that did show treatment group
386 differences in working memory performance, these correlations were not significant
387 (M1/M4R: r = 0.15, p = 0.52; AChE: (r = 0.04, p = 0.83). Instead, T-maze performance of male
388 offspring was positively correlated to hippocampal ChAT protein levels (r = 0.41, p = 0.03)
389 (Figure 6G).

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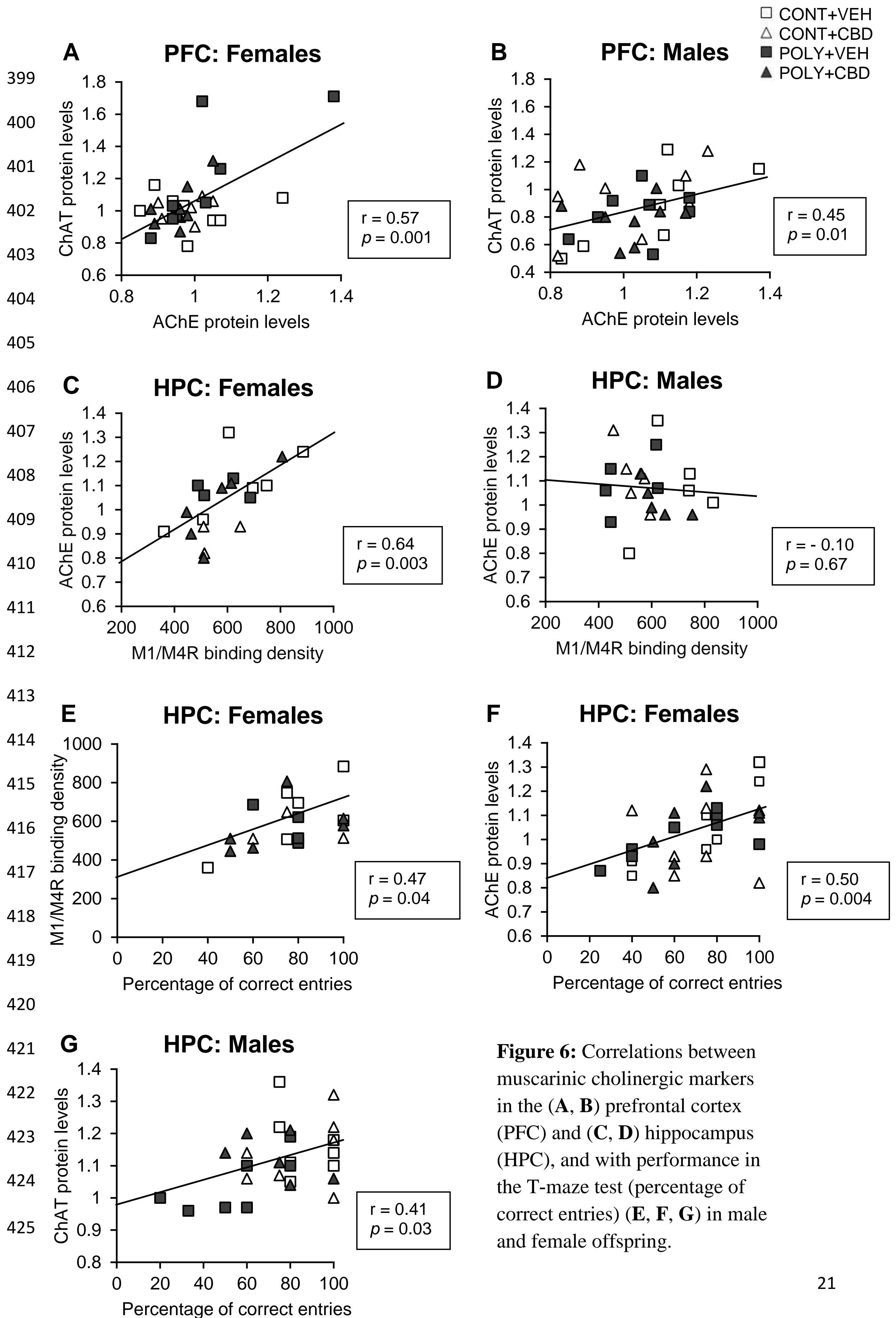

Figure 6: Correlations between muscarinic cholinergic markers in the (**A**, **B**) prefrontal cortex (PFC) and (**C**, **D**) hippocampus (HPC), and with performance in the T-maze test (percentage of correct entries) (**E**, **F**, **G**) in male and female offspring.

426 **4. DISCUSSION**

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

438

439 Our finding of an M1/M4R downregulation in the PFC and HPC of male poly I:C offspring
440 compared to offspring born to control dams is consistent with numerous post-mortem studies
441 that have reported decreased levels of M1/M4R binding density in the schizophrenia brain,
442 including the PFC [39-41], anterior and posterior cingulate cortices [42, 43], superior temporal
443 gyrus [44], HPC [39, 45, 46] and caudate-putamen [47]. This result suggests that the poly I:C
444 model mimics muscarinic dysfunction in a manner that is relevant to human schizophrenia;
445 however, we also found that M1/M4R binding density was unaltered in the brains of female
446 offspring. The existence of sex differences in the M1/M4R in the human schizophrenia brain
447 is unclear. Reduced [^{3}H]pirenzepine binding to M1/M4R in the HPC and PFC has been
448 reported in the post-mortem schizophrenia brain in studies using predominantly (70-80%) male
449 tissue cohorts [39, 40]; however, decreased M1/M4R binding density has also been reported in
450 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,

476 hippocampal M1/M4R binding density and AChE protein levels did not correlate with each
477 other, or with T maze performance. Taken together, these findings could suggest that tight
478 regulation of M1/M4R expression and ACh metabolism in the HPC is important for functional
479 working memory. In other studies, decreased ChAT levels and M1R G protein-coupling were
480 reported in the PFC of Alzheimer's disease patients with severe cognitive impairment, with
481 M1R levels correlating to the rate of cognitive decline [50]. Decreased muscarinic receptor G
482 protein-coupled signalling in the PFC and HPC correlated to reduced spatial memory in aged
483 rats [51] and decreased ChAT was correlated to age-related learning and memory deficits in
484 mice [52]. Spatial working memory tasks that involve the HPC appear dependent on adequate
485 cholinergic neurotransmission, particularly M1Rs that are expressed on excitatory pyramidal
486 neurons [53]; therefore, hippocampal M1R downregulation may explain, in-part, the working
487 memory deficits that we observed in male offspring.

488

489 Down-regulated M1/M4R binding density in the poly I:C model could represent reduced
490 cholinergic neurotransmission or a compensatory decrease in receptor density due to hyper-
491 cholinergic tone. However, while the mechanism underlying the CBD-induced alterations to
492 muscarinic receptor density is unclear, one study recently reported that systemic administration
493 of CBD to mice dose-dependently increased ACh levels in the forebrain within hours [24].
494 Indeed, we found that levels of the ACh synthesising enzyme, ChAT, were reduced in the HPC
495 of poly I:C offspring, whereas CBD treatment resulted in control-like ChAT levels. On the
496 other hand, the degrading enzyme, AChE, was unaltered in the HPC or PFC of any of the
497 treatment groups. Therefore, the downregulated M1/M4R binding density in the poly I:C model
498 is likely to have represented reduced cholinergic tone (ACh and M1/M4R signalling), whereas
499 CBD alters muscarinic receptor density by restoring normal levels of ACh synthesis, not
500 preventing degradation. To that effect, we observed a positive correlation between ChAT and

501 percentage of correct entries in the T Maze in male offspring, but no significant correlation
502 between M1/M4R or AChE levels and cognitive performance in males. We also observed a
503 significant positive correlation between ChAT and AChE protein levels in the PFC of both
504 male and female offspring, suggesting that acetylcholine synthesis and metabolism is tightly
505 regulated in this brain region. Our finding that CBD did not reduce AChE protein expression
506 suggests that this molecule may not play a major role in CBD-induced cognitive performance;
507 however, AChE enzymatic activity may be worth examining in future studies. The CB1R
508 agonist Δ9-THC is a competitive inhibitor of AChE, *in-vitro* [54], but Δ9-THC effects on
509 cognition can be either deleterious or beneficial. On the other hand, CBD may have a different
510 mechanism of action as it did not alter AChE in the present study and was previously identified
511 as a CB1R negative allosteric modulator, ie binds to an allosteric site on the receptor to reduce
512 its activity, while traditional agonists/antagonists bind to the orthosteric site [55, 56].
513 Interestingly, cholinergic neurons projecting from the septum to the HPC release ACh, which
514 increases production of post-synaptic endocannabinoids (via the M1R [57]) that regulate
515 hippocampal excitation by controlling major inhibitory and excitatory neurotransmitters,
516 GABA and glutamate, respectively [58]. Hippocampal excitation then affects signalling to
517 higher brain regions, including the PFC, and is thought to contribute to the psychotic and
518 cognitive symptoms of schizophrenia [59, 60]. Indeed, we previously reported imbalances in
519 the level of glutamatergic NMDAR binding density in the PFC of female poly I:C offspring,
520 while CBD treatment restored levels to that of the controls [17]. Taken together, CBD may
521 alter signalling in the HPC and PFC through interactions with the cholinergic muscarinic,
522 glutamatergic, GABAergic and endogenous cannabinoid systems to influence cognitive
523 behaviour. However, further studies are required to confirm this mechanism.
524

525 Given that CBD can improve cognitive function in a range of disease states that manifest
526 impairment [9], its pro-cognitive effects may be attributed to a number of mechanisms. For
527 example, one study showed that CBD is a dopamine D2 receptor (D2R) partial agonist, similar
528 to the antipsychotic drug, aripiprazole [61]. Differing to risperidone and olanzapine, which
529 have been associated with impaired cognition in some schizophrenia patients [62], aripiprazole
530 improves cognition, including processing speed, attention and memory [63]. D2R activation
531 has also been shown to increase hippocampal ACh levels in rats [64]. Therefore, the D2R
532 partial agonist activity of CBD may be a contributing mechanism in its pro-cognitive effects
533 via cholinergic signalling in the poly I:C model. CBD is also anti-inflammatory,
534 neuroprotective and can stimulate neurogenesis [reviewed in 9, 10]. In a mouse model of
535 cerebral malaria, CBD improved cognitive performance that was associated with increased
536 neurogenic markers, such as brain-derived neurotrophic factor (BDNF), and reduced pro-
537 inflammatory cytokines, such as tumour necrosis factor (TNF)-α, in the HPC and PFC [65].
538 Therefore, future studies may consider investigating the role of these markers in the
539 mechanisms underlying the pro-cognitive effects of CBD in the poly I:C model. In addition,
540 while this study focussed on brain regions relevant to cognitive function, the mechanisms
541 underlying the improvements in social interaction in the poly I:C model of schizophrenia
542 following CBD treatment remain unknown [7] and further investigations focussing on regions
543 involved in social functioning, particularly glutamatergic and GABAergic signalling in the
544 amygdala [66], are required.
545
546 Our study and others [67], have shown that CBD is psychoactive (i.e. alters brain signalling
547 and changes behaviour), and is a promising novel therapeutic for a range of illnesses [9, 10].
548 However, the result that CBD treatment decreases M1/M4R binding density in the HPC and
549 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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