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Effect of cannabidiol on endocannabinoid, glutamatergic and GABAergic signalling markers in male offspring of a maternal immune activation (poly I:C) model relevant to schizophrenia

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Abstract

The mainstay treatment for schizophrenia is antipsychotic drugs (APDs), which are mostly effective against the positive symptoms (e.g. hallucinations), but provide minimal benefits for the negative symptoms (e.g. social withdrawal) and cognitive deficits. We have recently shown that treatment with the non-intoxicating phytocannabinoid, cannabidiol (CBD), can improve cognition and social interaction deficits in a maternal immune activation (MIA) model relevant to the aetiology of schizophrenia, however, the mechanisms underlying this effect are unknown. An imbalance in the main excitatory (glutamate) and inhibitory (GABA) neurotransmitter systems in the brain plays a role in the pathophysiology of schizophrenia. Therefore, the endocannabinoid system could represent a therapeutic target for schizophrenia as a regulator of glutamate and GABA release via the CB1 receptor (CB1R). This study investigated the effects of chronic CBD treatment on markers of glutamatergic, GABAergic and endocannabinoid signalling in brain regions implicated in social behaviour and cognitive function, including the prefrontal cortex (PFC) and hippocampus (HPC). Time-mated pregnant Sprague-Dawley rats ($n = 16$) were administered poly I:C (4 mg/kg, i.v.) or saline (control) on gestational day 15. Male offspring were injected with CBD (10 mg/kg, i.p.) or vehicle twice daily from postnatal day 56 for 3 weeks. The prefrontal cortex (PFC) and hippocampus (HPC) were collected for post-mortem receptor binding and Western blot analyses ($n = 8$ per group). CBD treatment attenuated poly I:C-induced deficits in cannabinoid CB1 receptor binding in the PFC and glutamate decarboxylase 67, the enzyme that converts glutamate to GABA, in the HPC. CBD treatment increased parvalbumin levels in the HPC, regardless of whether offspring were exposed to poly I:C in utero. Conversely, CBD did not affect N-methyl-d-aspartate receptor and gamma-aminobutyric acid (GABA) A receptor binding or protein levels of fatty acid amide hydrolase, the enzyme that degrades the endocannabinoid, anandamide. Overall, these findings show that CBD can restore cannabinoid/GABAergic signalling deficits in regions of the brain implicated in schizophrenia pathophysiology following maternal poly I:C exposure. These findings provide novel evidence for the potential mechanisms underlying the therapeutic effects of CBD treatment in the poly I:C model.

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51 **Abstract**

52 The mainstay treatment for schizophrenia is antipsychotic drugs (APDs), which are mostly
53 effective against the positive symptoms (e.g. hallucinations), but provide minimal benefits for
54 the negative symptoms (e.g. social withdrawal) and cognitive deficits. We have recently
55 shown that treatment with the non-intoxicating phytocannabinoid, cannabidiol (CBD), can
56 improve cognition and social interaction deficits in a maternal immune activation (MIA)
57 model relevant to the aetiology of schizophrenia, however, the mechanisms underlying this
58 effect are unknown. An imbalance in the main excitatory (glutamate) and inhibitory (GABA)
59 neurotransmitter systems in the brain plays a role in the pathophysiology of schizophrenia.
60 Therefore, the endocannabinoid system could represent a therapeutic target for schizophrenia
61 as a regulator of glutamate and GABA release via the CB1 receptor (CB1R). This study
62 investigated the effects of chronic CBD treatment on markers of glutamatergic, GABAergic
63 and endocannabinoid signalling in brain regions implicated in social behaviour and cognitive
64 function, including the prefrontal cortex (PFC) and hippocampus (HPC). Time-mated
65 pregnant Sprague-Dawley rats (n = 16) were administered poly I:C (4 mg/kg, i.v.) or saline
66 (control) on gestational day 15. Male offspring were injected with CBD (10 mg/kg, i.p.) or
67 vehicle twice daily from postnatal day 56 for 3 weeks. The prefrontal cortex (PFC) and
68 hippocampus (HPC) were collected for post-mortem receptor binding and Western blot
69 analyses (n = 8 per group). CBD treatment attenuated poly I:C-induced deficits in
70 cannabinoid CB1 receptor binding in the PFC and glutamate decarboxylase 67, the enzyme
71 that converts glutamate to GABA, in the HPC. CBD treatment increased parvalbumin levels
72 in the HPC, regardless of whether offspring were exposed to poly I:C in utero. Conversely,
73 CBD did not affect *N*-methyl-D-aspartate receptor and gamma-aminobutyric acid (GABA) A
74 receptor binding or protein levels of fatty acid amide hydrolase, the enzyme that degrades the
75 endocannabinoid, anandamide. Overall, these findings show that CBD can restore

76 cannabinoid/GABAergic signalling deficits in regions of the brain implicated in
77 schizophrenia pathophysiology following maternal poly I:C exposure. These findings provide
78 novel evidence for the potential mechanisms underlying the therapeutic effects of CBD
79 treatment in the poly I:C model.

80 **1. Introduction**

81

82 The prefrontal cortex (PFC) and hippocampus (HPC) are anatomically connected and
83 synchrony between the two regions is important for normal brain function (Li et al., 2015).
84 However, people with schizophrenia exhibit structural abnormalities in these brain regions,
85 including reduced hippocampal volume and cortical thinning in the PFC (Dietsche et al.,
86 2017; van Erp et al., 2016). In addition, patients show abnormal activity in the default mode
87 network (i.e. the network of brain regions that are active at rest) and during memory tasks
88 (e.g. evidenced by reduced gamma oscillations), suggesting that dysfunction in these regions
89 may underlie the symptomatology of the disorder, particularly the negative and cognitive
90 symptom domains (Gonzalez-Burgos and Lewis, 2012; Guo et al., 2017). Unfortunately, the
91 negative and cognitive symptoms tend to precede the onset of psychosis, are persistent over
92 the course of the disorder, and are associated with poor functional outcomes in patients
93 (Lindenmayer et al., 2013; Barch and Ceaser, 2012). Although antipsychotic drugs (APDs)
94 are generally effective at controlling the positive symptoms of schizophrenia (e.g.
95 hallucinations and delusions), the drugs have poor efficacy against the negative symptoms
96 (e.g. social withdrawal) (Lindenmayer et al., 2013) and cognitive deficits of schizophrenia
97 (Goff et al., 2011), and in some cases can worsen cognition (Hill et al., 2010). Despite the
98 introduction of newer ‘second generation’ APDs to the market, this drug class does not
99 exhibit superior efficacy to ‘first generation’ APDs (reviewed in MacKenzie et al., 2018).
100 Therefore, new therapeutic options that can address the negative and cognitive symptom
101 domains of schizophrenia are required.

102

103 Cannabidiol (CBD), the main non-intoxicating phytocannabinoid found in the cannabis plant,
104 has the potential to alleviate symptoms across a range of pathological conditions, including

105 epilepsy, chronic pain, anxiety and movement disorders (e.g. Parkinson's disease) (Crippa et
106 al., 2018; Osborne et al., 2017b). However, there are limited studies that have investigated the
107 chronic effects of CBD treatment in schizophrenia. CBD (800 mg, 4 weeks) significantly
108 improved symptoms (measured on the Positive and Negative Syndrome Scale (PANSS)) in
109 acute paranoid schizophrenia patients in a manner comparable to amisulpride, but had a more
110 favourable side effect profile (e.g. less body weight gain) (Leweke et al., 2012). Recent
111 clinical trials have explored the therapeutic potential of CBD as an adjunct to existing APD
112 medications of stable schizophrenia outpatients. After 6 weeks of treatment, PANSS scores of
113 the CBD-treated group (1000 mg/day, 6 weeks) significantly improved compared to placebo,
114 while cognition improved from baseline (assessed using Brief Assessment of Cognition in
115 Schizophrenia), but fell short of statistical significance against placebo ($p = 0.068$) (McGuire
116 et al., 2018). In contrast, a similar trial that used a lower dose of CBD (600 mg/day, 6 weeks)
117 found no improvement on PANSS or the MATRICS Consensus Cognitive Battery compared
118 to baseline (Boggs et al., 2018), suggesting that dosage may be a critical factor for the
119 efficacy of CBD in schizophrenia, particularly in APD-treated patients. Our laboratory
120 recently reported that CBD treatment improved cognition and social interaction in adult male
121 rat offspring exposed to maternal immune activation (MIA) using polyinosinic-polycytidilic
122 (poly I:C) acid (Osborne et al., 2017a), which mimics some of the positive and negative
123 symptoms, and cognitive deficits observed in schizophrenia (Meyer and Feldon, 2012).
124 However, the mechanisms by which CBD improves negative and cognitive phenotypes in
125 poly I:C offspring is unknown.

126

127 The endogenous cannabinoid (eCB) system plays an important role in various physiological
128 functions, including neuroprotection, synaptic plasticity, memory and reward processing. The
129 cannabinoid CB1 receptor (CB1R) is the main receptor of the eCB system in the brain with

130 high expression levels in regions involved in cognitive function, including the PFC and HPC
131 (Lu and Mackie, 2016). Multiple studies have implicated eCB system dysregulation in the
132 pathophysiology of schizophrenia, including alterations in CB1R expression (reviewed in
133 Ferretjans et al., 2012), its endogenous ligand anandamide (AEA) (De Marchi et al., 2003;
134 Giuffrida et al., 2004; Koethe et al., 2009; Leweke et al., 1999), and fatty acid amide
135 hydrolase (FAAH), the enzyme primarily responsible for the degradation of AEA (Bioque et
136 al., 2013; Takata et al., 2013). The eCB system is functionally linked to the major excitatory
137 (glutamatergic) and inhibitory (GABAergic) neurotransmitter systems in the brain, as
138 endogenous cannabinoids bind to the CB1R and dampen presynaptic glutamate and/or
139 GABA release (Viveros et al., 2012). An imbalance in glutamatergic and GABAergic
140 signalling in the brain has been implicated in the cognitive deficits of schizophrenia
141 (Gonzalez-Burgos and Lewis, 2012). Post-mortem schizophrenia studies report alterations in
142 the ionotropic glutamatergic *N*-methyl-D-aspartate receptor (NMDAR) and its obligatory
143 GluN1 subunit (Catts et al., 2016), and gamma-aminobutyric acid alpha receptor (GABA_AR)
144 (Gonzalez-Burgos and Lewis, 2008), as well as a reduction in glutamate decarboxylase 67
145 (GAD₆₇; the rate-limiting enzyme that converts glutamate to GABA) and the calcium binding
146 protein parvalbumin (PV), expressed on GABAergic interneurons (Cohen et al., 2015). The
147 interaction of CBD with the eCB, glutamatergic and GABAergic systems is not well
148 understood. CBD blocks the effects of CB1R/CB2 combined receptor agonists (McPartland
149 et al., 2015; Pertwee, 2008; Thomas et al., 2007), acting as a CB1R negative allosteric
150 modulator (NAM) *in vitro* (Laprairie et al., 2015) and may increase AEA levels by inhibiting
151 FAAH activity (Leweke et al., 2012). Studies show that CBD can prevent behavioural and
152 neurochemical deficits induced by NMDAR antagonism (Gomes et al., 2015a; Gururajan et
153 al., 2012), and can act as a positive allosteric modulator of the GABA_AR to increase
154 inhibitory tone (Bakas et al., 2017). Therefore, the aim of this study was to examine the

155 effects of CBD treatment on eCB (CB1R binding density and FAAH protein levels), as well
156 as glutamatergic (NMDAR binding and subunit levels) and GABAergic (GABA_AR binding
157 density, GAD₆₇ and PV protein levels) markers in the brains of poly I:C offspring that exhibit
158 cognitive deficits.

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160

161 **2. Experimental Procedures**

162

163 2.1 Ethics Statement

164 Experimental procedures were approved by the Animal Ethics Committee of the University
165 of Wollongong, NSW, Australia (AE15/05) and complied with the National Health and
166 Medical Research Council (NHMRC), Australian Code of Practice for the Care and Use of
167 Animals for Scientific Purposes (NHMRC, 2013). All efforts were made to minimise the
168 number and suffering of animals.

169

170 2.2 Animal Experiments

171 The detailed methods used for the animal experiments have been reported previously
172 (Osborne et al., 2017a) in accordance with the Animal Research: Reporting of *In Vivo*
173 Experiments (ARRIVE) guidelines (Kilkenny et al., 2010). Briefly, 16 time-mated pregnant
174 Sprague-Dawley rats (12 weeks old, gestational day (GD) 15; Animal Resources Centre,
175 Perth, WA) were administered poly I:C (4 mg/kg, i.v; Sigma-Aldrich, Castle Hill, Australia)
176 or saline (control) to the lateral tail vein. Offspring were not cross-fostered and were
177 maintained with their respective dams until weaning. After weaning (postnatal day (PN) 21),
178 male offspring were pair-housed with same-treatment littermates. Offspring were
179 administered CBD (10 mg/kg; i.p.; THC-Pharm GmbH, Frankfurt, Germany) dissolved in

180 Tween 80 and saline (vehicle, 1:16 (v/v); Chem Supply, Gillman, Australia), or vehicle
181 (control), twice daily at 12 hourly intervals from PN 56 to PN 80, equating to late
182 adolescence/early adulthood (Osborne et al., 2017a). The dose and duration of CBD
183 administration was based on studies that report improvement in models with cognitive
184 impairment following CBD treatment (Cassol et al., 2010; Barichello et al., 2012; Fagherazzi
185 et al., 2012; Schiavon et al., 2014). During the second week of treatment (i.e. PN72),
186 offspring underwent behavioural testing using the Novel Object Recognition, T-maze and
187 Social Interaction tests as reported previously (Osborne et al., 2017a). The day after the last
188 drug treatment, offspring were euthanased using carbon dioxide asphyxiation followed by
189 rapid decapitation, between 09:00 and 11:30 h to minimise the potential effects of circadian
190 rhythm variation on protein expression (Jasinska and Pyza, 2017). Whole brains were
191 removed, immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

192

193 2.3 Histological Procedures

194 Rat brains (n = 8 per group) were sectioned coronally into alternating 14 µM or 500 µM
195 sections using a cryostat (-17°C; Jung CM 3000, Leica Instruments GmbH, Nussloch,
196 Germany). Sections were collected from the PFC (containing the prelimbic and infralimbic
197 cortices; Bregma level: 4.2 mm to 2.56 mm) and HPC (containing the dorsal and ventral
198 subregions, Bregma level: - 4.36 mm to - 6.00 mm). Consecutive sections (2-3 per region, per
199 rat) were collected for receptor autoradiography experiments (14 µM) and thaw-mounted
200 onto PolysineTM slides (Sigma-Aldrich, Castle Hill, NSW, Australia) then stored at -20°C
201 until further analysis. Sections (2-3 per region, per rat) collected for Western blot
202 experiments (500 µM) were mounted on glass slides and regions of interest were
203 microdissected using a micropuncture kit, then stored at -80°C until further use. This

204 dissection protocol allowed investigation of receptor binding and protein levels from the
205 same animals.

206

207 2.4 *In situ* Receptor Autoradiography

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209 2.4.1 CB1R binding

210 CB1R binding density was examined using *in situ* receptor autoradiography methods
211 previously reported by our laboratory (Weston-Green et al., 2012a; Yu et al., 2013). Briefly,
212 slides (3 slides per rat, per treatment group) were air-dried and incubated in 50 mM Tris-HCl
213 buffer (pH 7.4) with 0.1% bovine serum albumin (BSA) for 15 min at room temperature. To
214 determine total binding, sections were incubated in 50 mM Tris-HCl buffer (with 0.1% BSA)
215 containing 10 nM [³H]SR141716A (43 Ci/mmol; PerkinElmer TM Life and Analytical
216 Sciences, Boston, USA), a selective inverse agonist for the CB1R, for 60 min at room
217 temperature. Non-specific binding was determined by incubating additional sections in 10
218 nM [³H]SR141716A in the presence of 100 μM CP-55940 (Sapphire Bioscience, Redfern,
219 NSW, Australia), a non-selective agonist of cannabinoid receptors. After incubation, slides
220 were washed twice for 30 min in ice-cold buffer, rinsed in cold milliQ water and air-dried
221 overnight.

222

223 2.4.2 NMDAR binding

224 NMDAR binding density was examined based on methods previously described by our
225 laboratory (du Bois et al., 2009; Newell et al., 2007). Briefly, sections were incubated in 30
226 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid (HEPES) buffer pH 7.5,
227 containing 100 μM glycine, 100 μM glutamate, 1 mM ethylenediaminetetraacetic acid
228 (EDTA) and 20 nM [³H]MK-801 (specific activity 22.5 Ci/mmol, PerkinElmer, Boston,

229 USA) for 2.5 hours at room temperature (RT). Non-specific binding was determined by
230 incubating sections in the same buffer containing [³H]MK-801 in the presence of 200 μM
231 unlabelled MK-801 (Sigma-Aldrich, Castle Hill, NSW, Australia). After incubation, sections
232 were washed twice in ice-cold 30 mM HEPES buffer containing 1 mM EDTA (pH 7.5) for
233 20 min each, rinsed in cold milliQ water and air-dried overnight.

234

235 2.4.3 GABA_AR binding

236 GABA_A receptor binding density was examined based on methods previously described by
237 our laboratory and others (du Bois et al., 2009; Xia and Haddad, 1992; Yu et al., 2013).
238 Briefly, sections were pre-incubated three times in 50 mM Tris-citrate (pH 7.0) buffer at 4°C
239 for 5 mins. Sections were incubated in 50 mM Tris-citrate buffer containing 50 nM [³H]
240 muscimol (specific activity 29.5 Ci/mmol, PerkinElmer, Boston, USA), a selective agonist
241 for the GABA_AR, for 45 min at 4°C. Non-specific binding was determined by incubating
242 sections in 50 mM Tris-citrate (pH 7.0) buffer containing [³H] muscimol and 100 μM GABA
243 (Sigma-Aldrich, Castle Hill, NSW, Australia). After incubation, sections were rinsed four
244 times for 2 sec each in 50 mM Tris-citrate buffer (4°C), rinsed in cold milliQ water and air-
245 dried overnight.

246

247 2.4.4 Quantification

248 Slides from CB1R, NMDAR and GABA_AR binding experiments were exposed to Amersham
249 Hyperfilm ECL (GE Healthcare Life Sciences, Parramatta, NSW, Australia) for 5 (NMDAR
250 and GABA_AR) or 8 weeks (CB1R) with a set of Amersham tritium standards. After
251 development, autoradiography films were de-identified, scanned using a GS-800 Imaging
252 Densitometer (Bio-Rad, Hercules, California, USA), and quantified (left and right
253 hemispheres) with Image J software (<https://imagej.nih.gov/ij>). Images were calibrated based

254 on the Rodbard curve obtained from the tritium standards to produce nCi/mg tissue
255 equivalent (TE) values. Specific binding density was estimated by subtracting non-specific
256 binding from total binding values. Values were converted to fmoles [³H] ligand per mg TE
257 taking into account the specific activity of the ligand, as we have previously reported
258 (Weston-Green et al., 2012a, 2012c, 2008). Tissue sections that were damaged were excluded
259 from analysis. Anatomical structures were confirmed using a standard rat brain atlas (Paxinos
260 and Watson, 2007).

261

262 2.5 Western Blot and Quantification

263 Brain samples containing the PFC and HPC (left and right hemispheres combined) were
264 homogenised in buffer containing 0.1 M Tris-HCl, 2 mM EDTA, 10% glycerol, 2% SDS, 0.5
265 mM PMSF, Protease Inhibitor Cocktail (P8340; Sigma-Aldrich, Australia) and Phosphatase
266 Inhibitor Cocktail 2 (P5726; Sigma-Aldrich, Australia) as previously described (Lum et al.,
267 2016). A DC assay kit was used to determine total protein concentration following the
268 manufacturer's instructions (Bio-Rad, Australia). Crude homogenates (10 µg protein; within
269 the linear range of detection of the primary antibodies, see Supplementary Figure 1) were
270 loaded into CriterionTM TGX Stain-FreeTM 4-20% gels (Bio-Rad, Australia) and underwent
271 electrophoresis in SDS buffer at 180V for 1 h. Gels were activated (GelDoc XR+ imaging
272 system; Bio-Rad, Australia) (Colella et al., 2012; Gürtler et al., 2013), proteins were
273 transferred on to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Australia) via
274 electrophoresis for 1 h at 100 V, and imaged (GelDoc XR+ imaging system; Bio-Rad,
275 Australia) to capture total protein in each lane (Colella et al., 2012; Gürtler et al., 2013).
276 Membranes were blocked in 5% milk (w/v) in Tris Buffered Saline with Tween 20® (TBST)
277 for 1 h at room temperature. To detect the proteins of interest, membranes were incubated
278 overnight at 4°C in the following primary antibodies: anti-FAAH (PFC: 1:2500, HPC: 1:5000

279 ; #ab54615, Abcam), anti-GluN1 (1:10 000; #MAB363, Millipore), anti-GAD₆₇ (1:7500
280 #MAB5406, Millipore), anti-PV (1:10 000; #Ab11427, Abcam). Membranes were washed in
281 TBST (5 x 5 min) and incubated at room temperature for 1 h with either horseradish
282 peroxidase (HRP)-conjugated goat anti-rabbit (1:5000; #AP307P, Millipore) or anti-mouse
283 secondary antibody (1:5000; #AP308P, Millipore). After washing in TBST (3 x 5 min),
284 membranes were incubated in Enhanced Chemiluminescence (ECL) reagents (Bio-strategy
285 Laboratory Products, Tingalpa, QLD Australia) and scanned using a Gel Imager (Amersham
286 600RB, GE Healthcare, Parramatta, NSW Australia). De-identified band signals were
287 quantified using Image Lab software (ver 6, Bio-Rad Laboratories Inc, California, USA). The
288 values for each signal were normalised to total protein in the respective lane to account for
289 loading variability (Colella et al., 2012; Gürtler et al., 2013). Values were then normalised to
290 an internal control sample (consisting of equal amounts of sample pooled together) to account
291 for inter-gel variability. Samples were assayed in duplicate at a minimum.

292

293 2.6 Statistical Analysis

294 Statistical analyses were performed using SPSS (Version 21.0, IBM Inc., Illinois, USA). Data
295 points that were $\pm 2SD$ from the mean were considered outliers and removed from analysis.
296 Data were tested for normality using Shapiro-Wilk tests. Two-way ANOVAs were used to
297 examine the effects of PRENATAL INFECTION (poly I:C vs. vehicle) and OFFSPRING
298 TREATMENT (CBD vs. vehicle) on receptor binding density and protein levels. Pairwise
299 comparisons (with Bonferroni adjustment) were used to examine differences between groups.
300 Data that were not normally distributed were transformed, retested for normality using
301 Shapiro-Wilk tests, and analysed using parametric testing. Variables that remained non-
302 normally distributed following transformation or that violated Levene's Test for Equality of
303 Variances were analysed using non-parametric Mann-Whitney U tests (indicated in the

304 Results section). Relevant comparisons were made between vehicle-treated control and poly
305 I:C offspring (CONT+VEH vs. POLY+VEH), vehicle and CBD-treated poly I:C offspring
306 (POLY+VEH vs. POLY+CBD), as well as vehicle and CBD-treated control offspring
307 (CONT+VEH vs. CONT+CBD). Where significant differences between the POLY+VEH and
308 POLY+CBD groups were observed, an additional comparison was made between the
309 CONT+VEH and POLY+CBD groups to determine if CBD restored the parameter to control
310 levels. *P* values less than 0.05 were considered statistically significant. Data are presented as
311 mean \pm standard error of the mean (SEM).

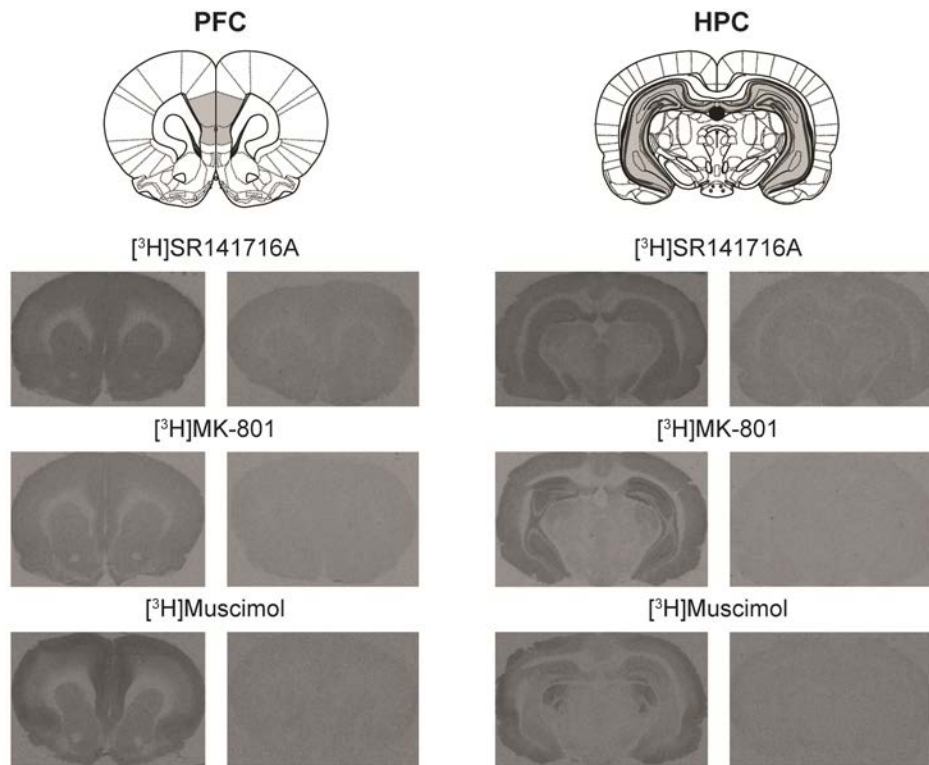
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314 **3. Results**

315 To determine the potential mechanisms underlying the beneficial effects of CBD, eCB,
316 NMDAR and GABAergic markers were analysed in the brains of control and poly I:C
317 offspring. Representative autoradiographs of CB1R ($[^3\text{H}]$ SR141716A), NMDAR ($[^3\text{H}]$ MK-
318 801) and GABA_AR ($[^3\text{H}]$ Muscimol) total binding density (as well as non-specific binding for
319 the respective ligands) in the PFC and HPC are shown (Figure 1). There was no difference in
320 receptor binding density along the dorsal-ventral axis of the HPC (Supplementary Figure 2);
321 therefore, the subregions were combined for analysis. Representative immunoblots showing
322 the expected bands for FAAH (Figure 2C, 2D), the obligatory GluN1 subunit of the NMDAR
323 (Figure 3C, 3D), GAD₆₇ (Figure 4C, 4D) and PV (Figure 4E, 4F) are displayed with their
324 respective graphs.

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341 **Figure 1:** Example autoradiographs showing cannabinoid CB1 receptor (CB1R), *N*-methyl-
342 *D*-aspartate receptor (NMDAR) and gamma-aminobutyric acid A receptor (GABA_AR)
343 binding in the adult rat brain. Schematic diagram adapted from a rat brain atlas (Paxinos and
344 Watson, 2007) showing the approximate Bregma level quantified for the prefrontal cortex
345 (PFC): 2.52 mm (containing the prelimbic and infralimbic cortices; shaded) and the
346 hippocampus (HPC): - 4.92 mm (shaded). Example autoradiographs demonstrate
347 [³H]SR141716A binding to CB1Rs, [³H]MK-801 binding to NMDARs and [³H]muscimol
348 binding to GABA_ARs in the PFC and HPC. Autoradiographs show total binding (left) and
349 non-specific binding (right) for each ligand. Example autoradiographs are from vehicle-
350 treated control offspring.

351
352

353 3.1 Effect of cannabidiol on endocannabinoid markers

354 CB1R binding density and FAAH protein levels were examined in the PFC and HPC to
355 determine if maternal poly I:C exposure could result in persistent alterations to eCB markers,
356 and whether chronic CBD treatment could reverse any changes. In the PFC, there was a

357 significant main effect of OFFSPRING TREATMENT ($F_{(1, 26)} = 6.481, p = 0.017$) and a
358 tendency for a PRENATAL INFECTION x OFFSPRING TREATMENT interaction ($F_{(1, 26)}$
359 $= 2.993, p = 0.095$), but no main effect of PRENATAL INFECTION ($F_{(1, 26)} = 1.679, p =$
360 0.206). Although the interaction did not reach statistical significance, visual inspection of the
361 graph showed that poly I:C offspring had lower CB1R binding density in the PFC compared
362 to control counterparts (-24.62%; POLY+VEH vs. CONT+VEH, $p = 0.042$) (Figure 2A).
363 CBD treatment restored CB1R binding deficits in poly I:C offspring (+46.16%; POLY+CBD
364 vs. POLY+VEH, $p = 0.006$) to control-like levels (POLY+CBD: 247.85 ± 15.19 vs.
365 CONT+VEH: $224.97 \pm 13.03; p = 0.275$) (Figure 2A). CBD administration did not
366 significantly alter CB1R binding density in the PFC of control offspring (CONT+CBD:
367 239.91 ± 19.92 vs. CONT+VEH: $224.97 \pm 13.03; p = 0.569$) (Figure 2A). The reduction in
368 CB1R binding density in poly I:C offspring appeared to be specific to the PFC, since there
369 were no significant effects of maternal poly I:C exposure or CBD treatment on CB1R binding
370 density in the HPC of offspring (PRENATAL INFECTION: $F_{(1, 23)} = 0.326, p = 0.574;$
371 OFFSPRING TREATMENT: $F_{(1, 23)} = 0.790, p = 0.383;$ PRENATAL INFECTION x
372 OFFSPRING TREATMENT: $F_{(1, 23)} = 0.321, p = 0.576$) (Figure 2B). FAAH protein levels
373 in the PFC did not differ significantly between treatment groups (PRENATAL INFECTION:
374 $F_{(1, 27)} = 0.112, p = 0.740;$ OFFSPRING TREATMENT: $F_{(1, 27)} = 1.610, p = 0.215;$
375 PRENATAL INFECTION x OFFSPRING TREATMENT: $F_{(1, 27)} = 0.690, p = 0.413$)
376 (Figure 2C). In line with the findings in the PFC, a two-way ANOVA showed no significant
377 effects on FAAH protein levels in the HPC (PRENATAL INFECTION: $F_{(1, 28)} = 0.066, p =$
378 $0.800,$ OFFSPRING TREATMENT: $F_{(1, 28)} = 0.356, p = 0.556,$ PRENATAL INFECTION x
379 OFFSPRING TREATMENT: $F_{(1, 28)} = 0.185, p = 0.671$) (Figure 2D).

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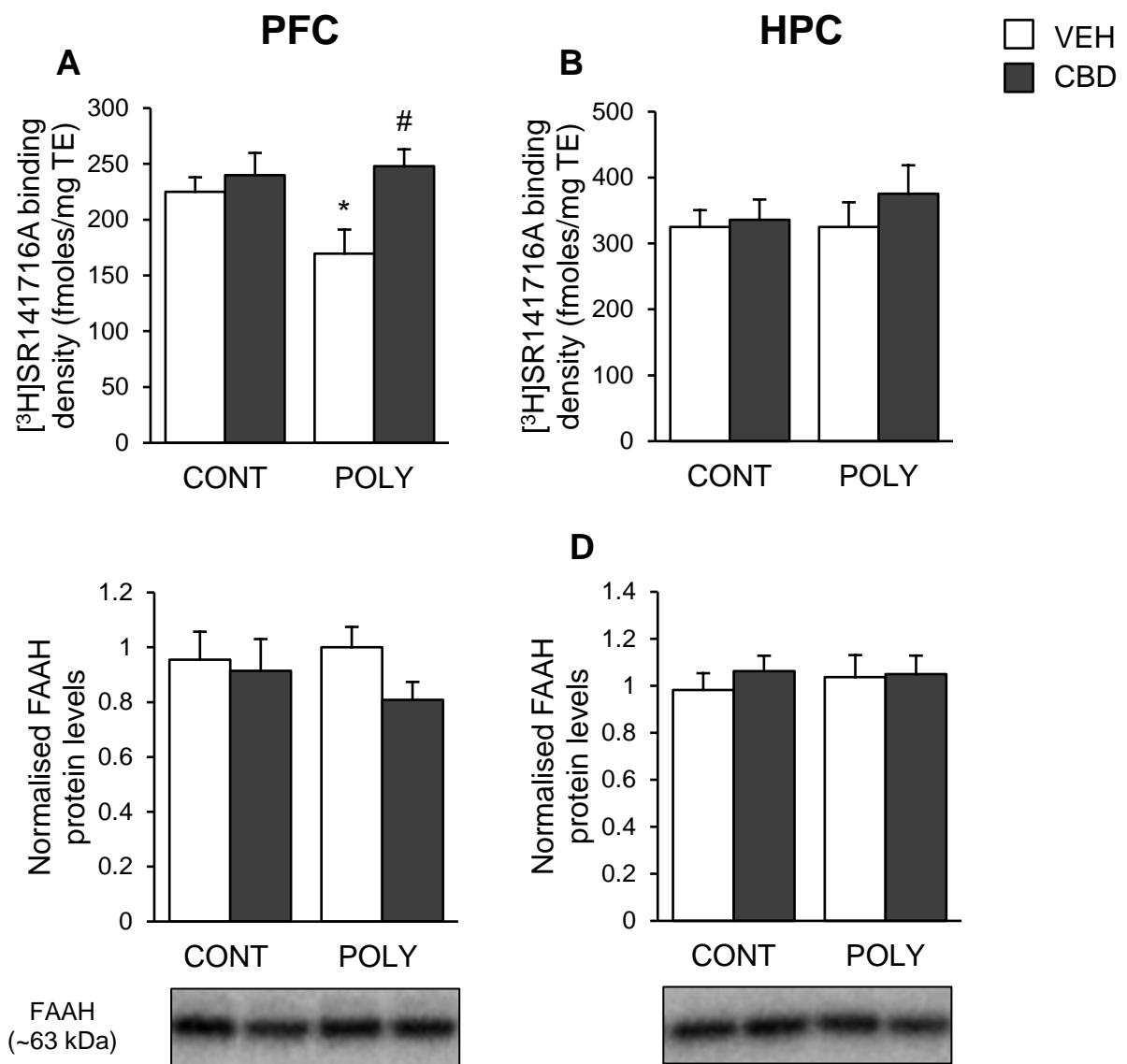


Figure 2: Cannabidiol (CBD) attenuated deficits in cannabinoid CB1R binding density in the prefrontal cortex (PFC) of male offspring exposed to maternal poly I:C infection. CB1R ($[^3\text{H}]$ SR141716A) binding density in the (A) PFC and (B) hippocampus (HPC) of offspring treatment groups. Normalised fatty acid amide hydrolase (FAAH) protein levels in the (C) PFC and (D) HPC with typical immunoblots for each treatment group shown underneath the graphs (normalised to total protein levels in the respective lanes). Data expressed as mean \pm SEM. $n = 6-8$ rats per group. * $p < 0.05$ vs. CONT+VEH, # $p < 0.05$ vs. POLY+VEH group. TE = tissue equivalent.

407 3.2 Effect of cannabidiol on the glutamatergic NMDAR

408 The effect of CBD treatment on NMDAR binding density and the obligatory GluN1 subunit
409 of the NMDAR were examined in the PFC and HPC of offspring. A two-way ANOVA
410 showed no significant effects of PRENATAL INFECTION ($F_{(1, 25)} = 0.054, p = 0.818$) or
411 OFFSPRING TREATMENT ($F_{(1, 25)} = 0.430, p = 0.518$) on NMDAR binding density in the
412 PFC, and no significant interaction between the factors ($F_{(1, 25)} = 0.224, p = 0.640$) (Figure
413 3A). A similar pattern was also observed in the HPC (PRENATAL INFECTION: $F_{(1, 23)} =$
414 $0.120, p = 0.732$; OFFSPRING TREATMENT: $F_{(1, 23)} = 2.256, p = 0.147$; PRENATAL
415 INFECTION x OFFSPRING TREATMENT: $F_{(1, 23)} = 0.712, p = 0.408$) (Figure 3B).
416 Reflecting the lack of change in binding density, GluN1 protein levels were not significantly
417 altered between treatment groups in the PFC (Figure 3C) or HPC (Figure 3D) (PFC:
418 PRENATAL INFECTION: $F_{(1, 27)} = 0.045, p = 0.833$; OFFSPRING TREATMENT: $F_{(1, 27)}$
419 $= 1.763, p = 0.195$; PRENATAL INFECTION x OFFSPRING TREATMENT: $F_{(1, 27)} =$
420 $1.524, p = 0.228$. HPC (Mann-Whitney U tests): POLY+VEH vs. CONT+VEH, $p = 0.600$;
421 POLY+CBD vs. POLY+VEH, $p = 0.916$; CONT+CBD vs. CONT+VEH, $p = 0.999$).

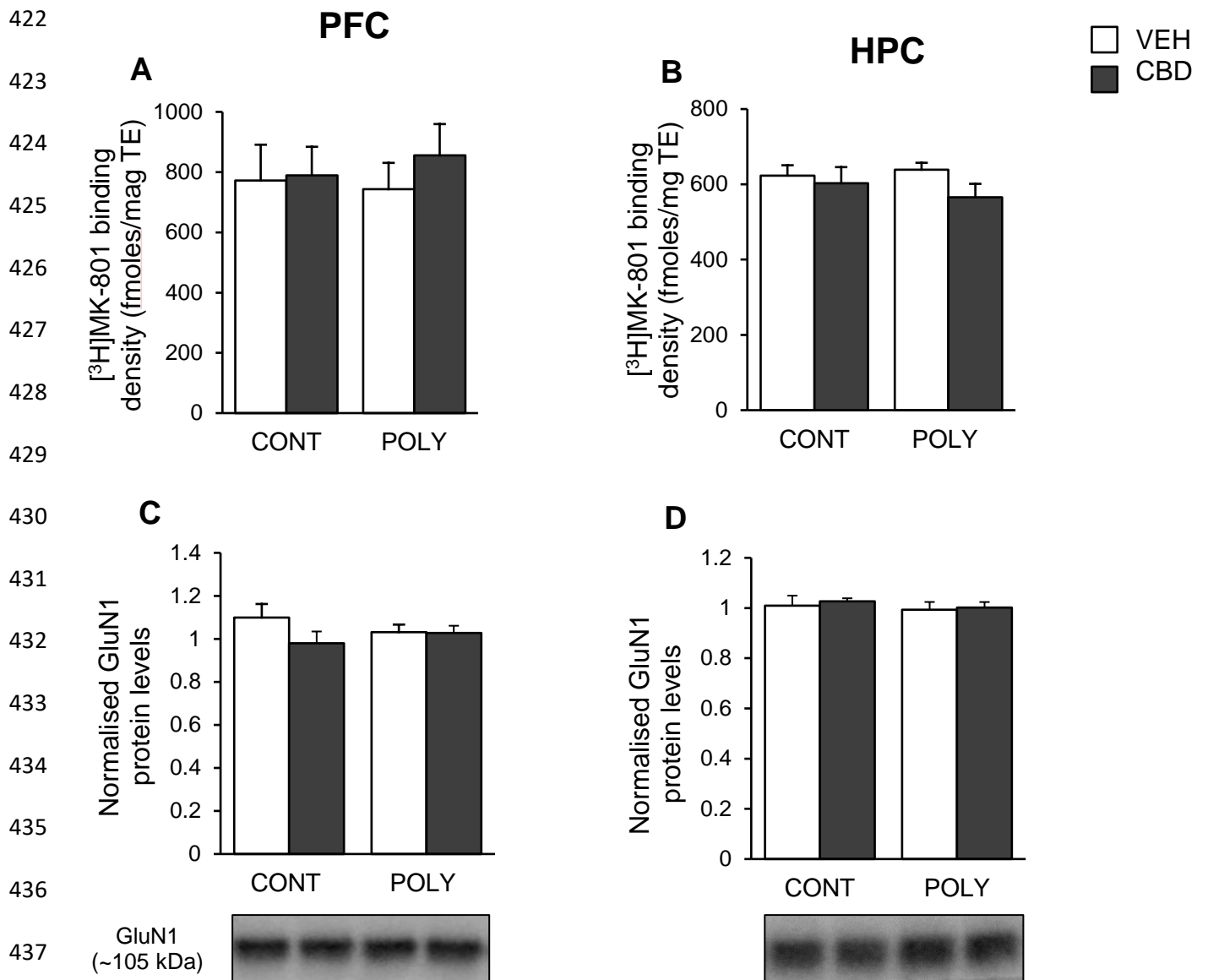


Figure 3: Cannabidiol (CBD) did not alter glutamatergic *N*-methyl-D-aspartate receptor (NMDAR) binding density or GluN1 protein levels in select brain regions of control (CONT) and poly I:C (POLY) offspring. NMDAR (^{3}H MK801) binding density in the (A) PFC and (B) HPC of offspring. Normalised GluN1 subunit protein levels (normalised to total protein levels in the respective lane) in the (C) PFC and (D) HPC, with typical GluN1 immunoblots (~105 kDa) shown underneath each graph. Data expressed as mean \pm SEM. $n = 6-8$ rats per group.

449

450 3.3 Effect of cannabidiol on GABAergic markers

451 GABA_AR binding density, as well as GAD₆₇ and PV protein levels were examined in the
452 PFC and HPC to determine whether CBD treatment could attenuate any changes in
453 GABAergic markers following maternal poly I:C exposure. Mann-Whitney U tests revealed
454 that poly I:C exposure did not significantly alter GABA_AR binding density in the PFC
455 (POLY+VEH vs. CONT+VEH: $p = 0.568$), and there was no significant impact of CBD
456 treatment on GABA_AR binding density in poly I:C (POLY+CBD vs. POLY+VEH: $p =$
457 0.668) or control offspring (CONT+CBD vs. CONT+VEH: $p = 0.317$) (Figure 4A).
458 Similarly, no significant changes in GABA_AR binding density were observed in the HPC
459 (PRENATAL INFECTION: $F_{(1, 22)} = 0.382$, $p = 0.543$; OFFSPRING TREATMENT: $F_{(1, 22)} =$
460 1.186 , $p = 0.288$; PRENATAL INFECTION x OFFSPRING TREATMENT: $F_{(1, 22)} =$
461 1.814 , $p = 0.192$) (Figure 4B). In the PFC, there was no significant difference in GAD₆₇
462 protein levels between groups (PRENATAL INFECTION: $F_{(1, 28)} = 0.244$, $p = 0.625$;
463 OFFSPRING TREATMENT: $F_{(1, 28)} = 0.149$, $p = 0.703$; PRENATAL INFECTION x
464 OFFSPRING TREATMENT: $F_{(1, 28)} = 0.364$, $p = 0.551$) (Figure 4C). Conversely, a two-way
465 ANOVA revealed significant main effects of PRENATAL INFECTION ($F_{(1, 27)} = 5.987$, $p =$
466 0.021) and OFFSPRING TREATMENT ($F_{(1, 27)} = 9.253$, $p = 0.005$) on relative GAD₆₇
467 protein levels in the HPC. There was no significant PRENATAL INFECTION x
468 OFFSPRING TREATMENT interaction ($F_{(1, 27)} = 0.600$, $p = 0.445$); however, visual
469 inspection of the means suggested individual group differences. Exploratory pairwise
470 comparisons showed a significant reduction in GAD₆₇ protein levels in poly I:C offspring (-
471 15.50%; POLY+VEH vs. CONT+VEH, $p = 0.028$), that was attenuated by CBD treatment
472 (+17.85%; POLY+CBD vs. POLY+VEH, $p = 0.013$), and did not differ from controls
473 (POLY+CBD: 1.04 ± 0.05 vs. CONT+VEH: 1.01 ± 0.05 , $p = 0.108$) (Figure 4D). In the PFC,

474 Mann-Whitney tests showed no significant difference in PV protein levels between groups
475 (CONT+VEH vs. CONT+CBD, $p = 0.208$; CONT+VEH vs. POLY+VEH, $p = 0.247$;
476 POLY+VEH vs. POLY+CBD, $p = 0.180$) (Figure 4E). In the HPC however, CBD treatment
477 significantly increased PV protein levels (+21.09 %, OFFSPRING TREATMENT: $F_{(1, 26)} =$
478 8.610 , $p = 0.007$) relative to vehicle treatment (CBD: 1.04 ± 0.05 vs. VEH: 0.82 ± 0.06) (Figure
479 4F). There was no main effect of PRENATAL INFECTION ($F_{(1, 26)} = 0.314$, $p = 0.580$) and
480 no significant interaction between the factors ($F_{(1, 26)} = 0.509$, $p = 0.482$).

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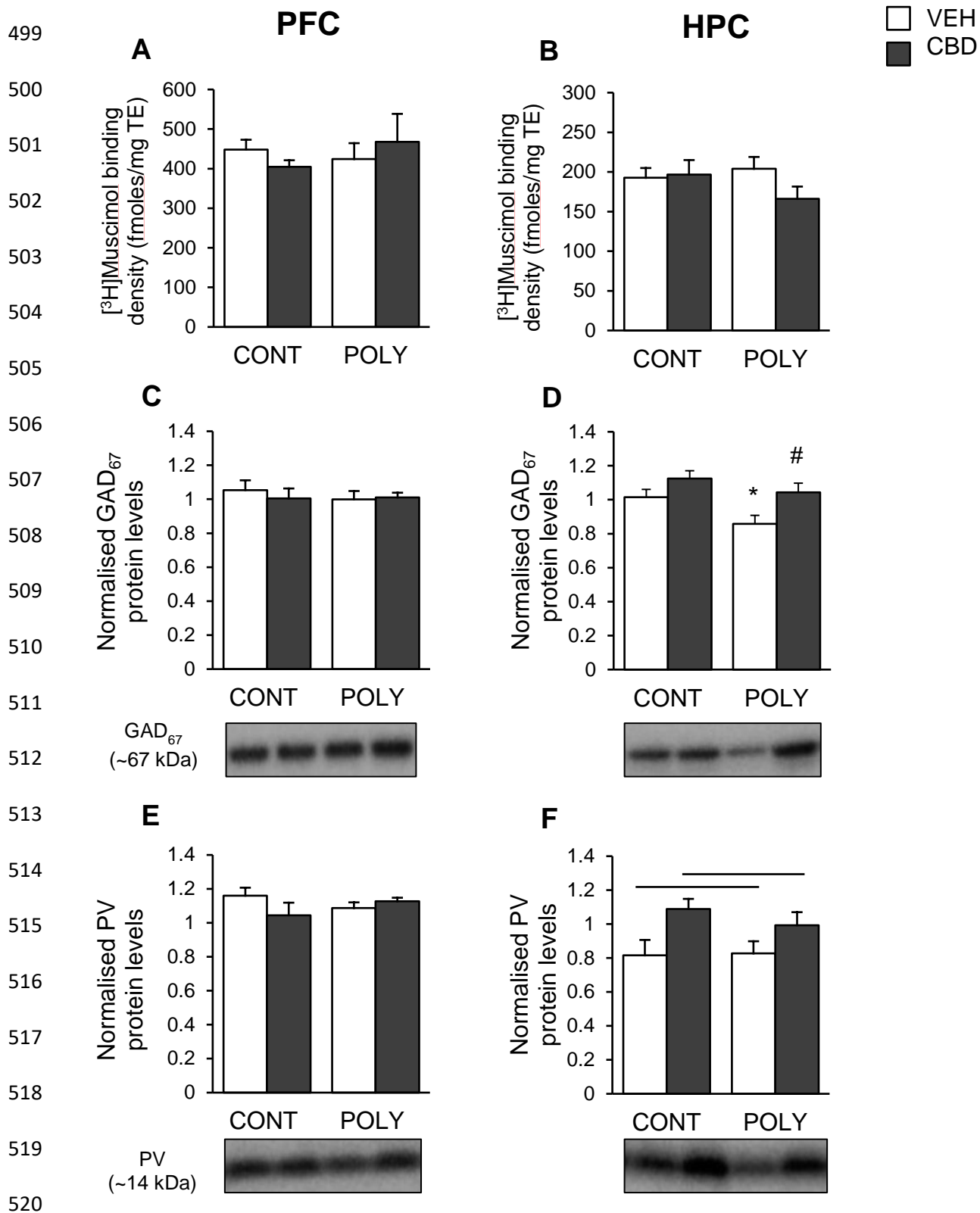
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521 **Figure 4:** Cannabidiol (CBD) treatment altered glutamate decarboxylase 67 (GAD₆₇) and
 522 pavalbumin (PV) protein levels in the hippocampus (HPC) of control (CONT) and poly I:C
 523 (POLY) offspring. GABA_AR ([³H]Muscimol) binding density in the (A) prefrontal cortex
 524 (PFC) and (B) hippocampus (HPC) of offspring. Normalised glutamate decarboxylase 67

525 (GAD₆₇) (C, D) and parvalbumin (PV) (E, F) protein levels (normalised to total protein
526 levels in the respective lanes) in the PFC and HPC with representative immunoblots shown
527 underneath the graphs. * $p < 0.05$ vs. CONT+VEH group, # $p < 0.05$ vs. POLY+VEH group;
528 lines indicate offspring treatment effect ($p < 0.01$ CBD vs. VEH). Data expressed as mean \pm
529 SEM. $n = 6-8$ rats per group.

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531

532 **4. Discussion**

533

534 We have previously shown that CBD treatment restored working and recognition memory, as
535 well as social interaction deficits in male poly I:C offspring (Osborne et al., 2017a), however,
536 the effects of CBD treatment on brain neurochemistry had not been characterised. The
537 present study examined the effect of CBD treatment on eCB, glutamatergic and GABAergic
538 markers in the PFC and HPC of male poly I:C offspring. We have shown that CBD treatment
539 restored poly I:C-induced deficits in CB1R binding density in the PFC and hippocampal
540 GAD₆₇ levels, and increased PV protein levels in the HPC regardless of whether offspring
541 were exposed to poly I:C in utero. This study provides the first evidence for the potential
542 molecular mechanisms underlying the therapeutic effects of CBD in the poly I:C model, and
543 may have implications for schizophrenia treatment.

544

545 In the present study, we found that CB1R binding density was reduced in the PFC, but not the
546 HPC of male poly I:C offspring that exhibit social interaction, recognition and working
547 memory deficits (Osborne et al., 2017a). In line with our findings, post-mortem studies of
548 predominantly male schizophrenia cohorts report alterations in CB1R expression in the PFC
549 (Eggan et al., 2010, 2008; Urigüen et al., 2009), but not the HPC (Dean et al., 2001).
550 Similarly, in other neurodevelopmental rodent models of schizophrenia, studies report
551 alterations in CB1R protein and mRNA expression in the PFC following gestational

552 methylazoxymethanol (MAM) administration (Stark et al., 2019; Gomes et al., 2018), with
553 no change in CB1R binding, protein or mRNA expression in the HPC of male
554 lipopolysaccharide- or MAM-exposed (in utero) offspring (Stark et al., 2019; Gomes et al.,
555 2018; Zavitsanou et al., 2013). However, this is the first study to report a corresponding
556 reduction in CB1R binding density in the PFC, a region critical to memory and social
557 behaviour, in male poly I:C offspring that also exhibit negative and cognitive phenotypes.
558 Our findings are similar to a recent study that reported social interaction and recognition
559 memory deficits in male offspring following gestational methylazoxymethanol (MAM)
560 exposure, with concurrent alterations in CB1R protein and mRNA expression (albeit
561 increases) in the PFC, but not the HPC of male offspring (Stark et al., 2019). It is unknown
562 how CBD rescues changes in CB1R expression in neurodevelopmental models. Recent *in*
563 *vitro* evidence suggests that CBD has an alternative mechanism of action to other
564 phytocannabinoids (e.g. THC), instead acting as a negative allosteric modulator of the CB1R
565 (Laprairie et al., 2015; Straiker et al., 2018; Tham et al., 2018). However, whether negative
566 allosteric modulation of the CB1R by CBD is responsible for the therapeutic effects observed
567 in the present study and others (e.g. Stark et al., 2019) is yet to be elucidated.

568

569 Deficits in GABAergic signalling are thought to underlie the pathophysiology of
570 schizophrenia, with post-mortem studies consistently reporting a reduction in GAD₆₇ and PV
571 expression in the cortex and HPC of patients (Guidotti et al., 2000; Zhang et al., 2002;
572 Thompson et al., 2011; Kimoto et al., 2014). The reduction in hippocampal GAD₆₇ levels
573 observed in the present study aligns with previous poly I:C studies that report reduced GAD₆₇
574 protein and mRNA expression, mainly in the dorsal HPC (Dickerson et al., 2014; Luoni et al.,
575 2017; Richetto et al., 2013), with no changes in the medial PFC (Dickerson et al., 2014).
576 Conversely, this is the first study to show that CBD can reverse deficits in hippocampal

577 GAD₆₇ protein levels in poly I:C offspring. GAD₆₇ is the rate-limiting enzyme responsible for
578 approximately 90% of GABA synthesis in the brain and can provide an indication of GABA
579 levels (Lazarus et al., 2015). Indeed, previous *in vitro* studies have reported that CBD can
580 elevate GABA levels in cortical (Banerjee et al., 1975) and striatal membranes (Sagredo et
581 al., 2007) by inhibiting GABA reuptake. While not impaired in poly I:C offspring in the
582 present study, PV protein levels in the HPC were significantly increased by CBD treatment.
583 The effect of CBD on hippocampal PV+ interneurons has not been reported in maternal
584 manipulation models of schizophrenia previously; however, CBD attenuated a reduction in
585 PV+ cells in the medial PFC following chronic administration of the NMDAR antagonist
586 MK-801 (Gomes et al., 2015a). Importantly, PV expression is activity-dependent, therefore,
587 taken together with recent *in vitro* findings that CBD can enhance inhibition elicited by PV+
588 and CCK+ GABAergic neurons (Khan et al., 2018), the results of the present study suggest
589 that CBD may increase inhibitory tone within the hippocampus.

590

591 In the present study, CBD treatment did not alter FAAH protein levels in the PFC or HPC.
592 FAAH is the primary enzyme responsible for the intracellular hydrolysis of AEA in neurons
593 (Lu and Mackie, 2016). AEA may play a protective role in schizophrenia, especially in the
594 early stages of the disorder (Giuffrida et al., 2004; Koethe et al., 2009); therefore, compounds
595 that limit AEA degradation (e.g. FAAH inhibitors) may be beneficial. Previous *in vitro*
596 investigations have identified CBD as a FAAH inhibitor (Elmes et al., 2015; Leweke et al.,
597 2012; Bisogno et al., 2001; De Petrocellis et al., 2011). Although we did not detect any
598 changes in FAAH protein levels following CBD treatment in the present study, we cannot
599 discount changes in enzymatic activity. Alternatively, CBD could interact with fatty acid
600 binding proteins, which are responsible for transporting AEA across the membrane for
601 intracellular hydrolysis by FAAH (Elmes et al., 2015). Further examination of AEA transport

602 and degradation may provide additional insight into the mechanisms underlying the
603 therapeutic benefits of CBD in the poly I:C model.

604

605 CBD treatment did not alter NMDAR (or protein levels of its obligatory GluN1 subunit) and
606 GABA_AR binding, which are primarily responsible for excitation and inhibition in the mature
607 brain, respectively. Our findings contrast with post-mortem studies in schizophrenia patients
608 that report a modest down-regulation in NMDAR binding (Catts et al., 2016), with a
609 corresponding up-regulation in GABA_AR binding density (Benes et al., 1996a, 1996b;
610 Verdurand et al., 2013). Alterations in individual receptor subunits have also been reported,
611 including down-regulation of the GluN1 subunit of the NMDAR (Catts et al., 2015; Weickert
612 et al., 2013), as well as specific α subunits of the GABA_AR in patients (Beneyto et al., 2011;
613 Volk et al., 2002) and poly I:C offspring (Meyer et al., 2008; Richetto et al., 2014). This is
614 the first study to report that CBD treatment does not alter NMDAR and GABA_AR binding
615 density in male poly I:C offspring that do not exhibit binding deficits in these receptors.
616 Literature investigating the effects of CBD treatment on these neurochemical markers in
617 other preclinical schizophrenia models is limited. In line with the present study, CBD did not
618 alter NMDAR or GABA_AR density in a genetic mouse model of schizophrenia-like
619 phenotypes (Neuregulin 1 transmembrane domain heterozygous mutant mice) that did not
620 exhibit binding deficits (Long et al., 2012), but did restore GluN1 gene expression in the PFC
621 of rodents exposed to NMDAR antagonism (MK-801) (Gomes et al., 2015a). Overall, the
622 findings of the present study suggest that CBD may not exert its therapeutic effects via the
623 NMDAR and GABA_ARs in poly I:C offspring. However, we cannot discount changes in
624 other subunits of the NMDAR (e.g. GluN2A or GluN2B) or GABA_AR (e.g. α subunits).
625 Alternatively, CBD could alter receptor activity or expression on specific neuronal

626 populations (e.g. PV+ interneurons, pyramidal cells) not detectable in the present study,
627 which could be addressed by future investigations.

628

629 Although further research is needed to characterise the neurochemical changes observed
630 following CBD treatment, this study provides the first insight into the potential mechanisms
631 underlying the beneficial effects of CBD in a MIA model of schizophrenia. Following on
632 from the previous behavioural investigation (Osborne et al., 2017a), the present study is
633 limited by the use male offspring only. The eCB system is known to exhibit sexual
634 dimorphism, particularly in CB1R expression and functionality (Rubino and Parolaro, 2011),
635 therefore, future studies are needed to determine if female poly I:C offspring show a similar
636 response to CBD treatment. Additionally, it is unclear whether the downregulation in CB1R
637 binding and GAD₆₇ levels in poly I:C offspring are a delayed result of the poly I:C stimulus;
638 GABAergic and eCB signalling is present in the brain from early prenatal development
639 (Danglot et al., 2006; Harkany et al., 2007), playing an important role in neuronal migration
640 and proliferation, and is vulnerable to neuroinflammation (Di Marzo et al., 2015). Given that
641 some behavioural phenotypes show delayed onset in the poly I:C model (reviewed in Meyer
642 and Feldon, 2012), tracking the neurochemical profile of offspring would elucidate whether
643 the deficits in CB1R binding and GAD₆₇ levels are present from gestation (i.e. after the
644 immune stimulus is delivered), or if the poly I:C insult primes the brain for altered maturation
645 during adolescence. Nevertheless, CBD treatment during adolescence/early adulthood was
646 able to rectify the behavioural (Osborne et al., 2017a) and neurochemical deficits observed in
647 poly I:C offspring.

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651 **Conclusions**

652 In the present study, CBD treatment reversed deficits in CB1R binding density in the PFC
653 and hippocampal GAD₆₇ protein levels in male poly I:C offspring. CBD also increased
654 hippocampal PV levels regardless of in utero poly I:C exposure. CBD had no effect on
655 FAAH protein levels, NMDAR or GABA_AR binding density in either brain region examined,
656 however, poly I:C offspring did not exhibit deficits in these markers. This is the first study to
657 characterise the neurochemical changes that occur following CBD treatment in a MIA model
658 of schizophrenia. Overall, our findings implicate eCB and GABAergic signalling markers in
659 the therapeutic effects of CBD in male poly I:C offspring, and this may have important
660 implications for schizophrenia treatment.

661

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677 **Contributors**

678 ALO, KWG, NS and XFH designed the study; ALO, JSL, IB and KWG performed the
679 experiments; XFH and KAN provided some experimental reagents; ALO analysed the data
680 and wrote the first draft of the manuscript; JSL, KWG, KAN, NS contributed to the
681 interpretation of the data and final manuscript. All authors have approved the final
682 manuscript.

683

684 **Conflict of Interest**

685 The authors declare no conflict of interest.

686

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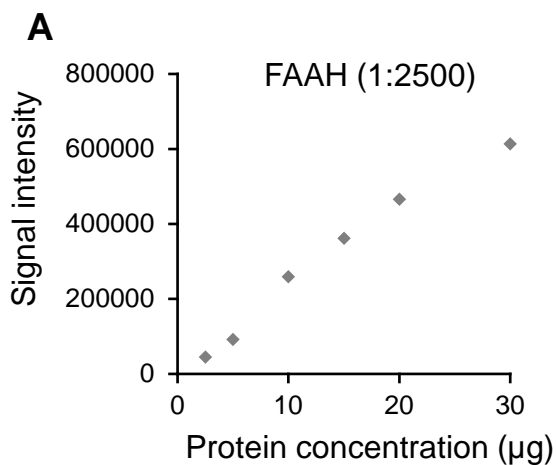
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1079 **Supplementary Material**

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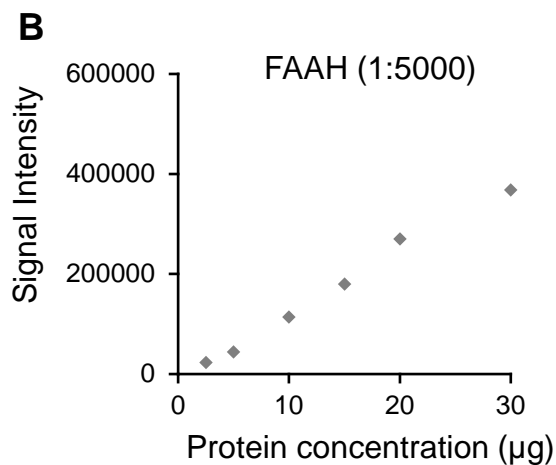
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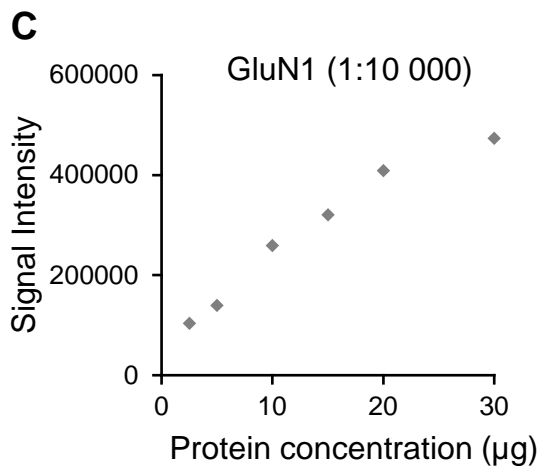
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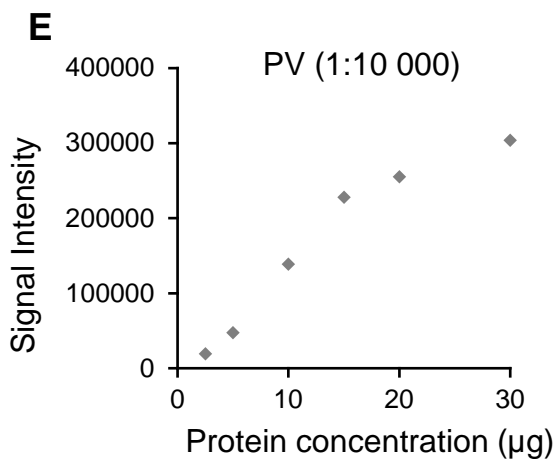
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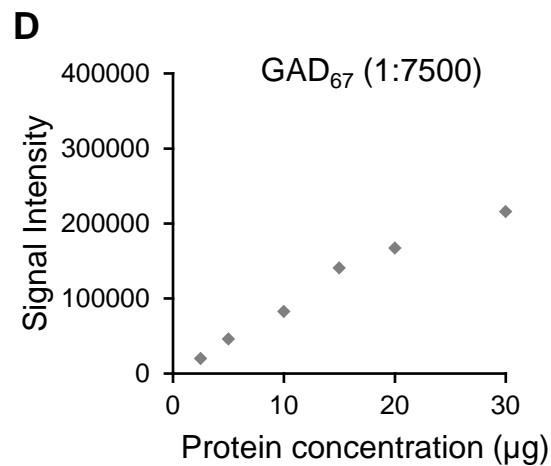
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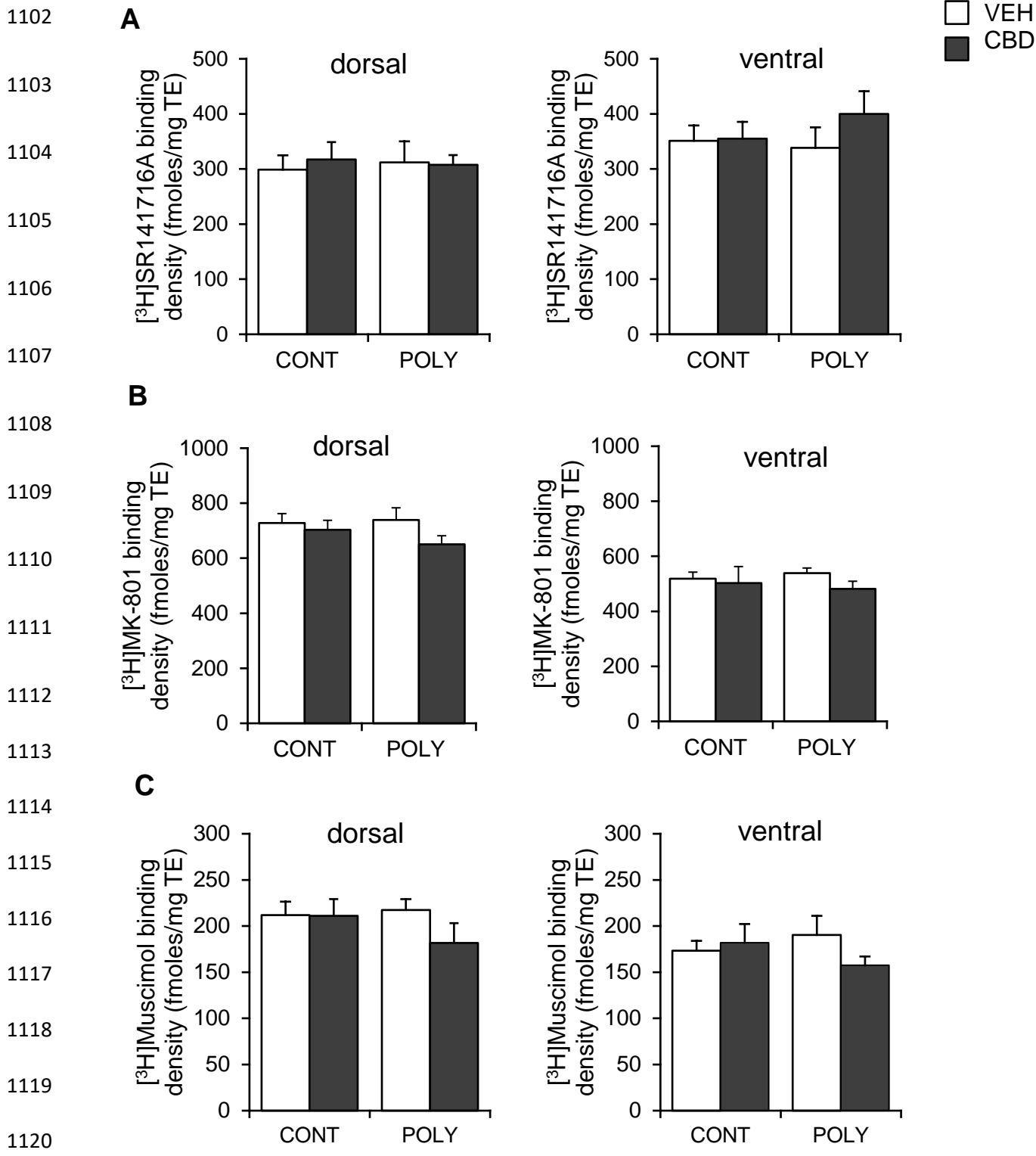
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1097 **Supplementary Figure 1:** Crude rat brain homogenate was prepared at increasing
1098 concentrations of total protein (2.5, 5, 10, 15, 20 and 30 µg) and immunoblotted for the
1099 primary antibodies of interest, A, B) fatty acid amide hydrolase (FAAH), C) GluN1 subunit,
1100 D) glutamate decarboxylase (GAD₆₇) and E) parvalbumin (PV), as previously described in
1101 the original manuscript.



1121 **Supplementary Figure 2:** No difference in (A) CB1R ([³H]SR141716A), (B) NMDAR
 1122 ([³H]MK801) or (C) GABA_AR ([³H]Muscimol) binding density in the dorsal (left) and
 1123 ventral (right) hippocampus of control (CONT) and poly I:C (POLY) offspring. Data
 1124 expressed as mean ± SEM. *n* = 5-7 rats per group.