ABC transporters P-gp and Bcrp do not limit the brain uptake of the novel antipsychotic and anticonvulsant drug cannabidiol in mice.

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Cannabidiol (CBD) is currently being investigated as a novel therapeutic for the treatment of CNS disorders like schizophrenia and epilepsy. ABC transporters such as P-glycoprotein (P-gp) and breast cancer resistance protein (Bcrp) mediate pharmacoresistance in these disorders. P-gp and Bcrp are expressed at the blood brain barrier (BBB) and reduce the brain uptake of substrate drugs including various current antipsychotics and anticonvulsants. It is therefore important to assess whether CBD may be prone to treatment resistance mediated by P-gp and Bcrp. Moreover, it has become common practice in the drug development of CNS agents to screen against ABC transporters to help isolate lead compounds with optimal pharmacokinetic properties. The current study aimed to assess whether P-gp and Bcrp impacts the brain transport of CBD by comparing CBD tissue concentrations in wild-type (WT) mice versus mice devoid of ABC transporter genes. P-gp knockout $(Abcb1a/b^{-/-})$, Bcrp knockout $(Abcg2^{-/-})$, combined P-gp/Bcrp knockout $(Abcb1a/b^{-/-}Abcg2^{-/-})$ and WT mice were injected with CBD, before brain and plasma samples were collected and determined at various time-points. CBD results were compared with the positive control risperidone and 9-hydroxy risperidone, antipsychotic drugs that are established ABC transporter substrates. Brain and plasma concentrations of CBD were not greater in P-gp, Bcrp or P-gp/Bcrp knockout mice than WT mice. In comparison, the brain/plasma concentration ratios of risperidone and 9-hydroxy risperidone were profoundly higher in P-gp knockout mice than WT mice. These results suggest that CBD is not a substrate of P-gp or Bcrp and may be free from the complication of reduced brain uptake by these transporters. Such findings provide favorable evidence for the therapeutic development of CBD in the treatment of various CNS disorders.

1 **ABC transporters P-gp and Bcrp do not limit the brain uptake of the novel antipsychotic**
2 **and anticonvulsant drug cannabidiol in mice.**

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32 **Abbreviations**

33 CBD, cannabidiol; THC, Δ^9-tetrahydrocannabinol; ABC transporters, adenosine triphosphate

34 (ATP) binding cassette transporters; P-gp, P-glycoprotein; Bcrp, breast cancer resistance protein;

35 WT, wild-type; 9-OH risperidone, 9-hydroxy risperidone; BBB, blood brain barrier; FVB, friend

36 virus B-type mice; s.c, subcutaneous; EDTA, ethylenediaminetetraacetic acid; LC-MS/MS,

37 liquid chromatography and tandem mass spectrometry (triple quadrupole mass spectrometer);

38 QC, quality control; SPE, solid phase extraction; LOQ, limits of quantification; CB_1, cannabinoid

39 1 receptor.

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Abstract

47   Cannabidiol (CBD) is currently being investigated as a novel therapeutic for the treatment of

48   CNS disorders like schizophrenia and epilepsy. ABC transporters such as P-glycoprotein (P-gp)

49   and breast cancer resistance protein (Bcrp) mediate pharmacoresistance in these disorders. P-gp

50   and Bcrp are expressed at the blood brain barrier (BBB) and reduce the brain uptake of substrate

51   drugs including various current antipsychotics and anticonvulsants. It is therefore important to

52   assess whether CBD may be prone to treatment resistance mediated by P-gp and Bcrp.

53   Moreover, it has become common practice in the drug development of CNS agents to screen

54   against ABC transporters to help isolate lead compounds with optimal pharmacokinetic

55   properties. The current study aimed to assess whether P-gp and Bcrp impacts the brain transport

56   of CBD by comparing CBD tissue concentrations in wild-type (WT) mice versus mice devoid of

57   ABC transporter genes. P-gp knockout ($Abcb1a/b^{-/-}$), Bcrp knockout ($Abcg2^{-/-}$), combined P-

58   gp/Bcrp knockout ($Abcb1a/b^{-/-}Abcg2^{-/-}$) and WT mice were injected with CBD, before brain and

59   plasma samples were collected and determined at various time-points. CBD results were

60   compared with the positive control risperidone and 9-hydroxy risperidone, antipsychotic drugs

61   that are established ABC transporter substrates. Brain and plasma concentrations of CBD were

62   not greater in P-gp, Bcrp or P-gp/Bcrp knockout mice than WT mice. In comparison, the

63   brain/plasma concentrations of risperidone and 9-hydroxy risperidone were profoundly higher in

64   P-gp knockout mice than WT mice. These results suggest that CBD is not a substrate of P-gp or

65   Bcrp and may be free from the complication of reduced brain uptake by these transporters. Such

66   findings provide favorable evidence for the therapeutic development of CBD in the treatment of

67   various CNS disorders.

1. Introduction

68 Cannabidiol (CBD), a non-psychoactive constituent of cannabis, displays much potential as a

69 novel therapeutic treatment for various CNS disorders including schizophrenia and epilepsy

70 (Bumb, Enning & Leweke, 2015; Iseger & Bossong, 2015; Longo, Friedman & Devinsky, 2015).

71 CBD has anticonvulsant effects in animal models of epilepsy and schizophrenia (Arnold,

72 Boucher & Karl, 2012; Jones et al., 2012; Jones et al., 2010; Mao et al., 2015). Anecdotal reports

73 and early clinical findings support CBD's ability to reduce seizure rates in humans with a good

74 safety profile (Rosenberg et al., 2015). Phase 3 clinical trails are currently investigating CBD in

75 the treatment of epilepsy, most notably in severe and pharmacoresistant childhood epilepsies.

76 There is also promising evidence that CBD is a novel antipsychotic, with a phase 2 clinical trial

77 showing CBD reduced symptoms in schizophrenia patients with comparable efficacy to a

78 conventional antipsychotic drug without producing extrapyramidal side-effects, sedation or

79 weight gain (Bumb, Enning & Leweke, 2015; Iseger & Bossong, 2015; Leweke et al., 2012).

80 Numerous further clinical trials are currently underway examining the efficacy of CBD in

81 treating schizophrenia. The precise pharmacodynamic mechanisms responsible for CBD's

82 anticonvulsant and antipsychotic efficacy are hotly debated and may involve inhibition of

83 degradation of the endocannabinoid anandamide. Although there are numerous other contenders

84 as CBD is a promiscuous drug that interacts with multiple drug targets including G-protein-

85 coupled receptor 55 (GPR55), transient receptor potential vanilloid type 1 (TRPV1) channels,

86 and adenosine transporters (Leweke et al., 2012; McPartland et al., 2015; Rosenberg et al. 2015).

87 Resistance to treatment is a major stumbling block in the clinical management of epilepsy

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88 and schizophrenia. Approximately 30% of both schizophrenia and epilepsy patients do not

89 respond adequately to drug therapy (Hoosain et al., 2015; van Os & Kapur, 2009) and ATP-

90 binding cassette (ABC) transporters play a role in treatment-resistance (Bebawy & Chetty, 2008;

91 Brandt et al., 2006). ABC transporters are a large superfamily of proteins that actively transport

92 substrates across biological membranes and thus influence the disposition of substrate drugs

93 (Hee Choi & Yu, 2014; Kathawala et al., 2015). The best characterized ABC transporters are P-

94 glycoprotein (P-gp, *Abcb1*) and breast cancer resistance protein (Bcrp, *Abcg2*), which are both

95 efflux pumps localized at various pharmacological barriers in the body including the blood brain

96 barrier (Löscher & Potschka, 2005). Many antipsychotic and anticonvulsant drugs are substrates

97 of P-gp, which strongly limits the brain accumulation of these agents by extruding the drugs

98 from the brain parenchyma back into the blood (Boulton et al., 2002; Doran et al., 2005; Luna-

99 Tortós, Fedrowitz & Löscher, 2008; Zhang et al., 2012). Few studies have examined the

100 substrate profile of CNS drugs for Bcrp, although lamotrigine has recently been demonstrated to

101 be a substrate of human and mouse Bcrp (Römermann, Helmer & Löscher, 2015).

102   There is evidence that genetic variation in P-gp influences treatment response to

103 antipsychotic and antiepileptic drugs such as olanzapine, risperidone, paliperidone (9-hydroxy

104 risperidone, the active metabolite of risperidone), phenobarbital and phenytoin (French, 2013;

105 Wolking et al., 2015). Furthermore, P-gp and Bcrp are upregulated at the BBB in epilepsy and

106 schizophrenia and contribute to pharmacoresistance by limiting the brain uptake and efficacy of

107 anticonvulsant and antipsychotic drugs (Aronica et al., 2005; Bauer et al., 2014; de Klerk et al.,

108 2010; Lazarowski et al., 2007; Van Vliet et al., 2005). Drugs that are not substrates of ABC

109 transporters will then make better therapeutics, as they will be immune to drug resistance

110 mediated by these proteins. Indeed, it has become common practice in drug development to

111   screen against ABC transporters to help isolate lead compounds that are less likely to fail in

112   clinical trials due to suboptimal pharmacokinetic properties. It is therefore important to establish

113   whether CBD is an ABC transporter substrate. We have shown that the main psychoactive

114   constituent of cannabis, Δ^9-tetrahydrocannabinol (THC) is a substrate of both P-gp and Bcrp

115   (Spiro et al., 2012). CBD, an isomer of THC, inhibits both P-gp and Bcrp transport (Feinshtein et

116   al., 2013b; Zhu et al., 2006), although results have not been consistent for P-gp (Holland et al.,

117   2006). Inhibitors are often substrates so there is a need to clarify whether CBD is a substrate of

118   P-gp or Bcrp and whether this has implications for the brain uptake of the compound.

119   The present study assesses whether CBD is a substrate of P-gp and Bcrp by utilising mice

120   devoid of these ABC transporter genes singly or in combination. If CBD accumulates at greater

121   concentrations in the brain of ABC transporter knockout animals than wild-type mice under the

122   same experimental conditions, then this provides evidence that it is an ABC transporter substrate.

123   The results of CBD are compared with a positive control risperidone and 9-hydroxy risperidone,

124   as these antipsychotic drugs are established P-gp substrates (Doran et al., 2005). Our results will

125   be useful in the evaluation of CBD as a therapeutic agent for CNS disorders from a

126   pharmacokinetic perspective. If P-gp or Bcrp do not limit the brain concentrations of CBD, it

127   implies that CBD might be free from drug resistance that is mediated by these ABC transporters.

128

2. Materials and methods

2.1. Animals

129   We used male wild-type (WT, FVB background strain), P-gp knockout (*Abcb1a/b$^{-/-}$*), Bcrp

130   knockout *(Abcg2$^{-/-}$)* and combined P-gp/Bcrp knockout (*Abcb1a/b$^{-/-}$Abcg2$^{-/-}$*) mice aged between

131   4-5 months weighing between 25-30 g (Taconic farms, New York, USA). P-gp, Bcrp and P-

132 gp/Bcrp knockout mice were developed by Professor Alfred Schinkel and colleagues at the

133 Netherlands Cancer Institute, Amsterdam (Jonker et al., 2002; Schinkel et al., 1995; Schinkel et

134 al., 1997). Mice were housed in groups of 4-6 mice per cage and kept under a standard 12 h

135 light/dark schedule. Food and water were available *ad libitum* and all cages contained various

136 forms of environmental enrichment such as a mouse house igloo and running wheel, a paper roll,

137 a climbing ring, tissue paper and sunflower seeds. The University of Sydney's Animal Ethics

138 Committee approved all experimental procedures undertaken (Protocol number: K21/1-

139 2013/3/5924) and all procedures were in accordance with the Australian Code of Practice for the

140 Care and Use of Animals for Scientific Purposes.

141

2.2. Drug treatment

142 CBD (THC Pharm, Frankfurt, Germany) was dissolved in a mixture of ethanol, Tween 80, and

143 saline (1:1:18) (Long et al., 2013; Todd & Arnold, 2016) and administered via subcutaneous (s.c)

144 injection at a dose of 10 mg/kg (Doran et al., 2005; Pacchioni et al., 2010). Risperidone (Sequioa

145 Pharmaceuticals, United Kingdom) was dissolved in a solution of 0.9% saline and 1% acetic acid

146 and injected s.c. at 3 mg/kg. All drugs were freshly prepared before use and made at an injection

147 volume of 10 ml/kg of body weight. At numerous time-points post-injection of CBD (1, 2 and 3

148 h) and risperidone (1 and 3 h), P-gp knockout, Bcrp knockout, P-gp/Bcrp knockout and WT mice

149 were lightly anesthetised with isoflurane and blood collected via cardiac puncture. Blood

150 samples were stored in ethylenediaminetetraacetic acid (EDTA) coated tubes to avoid

151 coagulation and kept on ice before separation of plasma (Spiro et al., 2012). To separate the

152 plasma from the blood, samples were centrifuged at 3000 rpm for 10 min at 4°C and the plasma

153 collected in clean eppendorf tubes (Wang et al., 2004). The brains were immediately extracted

154   and snap frozen in liquid nitrogen. Both the brain and plasma samples were stored at -80°C

155   before LC-MS/MS analysis.

156

2.3. Quantification of CBD in brain and blood samples

157   CBD was extracted using a previously outlined method from our group (Johnston et al., 2014). In

158   brief, a deuterated CBD-D_3 internal standard solution was added to every brain or plasma sample

159   (see Figure 1). Calibration standards and quality control (QC) samples were prepared by spiking

160   drug-free mouse plasma or drug-free brain homogenates, at linear concentrations from 10-400

161   ng/g of CBD for brain analysis and 10-300 ng/ml of CBD for plasma analysis. The standards

162   were vortexed and treated identically to other samples. Half brains were homogenised in dH_2O at

163   a 1:6 ratio (w/v) with 1 mL brain homogenate. For plasma analysis, 0.5 mL of a sample was

164   used. Brain and plasma samples were prepared by slowly adding 2 mL ice-cold acetonitrile,

165   mixed thoroughly and centrifuged at 3000 rpm for 10 minutes. The acetonitrile was decanted

166   into clean tubes and all samples were evaporated using the Genevac EZ-2 evaporation system for

167   approximately 3-4 hours. After reconstituting the samples with 2 mL dH_2O the samples were

168   loaded onto Styre Screen® SSTHC063 solid-phase extraction (SPE) columns (60 mg/3 ml) from

169   United Chemical Technologies (Horsham, PA, USA). Columns were then washed with 1 mL

170   water/acetonitrile/NH_4OH (84:15:1) and dried thoroughly under vacuum (10 mm Hg) for 10-15

171   minutes. Samples were eluted from the column by adding 3 mL of hexane/ethyl acetate/glacial

172   acetic acid (49:49:2). Extracts were completely dried under a nitrogen gas stream at 60°C for 5-

173   10 minutes and reconstituted with 50 μl initial mobile phase (40 % methanol and 60 % 10 mM

174   ammonium acetate) for analysis. All quantification was performed using a Shimadzu 8030 triple

175   quadrupole mass spectrometer. The mobile phase consisted of (A) 10 mM ammonium acetate in

176   water and (B) methanol. The limits of quantification (LOQ) for plasma analysis were 1.5 ng/ml

177   and 11.5 ng/g for brain analysis.

178

2.4. Quantification of risperidone and 9-hydroxy risperidone in brain and blood samples

179   For plasma analysis, 10 μl of methyl-risperidone (10 μM) internal standard (IS) solution and 0.5

180   mL of PO$_4$ buffer (pH 5.0) were added to each 0.1 mL sample of plasma. Calibration standards

181   were prepared by spiking drug-free mouse plasma at concentrations of 2-200 ng/ml for

182   risperidone and 9-hydroxy risperidone. The standards were vortexed and treated identically to

183   other plasma samples. For extraction, plasma samples underwent SPE using Varian SPEC 3 mL

184   MP3 (15 mg) microcolumns from Agilent (Santa Clara, CA, USA). Columns were first

185   conditioned by adding 0.5 mL methanol followed by 0.5mL 0.1M PO$_4$ buffer (pH 5.0). Plasma

186   samples were then loaded onto the column. The columns were washed in 0.5 mL of acetic acid

187   (1 M) and 0.5 mL methanol and then dried under vacuum for approximately 2 minutes. The

188   samples   were   eluted   from   the   column   using   1   mL   of   freshly   prepared

189   dichloromethane/isopropanol/ammonia (80:20:2). The elutant was evaporated to dryness using a

190   SpeedVac centrifugal evaporator. Samples were reconstituted with 200 μl of 50% acetonitrile.

191   For brain analysis, brains were dissected in half, weighed and one half of the brain was

192   homogenised in dH$_2$O at a 1:2 ratio (w/v). All samples and calibrators received 50 μl of 100 nM

193   internal standard. Calibrators were made up of 1.5 mL of 0.1 M phosphate buffer (pH 6) spiked

194   with linear concentrations of 1-100 nM of risperidone and 9-hydroxy risperidone. Sample

195   homogenates were centrifuged at 14,000 g for 10 minutes and the supernatant collected. The

196   brain supernatant underwent identical preparation and extraction as plasma samples and the

197   elutant evaporated accordingly. Samples were reconstituted with 50 μl of mobile phase A. The

198   linear gradient solutions consisted of mobile phase (A) 5 mM ammonium formate (pH 6) and (B)

199   90% acetonitrile. All quantification was performed using triple quadrupole liquid

200   chromatography-mass spectrometry (Agilent 6460). The LOQ for brain analysis was 1.5 ng/g

201   and 1 ng/ml for plasma analysis.

202

2.5. Statistical analysis

203   Two way ANOVA with factors of genotype (including WT, P-gp, Bcrp and P-gp/Bcrp knockout

204   mice) and time were performed on brain and plasma concentrations as well as brain/plasma

205   ratios of CBD, risperidone and 9-hydroxy risperidone. In the instance of finding an overall main

206   effect of genotype or genotype by time interaction, Tukey's post-hoc analysis was used to

207   individually compare WT mice to P-gp knockout, Bcrp knockout, or P-gp/Bcrp knockout mice.

208   In addition, P-gp knockout mice were compared to P-gp/Bcrp knockout mice. The latter

209   comparison is important, as compensation with transporters has been reported in knockout mice

210   studies (Tang et al., 2013; Vlaming et al., 2006)**.** For example, double knockout of P-gp and Bcrp

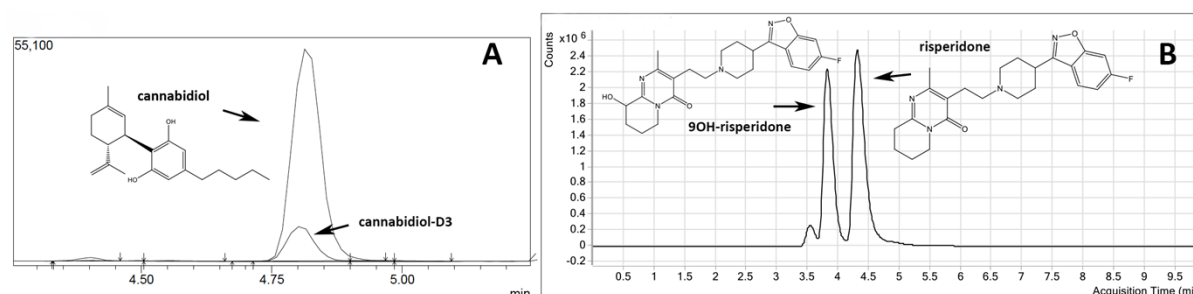
211   may significantly increase the brain uptake of drugs when no altered disposition was observed in

212   single P-gp knockout or Bcrp knockout mice (Tang et al., 2013). This has been attributed to Bcrp

213   being induced in response to P-gp knockout and vice versa. Differences were deemed

214   statistically significant when $P < 0.05$.

3. Results

3.1. P-gp or Bcrp knockout did not increase brain or plasma concentrations of CBD

215 **Figure 1. Representative chromatograms and molecular structures of tested compounds** A) CBD and internal standard
216 CBD-D3 B) Risperidone and 9-OH risperidone

217
218 Representative chromatogram traces of CBD and the internal standard CBD-D3 are shown in

219 Figure 1A. Concentrations of CBD in brain and plasma (Fig. 2) were not altered in P-gp, Bcrp or

220 P-gp/Bcrp knockout mice compared to WT mice as supported by overall two-way ANOVA with

221 no main effect of genotype or a genotype by time interaction. A significant main effect of time

222 was observed in both brain [$F_{(3,60)} = 30.5$, $P < 0.0001$] and plasma [$F_{(2,63)} = 34.8$, $P < 0.0001$]

223 samples, with CBD concentrations decreasing over the three hour time period in all genotypes.

224 Tukey's post-hoc comparisons confirmed that no significant differences were observed between

225 WT when individually compared to P-gp knockout, Bcrp knockout or P-gp/Bcrp knockout mice

226 at 1, 2 or 3 h time-points. Nor were there any significant differences between P-gp knockout and

227 P-gp/Bcrp knockout mice at any time-point.

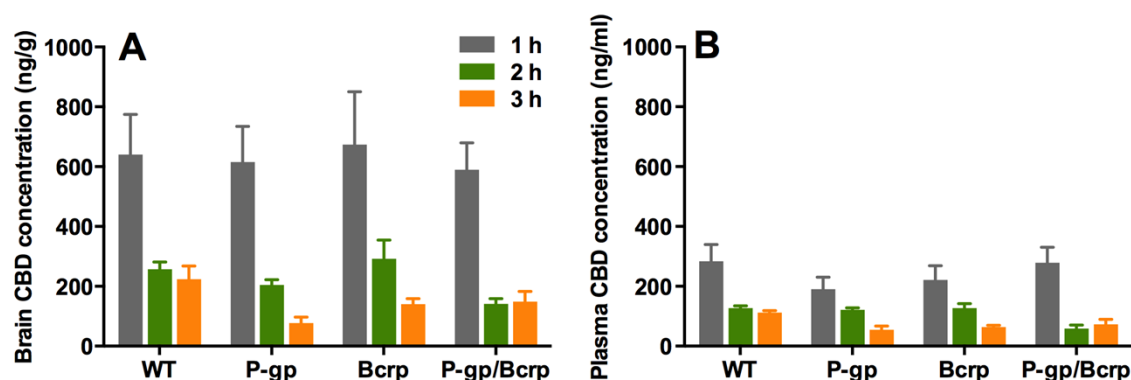



Figure 2. P-gp or Bcrp transporter knockout did not influence the CBD brain and plasma concentrations. Brain and plasma CBD samples were collected from WT and ABC transporter knockout mice. Samples were collected at 1, 2 and 3 h after an acute 10 mg/kg s.c. injection of CBD. A) CBD brain concentration B) CBD plasma concentration. CBD = cannabidiol, WT =

wild-type, P-gp = *Abcb1a/b*$^{(-/-)}$ (P-gp knockout), Bcrp = *Abcg2*$^{(-/-)}$ (Bcrp knockout), P-gp/Bcrp = *Abcb1a/b*$^{(-/-)}$*Abcg*$^{(-/-)}$ (P-gp/Bcrp combined knockout). Data represent mean + S.E.M.

3.2. P-gp or Bcrp knockout did not influence CBD brain/plasma ratios, whereas P-gp knockout profoundly increased risperidone and 9-hydroxy risperidone brain/plasma ratios

228   No differences were observed between P-gp, Bcrp or P-gp/Bcrp knockout and WT mice (Fig.

229   3A) in their brain/plasma CBD concentration ratios (overall a ratio of approximately 2), as two-

230   way ANOVA showed no main effect of genotype and no genotype by time interaction. This was

231   confirmed by post-hoc comparisons. As CBD did not show any altered disposition in ABC

232   transporter knockout mice, we sought to show, in our hands, that other known transporter

233   substrates, that is risperidone and 9-OH risperidone, display increased brain uptake in knockout

234   animals. This would prove that our negative finding with CBD was not a false negative due to

235   some procedural error.

236   Representative chromatogram traces of risperidone and 9-OH risperidone are shown in

237   Figure 1B. Two way ANOVA indicated a significant overall main effect of genotype in the

238   risperidone and 9-hydroxy risperidone brain/plasma concentration ratios [$F(3,39) = 34.62$, $P <$

239   0.0001; $F(3,39) = 32.17$, $P < 0.0001$ respectively] (Fig. 3B and 3C). The brain/plasma ratio for

240   risperidone and 9-hydroxy risperidone also tended to increase over time in all genotypes [main

241   effect of time: $F(1,39) = 5.66$, $P < 0.05$; $P(1,39) = 5.65$, $P < 0.05$ respectively]. Tukey's post-hoc

242   analysis showed P-gp knockout and P-gp/Bcrp knockout had significantly greater brain/plasma

243   risperidone concentration ratios than WT mice at both 1 h and 3 h time points respectively (Ps <

244   0.05). In the P-gp knockout and P-gp/Bcrp knockout mice the brain/plasma risperidone ratios

245   reached as high as 13 whereas the ratio for WT mice was less than 1. Brain/plasma concentration

246   ratios of 9-hydroxy risperidone in P-gp knockout mice and P-gp/Bcrp knockout mice were

247   significantly higher than the WT mice ratio at the 3 h time point (Ps < 0.05). In the P-gp

248 knockout and P-gp/Bcrp knockout mice the brain/plasma 9-hydroxy risperidone ratio reached as

249 high as 8 whereas the ratio for WT mice was less than 1. P-gp/Bcrp knockout mice achieved a

250 significantly higher brain/plasma concentration ratio for 9-hydroxy risperidone than WT mice at

251 1 h, however P-gp knockout mice failed to reach significance when compared to WT mice at this

252 timepoint. Although, P-gp knockout and P-gp/Bcrp knockout mice were not statistically different

253 in their brain/plasma concentration ratios for risperidone and 9-hydroxy risperidone at either the

254 1 or 3 h timepoints implying no cooperation between P-gp and Bcrp in the transport of these

255 drugs. Bcrp knockout did not display significantly different brain/plasma concentrations of

256 risperidone and 9-hydroxy risperidone to WT at either timepoint.

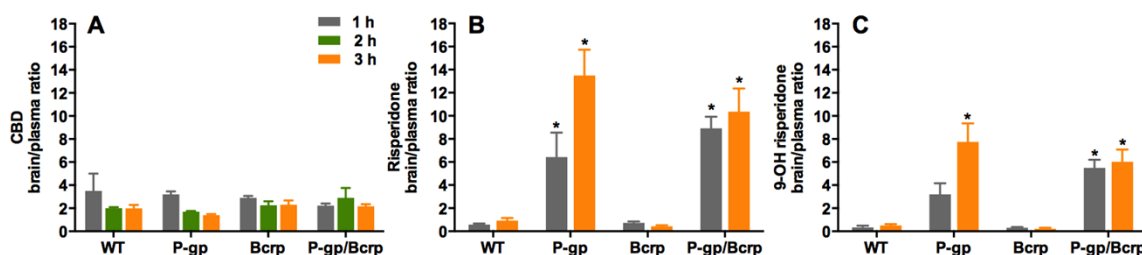



Figure 3. P-gp or Bcrp knockout did not alter CBD brain/plasma concentration ratios, whilst P-gp knockout profoundly increased risperidone and 9-hydroxy risperidone brain/plasma ratios. A) CBD brain/plasma concentration ratios B) risperidone brain/plasma concentration ratios C) 9-hydroxy risperidone brain/plasma concentration ratios. CBD = cannabidiol, 9-OH risperidone = 9-hydroxy risperidone, WT = wild-type, P-gp = *Abcb1a/b*$^{(-/-)}$ (P-gp knockout), Bcrp = *Abcg2*$^{(-/-)}$ (Bcrp knockout), P-gp/Bcrp = *Abcb1a/b*$^{(-/-)}$*Abcg*$^{(-/-)}$ (P-gp/Bcrp combined knockout). Tukey *post-hoc* analyses, *p<0.05 for comparisons between WT and knockout mice. Data represent mean + S.E.M.

4. Discussion

257 This study shows that the ABC transporters P-gp and Bcrp do not influence the brain uptake of

258 CBD, a novel antipsychotic and anticonvulsant drug. P-gp, Bcrp and P-gp/Bcrp knockout mice

259 did not accumulate greater brain or plasma concentrations of CBD compared to WT mice, nor

260 were the brain/plasma concentration ratios of CBD influenced by knockout of the ABC

261 transporter genes. By comparison, the known substrates of P-gp risperidone and 9-hydroxy

262 risperidone (Doran et al., 2005; Ejsing, Pedersen & Linnet, 2005; Kirschbaum et al., 2008; Wang

263   et al., 2004), displayed increased brain/plasma concentration ratios in P-gp knockout mice

264   compared to WT mice, reaching as high as approximately 13 and 8 times respectively whereas

265   WT mice only attained ratios < 1. Taken together these results suggest P-gp strongly regulates

266   the brain uptake of the antipsychotic drugs risperidone and 9-hydroxy risperidone but not CBD.

267   That P-gp and Bcrp did not influence the brain uptake of CBD is somewhat surprising

268   given our prior research showing these transporters regulate the brain concentrations of THC, the

269   main psychoactive constituent of cannabis (Spiro et al., 2012). CBD and THC are isomers with

270   very similar lipophilicity. However, CBD is formed when the central pyran ring of THC is

271   opened and the oxygen in the ring is converted into a free hydroxy group (Compton et al., 1992).

272   This subtle chemical modification yields remarkable differences in the pharmacological activity

273   of these drugs. For instance, THC is a partial agonist at CB_1 cannabinoid receptors and therefore

274   elicits profound psychotropic effects, whilst CBD has poor affinity for the orthosteric site of this

275   receptor and doesn't have appreciable psychoactivity (Laprairie et al., 2015; Long et al., 2010).

276   CBD has a growing list of distinctive properties to THC. For example, CBD inhibits the

277   anandamide degradative enzyme fatty acid amid hydrolase (FAAH) and activates TRPV1

278   receptors, actions not shared with THC (McPartland et al., 2015; Pertwee, 2008). Our data here

279   provides yet another example, this time a differential substrate binding character for ABC

280   transporter proteins.

281   Our finding that CBD is not a substrate of murine P-gp and Bcrp is consistent with *in*

282   *vitro* data with cells expressing human transporters. CBD did not stimulate ATPase activity in

283   insect membranes expressing human P-gp and Bcrp, unlike known P-gp and Bcrp substrates

284   verapamil and sulphasalazine respectively (Holland et al., 2007; Zhu et al., 2006). Therefore it

285   appears that our results here may generalize to human transporters, although future studies

286 assessing CBD transport by human ABC transporters using transwell assays would strengthen

287 this viewpoint. We and others have shown that both CBD and THC inhibit P-gp and Bcrp

288 (Feinshtein et al., 2013a; Feinshtein et al., 2013b; Holland et al., 2007; Spiro et al., 2012; Zhu et

289 al., 2006). While THC appears to be a competitive substrate, as it is also actively transported by

290 P-gp and Bcrp (Bonhomme-Faivre et al., 2008; Spiro et al., 2012), CBD's ability to inhibit the

291 transporters occurs in the absence of active transport. This phenomenon is not without precedent

292 as paracetamol and haloperidol both inhibit P-gp but are not actively transported as substrates

293 (Feng et al., 2008; Novak et al., 2013) and gefitinib inhibits Bcrp while not being a substrate

294 (Galetti et al., 2015).

295   Future studies are needed to examine whether CBD's ability to inhibit ABC transporters

296 alters the pharmacokinetics of co-administered drugs that are ABC transporters substrates like

297 risperidone. Interestingly co-administration of CBD with clobazam in children with refractory

298 epilepsy increased plasma concentrations of clobazam and its active metabolite norclobazam by

299 60 and 500% respectively (Geffrey et al., 2015). Whether norclozabam is an ABC transporter

300 substrate is unknown, however clobazam is a duel P-gp/Bcrp substrate opening the possibility

301 that these transporters may contribute to this drug interaction (Nakanishi et al., 2013). In addition,

302 CBD inhibition of ABC transporters appears relevant to pharmacokinetic interactions between

303 CBD and THC, as THC is a dual P-gp and Bcrp substrate, and CBD potentiates some of the

304 effects of THC via increasing brain THC concentrations (Klein et al., 2011; Spiro et al., 2012;

305 Todd & Arnold, 2015). Extended exposure to CBD has also been shown to influence the

306 expression of P-gp in cancer cells that is mediated by CB2 and TRPV1 receptors (Arnold et al.,

307 2012; Holland et al., 2006). Thus future studies assessing the impact of CBD on P-gp in brain

308 microvessels are warranted, as this may impact upon the pharmacokinetics of co-administered

309   substrate drugs following chronic CBD exposure. Another limitation of the current study is that

310   CBD metabolites were not examined, as no commercially available analytical standards for these

311   compounds were available. One metabolite of particular interest is 7-OH CBD which appears to

312   be an active anticonvulsant with greater potency than CBD (Jiang et al., 2011; Stott et al., 2015;

313   Ujváry & Hanuš, 2016). Future studies need to address whether CBD metabolites like 7-OH

314   CBD are ABC transporter substrates.

315   Our results suggest for the first time that risperidone and 9-hydroxy risperidone are not

316   Bcrp substrates and that there is no cooperation between P-gp and Bcrp in the transport of these

317   antipsychotic drugs. If these drugs were Bcrp substrates then the Bcrp knockout mice would

318   have displayed greater brain and plasma concentrations of these agents than WT mice. Further, if

319   these antipsychotic drugs were dual substrates of P-gp and Bcrp we would have expected the

320   double P-gp/Bcrp knockout mice to display greater brain and plasma concentrations than P-gp

321   knockout mice alone. Such observations have been made for other drugs, for example the Bcrp

322   substrates prazosin and mitoxantrone display greater brain or plasma concentrations in Bcrp

323   knockout mice than WT mice (Cisternino et al., 2004) and the dual P-gp and Bcrp substrates

324   sunitinib and dasatinib show greater brain concentrations in P-gp/Bcrp knockout mice than P-gp

325   or Bcrp knockout mice alone (Tang et al., 2013).

326   Cannabidiol is currently being assessed in randomized controlled trials as a novel

327   antipsychotic and anticonvulsant agent, supported by an array of preclinical and human data

328   (Arnold, Boucher & Karl, 2012; Devinsky et al., 2014; Leweke et al., 2012). ABC transporters

329   may play an important role in pharmacoresistance, which is a major stumbling block in the

330   successful treatment of schizophrenia and epilepsy. Indeed the ABC transporter substrate binding

331   character is routinely assessed in the development of novel CNS therapeutics to ensure adequate

332 brain uptake of the drug e.g. blonanserin (Inoue et al., 2012). As can be seen here, the brain

333 uptake of the commonly used antipsychotic drug risperidone and its active metabolite 9-hydroxy

334 risperidone is profoundly limited by P-gp. Similarly many anticonvulsant drugs such as

335 phenytoin, phenobarbital and clobazam are also ABC transporter substrates and subject to poor

336 brain uptake (Nakanishi et al., 2013; Zhang et al., 2012). Moreover, single nucleotide

337 polymorphisms (SNPs) in *MDR1* increase the risk of resistance or greater interindividual

338 response to antiepileptic and antipsychotic drugs (Bozina et al., 2008; Li et al., 2014; Shaheen et

339 al., 2014; Vijayan et al., 2012). Our data support that CBD may be free from the complication of

340 reduced brain uptake or varied interindividual response to drug therapy, at least that is mediated

341 by the ABC transporters P-gp and Bcrp. These findings provide evidence for the favourable

342 pharmacokinetic properties of CBD in the treatment of CNS disorders and help build the case for

343 the development of CBD as a therapeutic agent.

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