

ABC transporters P-gp and Bcrp do not limit the brain uptake of 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 concentrations 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.

ABC transporters P-gp and Bcrp do not limit the brain uptake of 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; CB₁, cannabinoid

39 1 receptor

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41 **Keywords**

42 Cannabidiol, P-glycoprotein, Breast cancer resistance protein, brain disposition, substrate,

43 risperidone

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Abstract

49 Cannabidiol (CBD) is currently being investigated as a novel therapeutic for the treatment of
 50 CNS disorders like schizophrenia and epilepsy. ABC transporters such as P-glycoprotein (P-gp)
 51 and breast cancer resistance protein (Bcrp) mediate pharmacoresistance in these disorders. P-gp
 52 and Bcrp are expressed at the blood brain barrier (BBB) and reduce the brain uptake of substrate
 53 drugs including various current antipsychotics and anticonvulsants. It is therefore important to
 54 assess whether CBD may be prone to treatment resistance mediated by P-gp and Bcrp.
 55 Moreover, it has become common practice in the drug development of CNS agents to screen
 56 against ABC transporters to help isolate lead compounds with optimal pharmacokinetic
 57 properties. The current study aimed to assess whether P-gp and Bcrp impacts the brain transport
 58 of CBD by comparing CBD tissue concentrations in wild-type (WT) mice versus mice devoid of
 59 ABC transporter genes. P-gp knockout *Abcb1a/b* (-/-), Bcrp knockout *Abcg2* (-/-), combined P-
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 61 and plasma samples were collected and determined at various time-points. CBD results were
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 63 that are established ABC transporter substrates. Brain and plasma concentrations of CBD were
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 67 and may be free from the complication of reduced brain uptake by these transporters. Such
 68 findings provide favorable evidence for the therapeutic development of CBD in the treatment of
 69 various CNS disorders.

1. Introduction

Cannabidiol (CBD), a non-psychoactive constituent of cannabis, displays much potential as a novel therapeutic treatment for various CNS disorders including schizophrenia and epilepsy (Bumb *et al.*, 2015; Iseger *et al.*, 2015; Longo *et al.*, 2015). CBD has anticonvulsant effects in animal models of epilepsy and schizophrenia (Arnold *et al.*, 2012; Jones *et al.*, 2012; Jones *et al.*, 2010; Mao *et al.*, 2015). Anecdotal reports and early clinical findings support CBD's ability to reduce seizure rates in humans with a good safety profile (Rosenberg *et al.*, 2015). Phase 3 clinical trials are currently investigating CBD in the treatment of epilepsy, most notably in severe and pharmaco-resistant childhood epilepsies. There is also promising evidence that CBD is a novel antipsychotic, with a phase 2 clinical trial showing CBD reduced symptoms in schizophrenia patients with comparable efficacy to a conventional antipsychotic drug without producing extrapyramidal side-effects, sedation or weight gain (Bumb *et al.*, 2015; Iseger *et al.*, 2015; Leweke *et al.*, 2012). Numerous further clinical trials are currently underway examining the efficacy of CBD in treating schizophrenia.

Resistance to treatment is a major stumbling block in the clinical management of epilepsy and schizophrenia. Approximately 30% of both schizophrenia and epilepsy patients do not respond adequately to drug therapy (Hoosain *et al.*, 2015; van Os *et al.*, 2009) and ABC transporters play a role in treatment-resistance (Bebawy *et al.*, 2008; Brandt *et al.*, 2006). These proteins transport substrates across biological membranes and thus influence the disposition of substrate drugs (Hee Choi *et al.*, 2014; Kathawala *et al.*, 2015). The best characterized ABC transporters are P-glycoprotein (P-gp, *Abcb1*) and breast cancer resistance protein (Bcrp, *Abcg2*),

both which are localized at various pharmacological barriers in the body including the blood brain barrier (Löscher *et al.*, 2005). Many antipsychotic and anticonvulsant drugs are substrates of P-gp, which strongly limits the brain accumulation of these agents by extruding the drugs from the brain parenchyma back into the blood (Boulton *et al.*, 2002; Doran *et al.*, 2005; Luna-Tortós *et al.*, 2008; Zhang *et al.*, 2012). Few studies have examined the substrate profile of CNS drugs for Bcrp.

There is evidence that genetic variation in P-gp influences treatment response to antipsychotic and antiepileptic drugs such as olanzapine, risperidone, paliperidone (9-hydroxy risperidone, the active metabolite of risperidone), phenobarbital and phenytoin (French, 2013; Wolking *et al.*, 2015). Furthermore some evidence suggests P-gp is upregulated at the BBB in epilepsy and schizophrenia thus limiting the brain uptake and efficacy of substrate anticonvulsant and antipsychotic drugs (Bauer *et al.*, 2014; de Klerk *et al.*, 2010). Drugs that are not substrates of ABC transporters will then make better therapeutics, as they will be immune to drug resistance mediated by these proteins. Indeed, it has become common practice in drug development to screen against ABC transporters to help isolate lead compounds that are less likely to fail in clinical trials due to suboptimal pharmacokinetic properties. It is therefore important to establish whether CBD is an ABC transporter substrate.

We have shown that the main psychoactive constituent of cannabis, THC is a substrate of both P-gp and Bcrp (Spiro *et al.*, 2012). CBD, an isomer of THC, inhibits both P-gp and Bcrp transport (Feinshtein *et al.*, 2013b; Zhu *et al.*, 2006), although results have not been consistent for P-gp (Holland *et al.*, 2006). Inhibitors are often substrates so there is a need to clarify whether CBD is a substrate of P-gp or Bcrp and whether this has implications for the brain uptake of the compound. The present study assessed whether CBD is a substrate of P-gp and

Bcrp by utilising mice devoid of these ABC transporter genes singly or in combination. If CBD were to accumulate at greater levels in the brain of knockout animals, then this provides evidence that it is an ABC transporter substrate. Further, it will help appreciate the extent that these transporters regulate the brain uptake of CBD (Doran *et al.*, 2005). We compared the results of CBD with a positive control risperidone and 9-hydroxy risperidone, as these antipsychotic drugs are established P-gp substrates.

2. Materials and methods

2.1. Animals

We used male wild-type (WT, FVB background strain), P-gp knockout *Abcb1a/b* (-/-), Bcrp knockout *Abcg2* (-/-), and combined P-gp/Bcrp knockout *Abcb1a/b*(-/-)*Abcg2*(-/-) mice aged between 4-5 months weighing between 25-30 g (Taconic farms, New York, USA). P-gp, Bcrp and P-gp/Bcrp knockout mice were developed by Professor Alfred Schinkel and colleagues at the Netherlands Cancer Institute, Amsterdam (Jonker *et al.*, 2002; Schinkel *et al.*, 1995; Schinkel *et al.*, 1997). Mice were housed in standard mouse cages in groups of 4-6 mice per cage. Food and water were available *ad libitum* and all cages contained various forms of environmental enrichment such as a mouse house igloo and running wheel, a paper roll, a climbing ring, tissue paper and sunflower seeds. The University of Sydney's Animal Ethics Committee approved all experimental procedures undertaken and all procedures were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2. Drug treatment

CBD (THC Pharm, Frankfurt, Germany) was dissolved in a mixture of ethanol, Tween 80, and saline (1:1:18) (Long *et al.*, 2013; Todd *et al.*, 2015) and administered via subcutaneous (s.c)

injection at a dose of 10 mg/kg. Risperidone (Sequoia Pharmaceuticals, United Kingdom) was dissolved in a solution of 0.9% saline and 1% acetic acid and injected s.c. at 3 mg/kg. All drugs were freshly prepared before use and made at an injection volume of 10 ml/kg of body weight. At numerous time-points post-injection of CBD (1, 2 and 3 h) and risperidone (1 and 3 h), *Abcb1a/b* (-/-), *Abcg2* (-/-), *Abcb1a/b*(-/-)*Abcg2*(-/-) and WT mice were lightly anesthetised with isoflurane and blood collected via cardiac puncture. Blood samples were stored in ethylenediaminetetraacetic acid (EDTA) coated tubes to avoid coagulation and kept on ice before separation of plasma. To separate the plasma from the blood, samples were centrifuged at 3000 rpm for 10 min at 4°C and the plasma collected in clean eppendorf tubes. The brains were immediately extracted and snap frozen in liquid nitrogen. Both the brain and plasma samples were stored at -80°C before LC-MS/MS analysis.

2.3. Quantification of CBD in brain and blood samples

CBD was extracted using a previously outlined method from our group (Johnston *et al.*, 2014). In brief, a deuterated D₃-CBD internal standard solution was added to every brain or plasma sample. Calibration standards and quality control (QC) samples were prepared by spiking drug-free mouse plasma or drug-free brain homogenates, at linear concentrations from 10-400 ng/g of CBD for brain analysis and 10-300 ng/ml of CBD for plasma analysis. The standards were vortexed and treated identically to other samples. Half brains were homogenised in dH₂O at a 1:6 ratio (w/v) with 1 mL brain homogenate. For plasma analysis, 0.5 mL of a sample was used. Brain and plasma samples were prepared by slowly adding 2 mL ice-cold acetonitrile, mixed thoroughly and centrifuged at 3000 rpm for 10 minutes. The acetonitrile was decanted into clean tubes and all samples were evaporated using the Genevac EZ-2 evaporation system for

approximately 3-4 hours. After reconstituting the samples with 2 mL dH₂O the samples were loaded onto Styre Screen® SSTHC063 solid-phase extraction (SPE) columns (60 mg/3 ml) from United Chemical Technologies (Horsham, PA, USA). Columns were then washed with 1 mL water/acetonitrile/NH₄OH (84:15:1) and dried thoroughly under vacuum (10 mm Hg) for 10-15 minutes. Samples were eluted from the column by adding 3 mL of hexane/ethyl acetate/glacial acetic acid (49:49:2). Extracts were completely dried under a nitrogen gas stream at 60°C for 5-10 minutes and reconstituted with 50 µl initial mobile phase (40 % methanol and 60 % 10 mM ammonium acetate) for analysis. All quantification was performed using a Shimadzu 8030 triple quadrupole mass spectrometer. The mobile phase consisted of (A) 10 mM ammonium acetate in water and (B) methanol. The limits of quantification (LOQ) for plasma analysis were 1.5 ng/ml and 11.5 ng/g for brain analysis.

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

For plasma analysis, 10 µl of methyl-risperidone (10 µM) internal standard (IS) solution and 0.5 mL of PO₄ buffer (pH 5.0) were added to each 0.1 mL sample of plasma. Calibration standards were prepared by spiking drug-free mouse plasma at concentrations of 2-200 ng/ml for risperidone and 9-hydroxy risperidone. The standards were vortexed and treated identically to other plasma samples. For extraction, plasma samples underwent SPE using Varian SPEC 3 mL MP3 (15 mg) microcolumns from Agilent (Santa Clara, CA, USA). Columns were first conditioned by adding 0.5 mL methanol followed by 0.5mL 0.1M PO₄ buffer (pH 5.0). Plasma samples were then loaded onto the column. The columns were washed in 0.5 mL of acetic acid (1 M) and 0.5 mL methanol and then dried under vacuum for approximately 2 minutes. The samples were eluted from the column using 1 mL of freshly prepared

dichloromethane/isopropanol/ammonia (80:20:2). The elutant was evaporated to dryness using a SpeedVac centrifugal evaporator. Samples were reconstituted with 200 µl of 50% acetonitrile.

For brain analysis, brains were dissected in half, weighed and one half of the brain was homogenised in dH₂O at a 1:2 ratio (w/v). All samples and calibrators received 50 µl of 100 nM internal standard. Calibrators were made up of 1.5 mL of 0.1 M phosphate buffer (pH 6) spiked with linear concentrations of 1-100 nM of risperidone and 9-hydroxy risperidone. Sample homogenates were centrifuged at 14,000 g for 10 minutes and the supernatant collected. The brain supernatant underwent identical preparation and extraction as plasma samples and the elutant evaporated accordingly. Samples were reconstituted with 50 µl of mobile phase A. The linear gradient solutions consisted of mobile phase (A) 5 mM ammonium formate (pH 6) and (B) 90% acetonitrile. All quantification was performed using triple quadrupole liquid chromatography-mass spectrometry (Agilent 6460). The LOQ for brain analysis was 1.5 ng/g and 1 ng/ml for plasma analysis.

2.5. Statistical analysis

Two way ANOVA with factors of genotype (including WT, P-gp, Bcrp and P-gp/Bcrp knockout mice) and time were performed on brain and plasma concentrations as well as brain/plasma ratios of CBD, risperidone and 9-hydroxy risperidone. In the instance of finding an overall main effect of genotype, further two-way ANOVAs of specific genotype by time were conducted which separately compared WT mice to either 1) P-gp knockout, 2) Bcrp knockout, 3) P-gp/Bcrp knockout mice, or 4) P-gp versus P-gp/Bcrp knockout mice. Differences were deemed statistically significant when $P < 0.05$.

3. Results

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

Concentrations of CBD in brain and plasma (Fig. 1) were not altered in P-gp, Bcrp or P-gp/Bcrp knockout mice compared to WT mice as supported by overall two-way ANOVA. A significant main effect of time was observed in both brain [$F(3,60) = 30.5$, $P < 0.0001$] and plasma [$F(2,63) = 34.8$, $P < 0.0001$] samples, with CBD concentrations decreasing over the three hour time period in all genotypes. Two-way ANOVAs comparing individual ABC transporter genotypes of the mice to WT controls showed no significant main effects of genotype, nor any interaction of genotype by time for brain and plasma CBD concentrations.

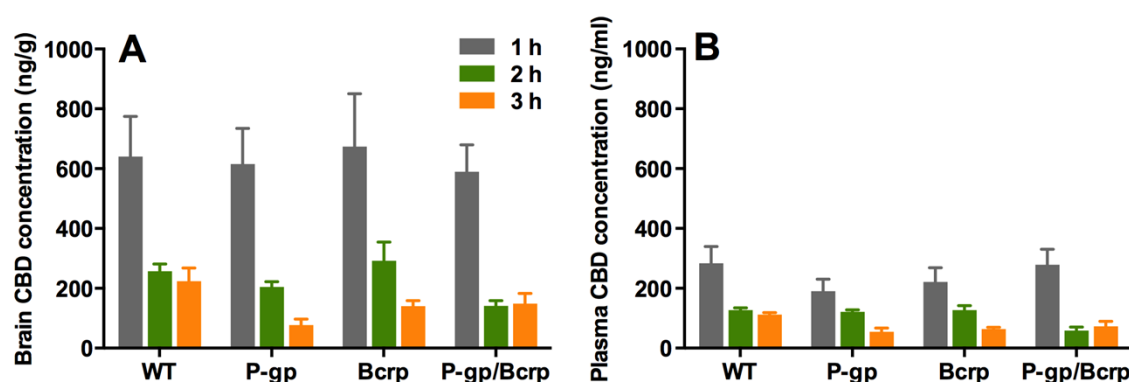


Figure 1. P-gp or Bcrp transporter knockout did not influence the CBD brain and plasma concentrations. Brain and plasma CBD samples were collected from WT and ABC transporter knockout mice. Samples were collected at 1, 2 and 3 h after an acute 10 mg/kg s.c. injection of CBD. **A)** CBD brain concentration **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* (-/-)*Abcg2* (-/-), (P-gp/Bcrp combined knockout). Data represent mean + S.E.M.

3.2. P-gp but not Bcrp knockout increased brain concentrations of risperidone and 9-hydroxy risperidone

As CBD did not show any altered disposition in ABC transporter knockout mice, we sought to show, in our hands, that other known transporter substrates do display increased brain uptake in knockout animals. Brain concentrations of risperidone and 9-hydroxy risperidone varied across genotypes (Fig. 2A and 2C), with two way ANOVA indicating main effects of genotype on brain

205 concentration of both risperidone and 9-hydroxy risperidone [$F(3,43) = 31.5$, $P < 0.0001$; $F(3,43)$
 206 $= 30.7$, $P < 0.0001$ respectively]. A significant main effect of genotype was observed for
 207 risperidone plasma concentration [$F(3,39) = 6.8$, $P < 0.001$] but not for 9-hydroxy risperidone (P
 208 > 0.05) (Fig. 2B and 2D). Risperidone and 9-hydroxy risperidone brain concentrations
 209 significantly decreased over time [main effect of time: $F(1,43) = 12.8$, $P < 0.001$; $F(1,43) = 8.4$,
 210 $P < 0.01$ respectively] as did risperidone plasma concentrations [$F(1,39) = 101.4$, $P < 0.0001$].
 211 The 9-hydroxy risperidone plasma concentration did not change over time ($P > 0.05$).

212 Individual two way ANOVAs indicated main effects of genotype for P-gp knockout and
 213 combined P-gp/Bcrp knockout mice who had greater brain concentrations than WT mice of
 214 risperidone [$F(1,20) = 37.6$, $P < 0.0001$; ($F(1,24) = 65.3$, $P < 0.0001$ respectively] and 9-hydroxy
 215 risperidone [$F(1,20) = 31.4$, $P < 0.0001$; $F(1,24) = 87.3$, $P < 0.0001$ respectively]. Plasma
 216 concentrations of risperidone were greater in P-gp knockout compared to WT mice [main effect
 217 of genotype: $F(1,18) = 7.6$, $P < 0.01$], however no overall differences were observed in 9-
 218 hydroxy risperidone plasma concentrations. Bcrp knockout mice had significantly less
 219 risperidone brain concentration compared to WT mice [main effect of genotype: $F(1,23) = 5.2$, P
 220 < 0.05] and combined P-gp/Bcrp knockout mice had significantly lower risperidone plasma
 221 concentration compared to P-gp knockout mice [main effect of genotype: $F(1,19) = 8.4$, $P <$
 222 0.01].

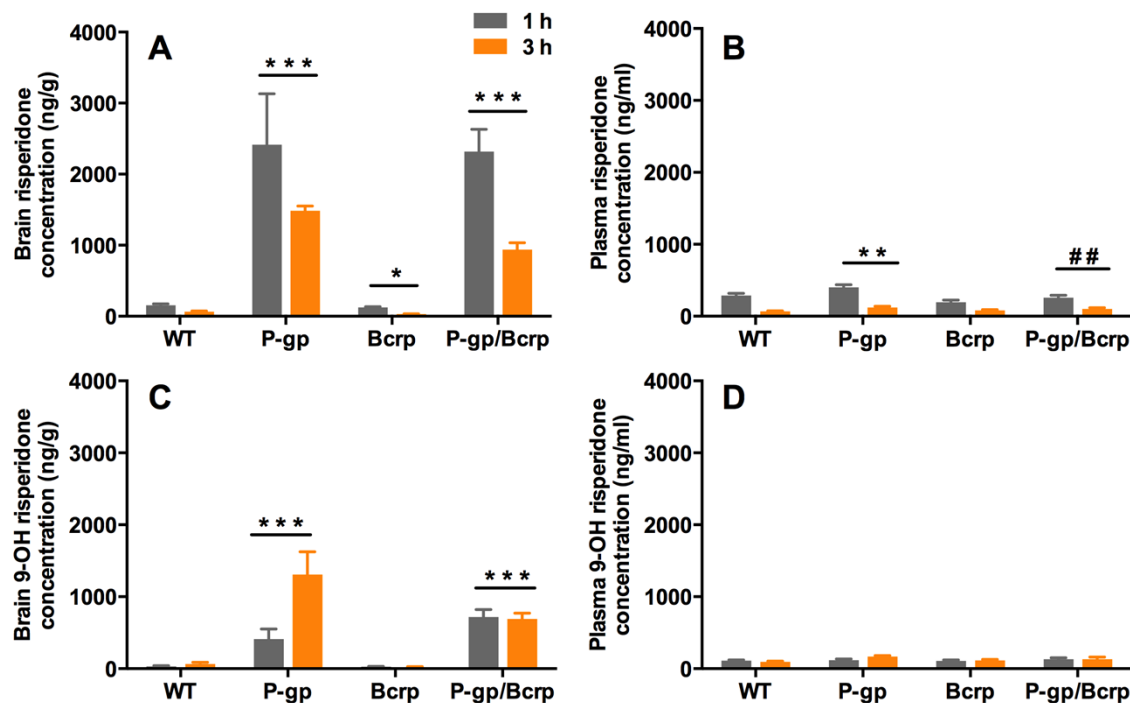


Figure 2. P-gp profoundly influences the brain uptake of risperidone and 9-hydroxy risperidone. Brain and plasma risperidone samples were collected from mice devoid of ABC transporter genes. Samples were collected at 1 and 3 h after an acute 3 mg/kg s.c injection of risperidone. **A)** Risperidone brain concentration **B)** Risperidone plasma concentration **C)** 9-hydroxy risperidone brain concentration **D)** 9-hydroxy risperidone plasma concentration. 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* (-/-)*Abcg2* (-/-), (P-gp/Bcrp combined knockout). Data represent mean + S.E.M. Two way ANOVA analysis between genotypes, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for comparisons between WT and knockout mice. ## $p < 0.01$ for comparison between P-gp knockout and P-gp/Bcrp combined knockout.

3.3. 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

No differences were observed between P-gp, Bcrp or P-gp/Bcrp knockout and WT mice (Fig. 3A) in their brain/plasma CBD concentration ratios (overall a ratio of approximately 2). In comparison, two way ANOVA indicated a significant overall genotype effect in the risperidone and 9-hydroxy risperidone brain/plasma concentration ratios [$F(3,39) = 34.62$, $P < 0.0001$; $F(3,39) = 32.17$, $P < 0.0001$ respectively] (Fig. 3B and 3C). The ratio for risperidone and 9-hydroxy risperidone also tended to increase over time between genotypes [main effect of time: $F(1,39) = 5.66$, $P < 0.05$; $P(1,39) = 5.65$, $P < 0.05$ respectively]. Individual two way ANOVAs indicated main effects of genotype for both P-gp knockout and P-gp/Bcrp knockout, who

231 compared to WT mice (ratios less than 1) had significantly greater brain/plasma risperidone
 232 concentration ratios (reaching as high as 14) [$F(1,18) = 41.1$, $P < 0.0001$; $F(1,21) = 58.8$, $P <$
 233 0.0001 respectively] and 9-hydroxy risperidone concentration ratios (reaching as high as 8)
 234 [$F(1,18) = 32.5$, $P < 0.0001$; $F(1,21) = 61.1$, $P < 0.0001$]. However, there was no difference
 235 between P-gp knockout and P-gp/Bcrp knockout mice in the brain/plasma concentration ratios
 236 for risperidone and 9-hydroxy risperidone, implying no cooperation between P-gp and Bcrp in
 237 the transport of these drugs.

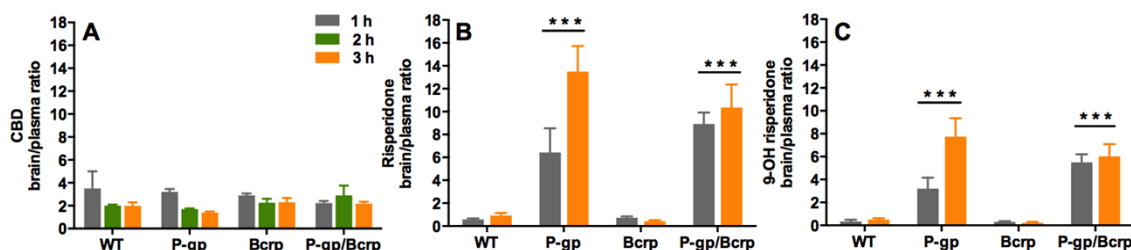


Figure 3. P-gp or Bcrp knockout did not alter CBD brain/plasma concentration ratios, whilst P-gp knockout profoundly increased risperidone and 9-hydroxy risperidone brain/plasma ratios. A) CBD brain/plasma concentration ratios B) risperidone brain/plasma concentration ratios C) 9-hydroxy risperidone 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* (-/-)*Abcg2* (-/-), (P-gp/Bcrp combined knockout). Data represent mean + S.E.M. Two way ANOVA analysis between genotypes, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for comparisons between WT and knockout mice.

4. Discussion

238 This study shows that the ABC transporters P-gp and Bcrp do not influence the brain uptake of
 239 CBD, a novel antipsychotic and anticonvulsant drug. P-gp, Bcrp and P-gp/Bcrp knockout mice
 240 did not accumulate greater brain or plasma concentrations of CBD compared to WT mice. By
 241 comparison, the known substrates of P-gp risperidone and 9-hydroxy risperidone (Ejsing *et al.*,
 242 2005; Kirschbaum *et al.*, 2008; Wang *et al.*, 2004), displayed increased brain and plasma

concentrations in P-gp knockout mice compared to WT mice. Moreover, the brain/plasma concentration ratios of risperidone and 9-hydroxy risperidone were dramatically increased in P-gp knockout mice compared to WT mice reaching as high as approximately 14 and 8 times respectively whereas WT mice only attained ratios < 1 . No differences were observed between P-gp or Bcrp knockout mice and WT mice in the brain/plasma concentration ratios of CBD. Taken together these results suggest P-gp strongly regulates the brain uptake of the antipsychotic drugs risperidone and 9-hydroxy risperidone but not CBD.

That P-gp and Bcrp did not influence the brain uptake of CBD is somewhat surprising given our prior research showing these transporters regulate the brain concentrations of THC, the main psychoactive constituent of cannabis (Spiro *et al.*, 2012). CBD and THC are isomers with very similar lipophilicity. However, CBD is formed when the central pyran ring of THC is opened and the oxygen in the ring is converted into a free hydroxy group (Compton *et al.*, 1992). This subtle chemical modification yields remarkable differences in the pharmacological activity of these drugs. For instance, THC is a partial agonist at CB₁ cannabinoid receptors and therefore elicits profound psychotropic effects, whilst CBD has poor affinity for the orthosteric site of this receptor and doesn't have appreciable psychoactivity (Laprairie *et al.*, 2015; Long *et al.*, 2010). CBD has a growing list of distinctive properties to THC (McPartland *et al.*, 2015), and our data here provides yet another example, this time a differential substrate binding character for ABC transporter proteins.

Our finding that CBD is not a substrate of murine P-gp and Bcrp is consistent with *in vitro* data with cells expressing human transporters. CBD did not stimulate ATPase activity in insect membranes expressing human P-gp and Bcrp, unlike known P-gp and Bcrp substrates verapamil and sulphasalazine respectively (Holland *et al.*, 2007; Zhu *et al.*, 2006). Therefore it

appears that our results here may generalize to human transporters, although future studies assessing CBD transport by human ABC transporters using transwell assays would strengthen this viewpoint. We and others have shown that both CBD and THC inhibit P-gp and Bcrp (Feinshtein *et al.*, 2013a; Feinshtein *et al.*, 2013b; Holland *et al.*, 2007; Spiro *et al.*, 2012; Zhu *et al.*, 2006). While THC appears to be a competitive substrate, as it is also actively transported by P-gp and Bcrp (Bonhomme-Faivre *et al.*, 2008; Spiro *et al.*, 2012), CBD's ability to inhibit the transporters occurs in the absence of active transport. This phenomenon is not without precedent as paracetamol and haloperidol both inhibit P-gp but are not actively transported as substrates (Feng *et al.*, 2008; Novak *et al.*, 2013) and gefitinib inhibits Bcrp while not being a substrate (Galetti *et al.*, 2015).

Our results suggest for the first time that risperidone and 9-hydroxy risperidone are not Bcrp substrates and that there is no cooperation between P-gp and Bcrp in the transport of these antipsychotic drugs. If these drugs were Bcrp substrates then the Bcrp knockout mice would have displayed greater brain and plasma concentrations of these agents than WT mice. Further, if these antipsychotic drugs were dual substrates of P-gp and Bcrp we would have expected the double P-gp/Bcrp knockout mice to display greater brain and plasma concentrations than P-gp knockout mice alone. Such observations have been made for other drugs, for example the Bcrp substrates prazosin and mitoxantrone display greater brain or plasma concentrations in Bcrp knockout mice than WT mice (Cisternino *et al.*, 2004) and the dual P-gp and Bcrp substrates sunitinib and dasatinib show greater brain concentrations in P-gp/Bcrp knockout mice than P-gp or Bcrp knockout mice alone (Tang *et al.*, 2013).

Intriguingly the risperidone concentration observed in the brain of Bcrp knockout mice was slightly but significantly lower than the concentration seen in WT mice. Further, the P-

gp/Bcrp knockout mice displayed lower plasma concentrations of risperidone than P-gp knockout mice. One possible explanation for this is that Bcrp knockout promoted a compensatory alteration in the expression of other undefined transporters which risperidone may be a substrate. For instance, the upregulation of another efflux transporter or the downregulation of an influx transporter could reduce active transport of risperidone leading to a decreased brain concentration. One study has addressed whether ABC transporter knockout mice display compensatory alterations in the expression of alternate transporters or enzymes (Agarwal *et al.*, 2012). Using a proteomic approach in brain capillary endothelial cells, P-gp, Bcrp or combined P-gp/Bcrp knockout did not influence the expression of 29 proteins, including well-characterised ABC transporters and solute carrier transporters. However, it is still an open possibility that Bcrp knockout or P-gp/Bcrp knockout induces compensation in the expression of transporters in peripheral tissues, which could be examined in a future study. In any case, the implications of any compensatory changes for risperidone brain and plasma distribution is minimal, which is reinforced by the observation that the subtle changes in brain and plasma concentrations alone did not translate to any differences in brain/plasma ratios in WT versus Bcrp knockout mice or P-gp versus P-gp/Bcrp knockout mice.

Cannabidiol is currently being assessed in randomized controlled trials as a novel antipsychotic and anticonvulsant agent, supported by an array of preclinical and human data (Arnold *et al.*, 2012; Devinsky *et al.*, 2014; Leweke *et al.*, 2012). ABC transporters may play an important role in pharmacoresistance, which is a major stumbling block in the successful treatment of schizophrenia and epilepsy. Indeed the ABC transporter substrate binding character is routinely assessed in the development of novel CNS therapeutics to ensure adequate brain uptake of the drug e.g. blonanserin (Inoue *et al.*, 2012). As can be seen here, the brain uptake of

312 the commonly used antipsychotic drug risperidone and its active metabolite 9-hydroxy
 313 risperidone is profoundly limited by P-gp. Similarly many anticonvulsant drugs such as
 314 phenytoin and phenobarbital are also ABC transporter substrates and subject to poor brain uptake
 315 (Zhang *et al.*, 2010; Zhang *et al.*, 2012). Moreover, single nucleotide polymorphisms (SNPs) in
 316 *MDR1* increase the risk of resistance or greater interindividual response to antiepileptic and
 317 antipsychotic drugs (Bozina *et al.*, 2008; Li *et al.*, 2014; Shaheen *et al.*, 2014; Vijayan *et al.*,
 318 2012). Our data support that CBD may be free from the complication of reduced brain uptake or
 319 varied interindividual response to drug therapy, at least that is mediated by the ABC transporters
 320 P-gp and Bcrp. These findings provide evidence for the favourable pharmacokinetic properties of
 321 CBD in the treatment of CNS disorders and help build the case for the development of CBD as a
 322 therapeutic agent.

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