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RESEARCH PAPER

Cannabidiol ameliorates cognitive and motor impairments in bile-duct ligated mice via 5-HT$_{1A}$ receptor activation

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Background and purpose: We aimed to demonstrate the involvement of 5-HT$_{1A}$ receptors in the therapeutic effect of cannabidiol, a non-psychoactive constituent of *Cannabis sativa*, in a model of hepatic encephalopathy induced by bile-duct ligation (BDL) in mice.
Experimental approach: Cannabidiol (5 mg·kg^{-1}; i.p.) was administered over 4 weeks to BDL mice. Cognition and locomotion were evaluated using the eight-arm maze and the open field tests respectively. Hippocampi were analysed by RT-PCR for expression of the genes for tumour necrosis factor-α receptor 1, brain-derived neurotrophic factor (BDNF) and 5-HT$_{1A}$ receptor. *N*-(*2*-(4-(*2*-methoxy-phenyl)-1-piperazin-1-yl)ethyl)-*N*-(*2-pyridyl*) cyclohexanecarboxamide (WAY-100635), a 5-HT$_{1A}$ receptor antagonist (0.5 mg·kg^{-1}), was co-administered with cannabidiol. Liver function was evaluated by measuring plasma liver enzymes and bilirubin.
Key results: Cannabidiol improved cognition and locomotion, which were impaired by BDL, and restored hippocampal expression of the tumour necrosis factor-α receptor 1 and the BDNF genes, which increased and decreased, respectively, following BDL. It did not affect reduced 5-HT$_{1A}$ expression in BDL mice. All the effects of cannabidiol, except for that on BDNF expression, were blocked by WAY-100635, indicating 5-HT$_{1A}$ receptor involvement in cannabidiol's effects. Cannabidiol did not affect the impaired liver function in BDL.
Conclusions and implications: The behavioural outcomes of BDL result from both 5-HT$_{1A}$ receptor down-regulation and neuroinflammation. Cannabidiol reverses these effects through a combination of anti-inflammatory activity and activation of this receptor, leading to improvement of the neurological deficits without affecting 5-HT$_{1A}$ receptor expression or liver function. BDNF up-regulation by cannabidiol does not seem to account for the cognitive improvement.
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Keywords: cognition; activity; inflammation; bile-duct ligation; gene expression

Abbreviations: 2-AG, 2-arachidonoylglycerol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; BDL, bile-duct ligation; BDNF, brain-derived neurotrophic factor; CB$_1$/CB$_2$, cannabinoid receptor 1/2; CBD, cannabidiol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HE, hepatic encephalopathy; TNFRSF1A, TNF-α receptor 1; WAY-100635, *N*-(*2*-(4-(*2*-methoxy-phenyl)-1-piperazin-1-yl)ethyl)-*N*-(*2-pyridyl*) cyclohexanecarboxamide; ZM241385, 4-(2-{[5-amino-2-(2-furyl)[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-7-yl]amino}ethyl)phenol

Introduction

Hepatic encephalopathy (HE), a neuropsychiatric complication occurring in both acute and chronic liver failure, is a major clinical problem in the treatment of patients with liver insufficiency. Among the symptoms characterizing this disorder are shortened attention span, impaired muscular coordination and asterixis (flapping tremor), which may progress to stupor and coma. Pathologically, it is characterized by an abnormal morphology of astrocytes, termed 'Alzheimer type II astrocytosis', in which astrocytes exhibit large swollen nuclei, prominent nucleoli and margination of the chromatin pattern (Butterworth *et al.*, 1987). The importance of HE is highlighted by the increasing number of patients waiting for liver transplantation due to liver failure. Its pathogenesis is a

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complex process involving several mechanisms. For many years, research in this area focused mainly on neurotoxins, especially ammonia, believed to be involved in the development of the abnormal mental state, but it is now realized that multi-organ alterations and many neurotransmitter systems also play a role, among which are the monoaminergic (Mousseau *et al.*, 1997), opioidergic (Yurdaydin *et al.*, 1998) and GABA-ergic (Jalan *et al.*, 2000) systems. Systemic inflammatory response may also contribute to this disorder, as severity of HE in humans correlates with plasma levels of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-1β (Odeh *et al.*, 2004; Shawcross *et al.*, 2004). Cerebral inflammation was found to contribute to HE in acute liver failure in rats, because anti-inflammatory treatment ameliorated the symptoms (Jiang *et al.*, 2009).

Bile-duct ligation (BDL) is a common model of chronic liver disease (also termed 'cholestatic liver disease') in rats and, to a lesser extent, in mice. It mimics biliary liver disease in humans (Kountouras *et al.*, 1984). In this model, liver enzyme levels are elevated and liver fibrosis develops in rats (Liu *et al.*, 2006). Cognitive impairments are also observed (Huang *et al.*, 2004). Evidence for the role of inflammation in the BDL model comes from a study that demonstrated infiltration of peripheral TNF-α-secreting monocytes into mice brains 10 days after the ligation (Kerfoot *et al.*, 2006). This finding may be relevant to the cognitive dysfunction in HE, as systemic lipopolysaccharide-induced neuroinflammation leading to microglial activation was reported previously to be responsible for disrupted neurogenesis in the dentate gyrus region of the hippocampus (Monje *et al.*, 2003) and for a decrease in the levels of the neurotrophin, brain-derived neurotrophic factor (BDNF) in the hippocampus (Wu *et al.*, 2007). This region is known to play a crucial role in learning and memory (Feng *et al.*, 2001). Thus, animals with BDL may serve as a model of HE.

Endocannabinoids are a family of lipid messengers that bind to the cell surface receptors (cannabinoid receptors – CBr) targeted by Δ^9-tetrahydrocannabinol (Δ^9-THC), the major psychoactive constituent of the plant *Cannabis sativa*. Their synthesis in the CNS is triggered by an elevation of intracellular calcium levels (Stella, 2004), and they are involved in a wide range of intracellular events (Howlett *et al.*, 2002). Two major endocannabinoids have been identified and well characterized: arachidonoyl ethanolamide (anandamide) (Devane *et al.*, 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). They bind to CB_1 receptors on axon terminals, thus regulating ion channels activity and neurotransmitter release by a retrograde messenger mechanism (Van der Stelt and Di Marzo, 2005). CB_2 receptors exist mainly in the immune system, but recent publications have reported their existence also in the CNS, particularly following brain damage (Benito *et al.*, 2008; receptor nomenclature follows Alexander *et al.*, 2008). The levels of 2-AG and the expression of the CB_1 and CB_2 receptors are increased in the brains of mice with acute HE induced by the hepatotoxin thioacetamide (TAA) (Avraham *et al.*, 2006; Dagon *et al.*, 2007). Stimulation of CB_1 receptors enhances liver fibrosis, whereas that of CB_2 receptors suppresses it (Pacher *et al.*, 2006; Mallat and Lotersztajn, 2008).

Cannabidiol (CBD) is a non-psychoactive constituent of *C. sativa* known to be a weak antagonist of CB_1 and CB_2 receptors (Thomas *et al.*, 2007). However, it has been proposed to have many other mechanisms of actions such as adenosine reuptake inhibition (Carrier *et al.*, 2006; Mechoulam *et al.*, 2007) and activation of 5-HT_{1A} receptors (Mechoulam *et al.*, 2007), and direct agonism of these receptors (Russo *et al.*, 2005). It also has very strong anti-inflammatory activity both *in vivo*, as an anti-arthritic agent (Malfait *et al.*, 2000), and *in vitro*, manifested by inhibition of cytokine production in immune cells (Ben-Shabat *et al.*, 2006).

Previous work from our lab has demonstrated the therapeutic effects of CBD in BDL-induced HE (Magen *et al.*, 2009) and has highlighted the involvement of indirect activation of the A_{2A} adenosine receptors in these effects of CBD. In the present work, we aimed to determine whether the effects of CBD may also be mediated via 5-HT_{1A} receptors, as CBD has been shown to exert some of its therapeutic effects via these receptors (Mishima *et al.*, 2005; Campos and Guimarães, 2008; Resstel *et al.*, 2009). We also addressed the question whether their expression is altered in BDL, and whether CBD may reduce inflammatory markers in BDL.

Methods

Animals

Eight-week-old female Sabra mice (25–30 g) were assigned at random to five groups of 10 mice per cage. They received food and water *ad libitum*, except for the day before surgery, when food was deprived (see below). The food provided was Purina Chow and the mice were maintained on a 12 h light : dark cycle. Lights were on at 07.00 h and were off at 19.00 h. The mice were maintained in the animal facility (SPF unit) of the Hebrew University Hadassah Medical School, Jerusalem, and received humane care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals'.

Surgery

The mice were deprived of food 12 h prior to the surgery, with free access to water. Under anaesthesia of ketamine : xylazine mixed at a ratio of 9:1, respectively and given subcutaneously at a dose of 200 $mg \cdot kg^{-1}$ ketamine, a midline laparotomy (1 cm) was performed; the common bile duct was exposed and ligated twice with 6-0 silk sutures. Sham-operated mice were laparotomized without BDL. The abdomen was closed in layers, and the animals were allowed to recover on a heating pad. Dipyrone, 7.5 $mg \cdot kg^{-1}$, dissolved in drinking water was used as an analgesic after surgery.

Evaluation of cognitive function: eight-arm maze test

The eight-arm maze test was conducted as described previously, with minor modifications (Pick and Yanai, 1983). The mice were deprived of water 2 h prior to the test, and a reward of 50 μL of water was presented at the end of each arm. After injection of the drugs, each mouse was tested until it made entries into all eight arms or until it completed 24 entries, whichever came first. In case the mouse did not complete

Table 1 Primers used in this study, including accession numbers, sequences and their location in the transcript

Gene	*Accession number (NCBI)*	*Primer sequence*	*Primer location in the transcript*
GAPDH	NM 008084	F:5′-CTCTGCTCCTCCCTGTTCCA-3′	172-191
		R:5′-CTGGCACTGCACAAGAAGATG-3′	222-202
BDNF	NM 007540	F:5′-CACTGAGTCTCCAGGACAGCAA-3′	609-630
		R:5′-CTCTTCTCACCTGGTGGAACATT-3′	659-637
5-HT_{1A} receptor	NM 008308	F:5′-GACAGGCGGCAACGATACT-3′	621-639
		R:5′-CCAAGGAGCCGATGAGATAGTT-3′	796-775
TNF-α receptor 1 (TNFRSF1A)	NM 011609	F:5′-TGGTCCGATCATCTTACTTCATTC-3′	209-232
		R:5′-CTCAGGGCAGCAATTGACAA-3′	319-300

BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TNF-α, tumour necrosis factor-α.

visiting all eight arms within 24 trials, it was excluded from the data analysis. In cases where more than 2 min passed between an exit from one arm and an entry into the next, the mouse was also excluded. Food and water were given at the completion of the test. Maze performance was calculated each day for five consecutive days. Results are presented as area under the curve (AUC) utilizing the formula: (day 2 + day 3 + day 4 + day 5) − 4*(day 1). Hence, the lower the AUC, the better the cognitive function (Pick and Yanai, 1983).

Activity assessment: open field test

Activity was assessed in the open field (20 × 30 cm field divided into 12 squares of equal size) as described previously (Hanuš *et al*., 1999). After drug injection, locomotor activity was recorded by counting the number of crossings by the mice at 1 min intervals, within a period of 3 min. Results are presented as the mean number of crossings min^{-1}. The test was performed only once for each animal.

Quantitative RT-PCR analysis of TNF-α receptor 1 (TNFRSF1A), BDNF and 5-HT_{1A} receptor

Two hours after the last cognitive testing in the eight-arm maze, mice were killed by decapitation, brains were removed, and hippocampi were dissected out. Injection of all substances took place on that day, 3 h prior to death. Total hippocampal RNA was extracted using Tri reagent according to the manufacturer's instructions and reverse transcribed. RNA samples, which were not reverse transcribed, were amplified in the PCR in order to rule out the possibility of amplifying genomic DNA contamination that was present in the RNA extracted from the tissue.

Quantitative RT-PCR was carried out with Power SYBR Green PCR Master Mix, in 7900HT instrument (Applied Biosystems, Warrington, UK). Between three to five animals from each group were used for the analysis, with three repeats (triplicate) for each animal. Volume reaction was 15 μL, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Threshold cycle (Ct) was determined by SDS software for each of the samples tested, and the average Ct was calculated for each triplicate. ΔCt of each target gene was calculated by subtracting the average Ct for GAPDH of a given sample from the average Ct for the target gene of the same sample. Average ΔCt of a certain target gene in the sham group was subtracted from ΔCt of the same gene in samples from the treated groups (CBD, BDL, BDL + CBD, BDL + CBD + WAY) to yield ΔΔCt of this gene in the sample. The quantity of a specific target gene in a certain sample relative to the sham group was calculated as $2^{-\Delta\Delta Ct}$ using ΔΔCt determined for that sample.

Specific primers, including their accession numbers, sequences and location in the mRNA transcript for each gene are detailed in Table 1.

Serum liver enzyme and bilirubin levels

Serum for alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin measurements was obtained in glass tubes, centrifuged and analysed on the day of sampling using a Kone Progress Selective Chemistry Analyzer (Kone Instruments, Espoo, Finland). All serum samples were processed in the same laboratory using the same methods and the same reference values.

Experimental design

Mice were assigned at random to five groups, with 10–12 mice per group: (i) sham animals receiving vehicle (sham); (ii) sham animals receiving 5 $mg \cdot kg^{-1}$ CBD (5 $mg \cdot kg^{-1}$ CBD); (iii) BDL animals receiving vehicle (BDL); (iv) BDL animals receiving 5 $mg \cdot kg^{-1}$ CBD (BDL + 5 $mg \cdot kg^{-1}$ CBD); and (v) BDL animals receiving 5 $mg \cdot kg^{-1}$ CBD and 0.5 $mg \cdot kg^{-1}$ *N*-(*2*-(4-(*2*-methoxy-phenyl)-1-piperazin-1-yl)ethyl)-*N*-(*2-pyridyl*) cyclohexanecarboxamide (WAY-100635) (BDL + 5 $mg \cdot kg^{-1}$ CBD + 0.5 $mg \cdot kg^{-1}$ WAY-100635). Injections of all drugs were started one day after surgery and were performed over a period of 4 weeks. CBD was administered i.p. at a dose of 5 $mg \cdot kg^{-1} \cdot day^{-1}$ in 0.1 mL. The same solution without CBD was injected as vehicle. This dose was chosen based on preliminary experiments, which demonstrated that this dose caused the strongest effect, compared with 1 and 10 $mg \cdot kg^{-1}$ (data not shown), and on data from previous studies demonstrating this, thus arguing for a biphasic effect of CBD, similar to other cannabinoids (Parker *et al*., 2004).

WAY-100635 was injected s.c. at a dose of 0.5 $mg \cdot kg^{-1} \cdot day^{-1}$ in 0.3 mL. The dose was selected based on the literature, with some modifications (Resstel *et al*., 2009). Saline solution without WAY-100635 was injected as vehicle.

Three weeks post surgery, mice were tested in the eight-arm maze test for five consecutive days and once only in the open field test. After completion of the behavioural studies (4 weeks

post surgery), half of the mice in each group were killed for serum analysis of liver enzymes, and half for RT-PCR analysis.

Statistical analysis

Data are presented as mean ± SEM. One-way ANOVA was performed for statistical analysis of all results, with the following planned comparisons: sham versus 5 mg·kg^{-1} CBD; sham versus BDL; BDL versus BDL + 5 mg·kg^{-1} CBD; BDL + 5 mg·kg^{-1} CBD versus BDL + 5 mg·kg^{-1} CBD + 0.5 mg·kg^{-1} WAY-100635.

Drugs and materials

Cannabidiol was extracted from Cannabis resin (hashish) and purified as previously reported (Gaoni and Mechoulam, 1971). It was first dissolved in ethanol and then the same amount of Cremophor EL (Sigma-Aldrich, St. Louis, MO, USA) was added. This solution was then further diluted with saline so that the final solution was ethanol/cremophor/saline (1:1:18).

WAY-100635, the 5-HT$_{1A}$ antagonist, was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in saline to a concentration of 1 mg·mL^{-1}. This solution was further diluted with saline, so that the final concentration was 0.05 mg·mL^{-1} (drug nomenclature follows *Nomenclature of Organic Chemistry*, Sections A, B, C, D, E, F and H, 1979).

Ketamine hydrochloride (100 mg·mL^{-1}, Fort Dodge, IA, USA) and xylazine (20 mg·mL^{-1}, Biob, France) were ordered from the animal facility.

Power SYBR Green PCR Master Mix was obtained from Applied Biosystems. All primers were synthesized by Syntezza (Jerusalem, Israel).

Results

Cognitive function is impaired in BDL and is improved by CBD, and this effect is blocked by the 5-HT$_{1A}$ antagonist WAY-100635

Performance in the eight-arm maze test, 3 weeks post surgery, was significantly poorer in the BDL group than in the sham group (Figure 1), as reflected by the higher AUC values (6.8 ± 3.8 vs. −13.5 ± 4.5 respectively; ANOVA: $F_{(4,53)} = 4.95$, $P = 0.0018$; planned comparisons: $P = 0.002$). Chronic administration of 5 mg·kg^{-1} CBD for 21 days prior to the test fully restored cognitive function (−14.6 ± 3.4 vs. 6.8 ± 3.8 for BDL only, $P = 0.001$). CBD had no effect on the cognitive function of sham animals. WAY-100635 (0.5 mg·kg^{-1}), the 5-HT$_{1A}$ receptor antagonist, reversed the effect of CBD (AUC = 5.4 ± 3.2, $P = 0.001$ vs. BDL + CBD).

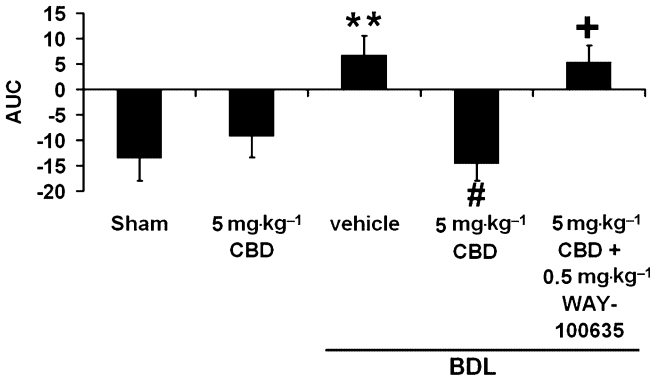

Figure 1 Cognitive function of the mice 3 weeks post surgery. Performance in the eight-arm maze test that was impaired in BDL mice was improved by 5 mg·kg^{-1} CBD, and this effect was blocked by WAY-100635. **$P < 0.01$ versus sham; #$P < 0.01$ versus BDL; +$P < 0.01$ versus BDL + 5 mg·kg^{-1} CBD. AUC, area under the curve; BDL, bile-duct ligation; CBD, cannabidiol; WAY-100635, *N*-(*2*-(4-(*2*-methoxy-phenyl)-1-piperazin-1-yl)ethyl)-*N*-(*2-pyridyl*) cyclohexanecarboxamide.

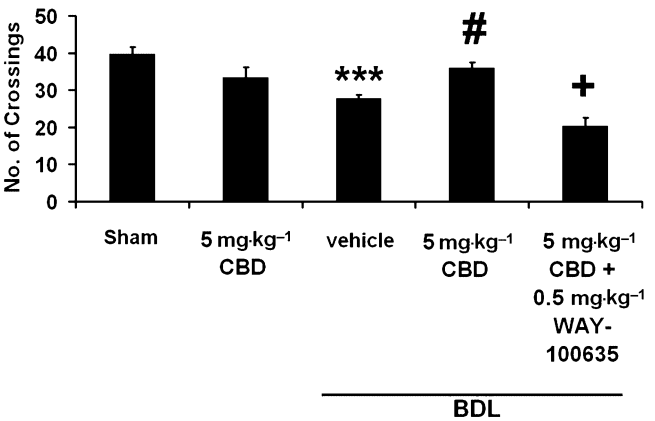

Figure 2 Motor activity of the mice 3 weeks post-surgery. The number of crossings that occurred in 1 min in the open field test decreased in bile-duct ligation (BDL) mice and was restored by 5 mg·kg^{-1} cannabidiol (CBD). The effect of CBD was blocked by *N*-(*2*-(4-(*2*-methoxy-phenyl)-1-piperazin-1-yl)ethyl)-*N*-(*2-pyridyl*) cyclohexanecarboxamide (WAY-100635). ***$P < 0.001$ versus sham; #$P < 0.001$ versus BDL; +$P < 0.001$ versus BDL + 5 mg·kg^{-1} CBD.

Locomotor function impaired by BDL is improved by CBD, and this effect is blocked by WAY-100635

The number of crossings 1 min^{-1} in the open field arena (Figure 2) for the BDL group was decreased compared with the sham group (27.7 ± 1.1 vs. 39.8 ± 1.8, respectively, $F_{(4,38)} = 13.3$, $P = 0.00001$; planned comparisons: $P < 0.001$). This decrease was reversed by CBD (36.8 ± 1.3 vs. 27.7 ± 1.1 for BDL only, $P < 0.001$). However, CBD had no effect on locomotor activity in sham mice ($P = 0.083$). WAY-100635, the 5-HT$_{1A}$ receptor antagonist, fully reversed the effect of CBD on locomotor activity (20.3 ± 2.4 vs. 36.8 ± 1.4 crossings min^{-1}, respectively; $P < 0.001$). WAY-100635 (0.5 mg·kg^{-1}) did not affect the cognitive or locomotor function of either the sham or the BDL mice untreated with CBD (data not shown).

Hippocampal TNFRSF1A mRNA increased and BDNF mRNA decreased following BDL and CBD reversed these changes; WAY-100635 blocked the effect of CBD on TNFRSF1A

The expression of TNFRSF1A in the hippocampus of BDL mice increased modestly but significantly, to 127 ± 7% of the sham expression level (Figure 3A; ANOVA: $F_{(4,22)} = 10.29$, $P < 0.0001$; planned comparisons: $P < 0.05$ vs. sham). CBD (5 mg·kg^{-1}) significantly decreased its expression, to 74 ± 8% of sham

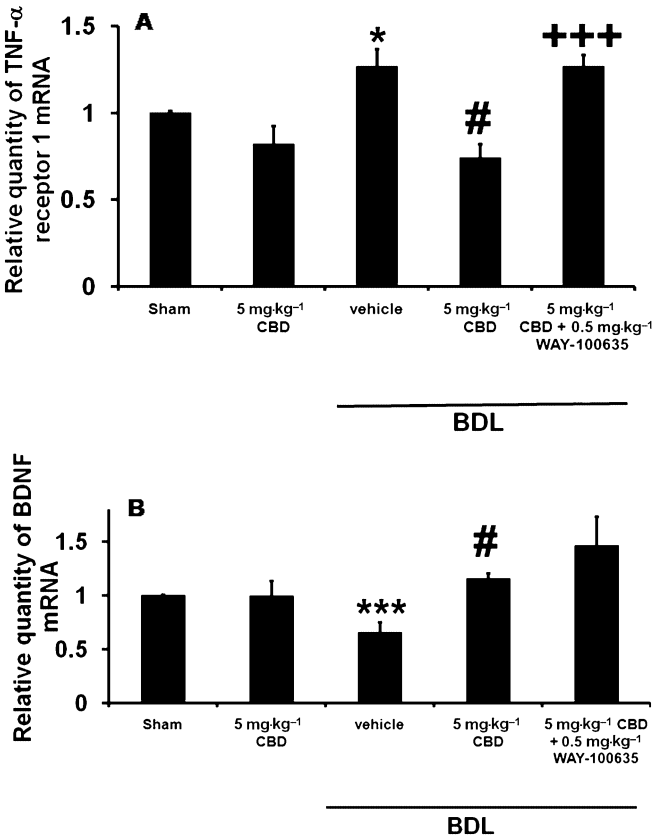

Figure 3 Hippocampal gene expression 4 weeks after bile-duct ligation (BDL) was altered. (A) Tumour necrosis factor (TNF)-α receptor 1 expression increased in the hippocampus of BDL mice; this was normalized after cannabidiol (CBD) administration, and this effect was reversed by *N*-(*2*-(4-(*2*-methoxy-phenyl)-1-piperazin-1-yl)ethyl)-*N*-(*2-pyridyl*) cyclohexanecarboxamide (WAY-100635). **P* < 0.05 versus sham; #*P* < 0.01 versus BDL; +++*P* < 0.01 versus BDL + 5 mg·kg^{-1} CBD. (B) Brain-derived neurotrophic factor (BDNF) expression decreased and was normalized by CBD. ****P* < 0.01 versus sham; #*P* < 0.01 versus BDL.

expression level ($P = 0.002$ vs. BDL only), and 0.5 mg·kg^{-1} WAY-100635 fully reversed this effect of CBD, increasing the expression to 127 ± 8% of the sham expression level, which was significantly different from BDL + 5 mg·kg^{-1} CBD ($P = 0.002$).

mRNA expression of BDNF in the hippocampus decreased following BDL surgery (Figure 3B) to 66 ± 9% of sham expression level (ANOVA: $F_{(4,23)} = 6.042$, $P = 0.002$; planned comparisons: $P = 0.009$ vs. sham). It was restored to normal (115 ± 5%) expression by CBD ($P = 0.001$ vs. BDL only). CBD did not affect BDNF mRNA expression in sham animals (100 ± 14% of sham expression level, $P = 0.977$ vs. sham). The effect of CBD was not abolished by the 5-HT$_{1A}$ receptor antagonist WAY-100635 (105 ± 7% of sham expression level, $P = 0.262$ vs. BDL + CBD).

Hippocampal expression of 5-HT$_{1A}$ receptor mRNA was reduced by BDL but unaffected by CBD

The expression of 5-HT$_{1A}$ receptors in the hippocampus decreased by 33% in BDL mice compared with sham mice (Figure 4; 67 ± 8% of sham expression level; ANOVA: $F_{(3,23)} =$ 11.39, $P < 0.001$; planned comparisons: $P < 0.05$ vs. sham). Interestingly, the expression level in CBD-treated sham mice decreased to 77 ± 5% of sham expression level ($P < 0.01$ sham). The expression level of 5-HT$_{1A}$ receptors in CBD-treated BDL mice did not change compared with untreated BDL mice (61 ± 3% of sham expression level, $P = 0.503$ vs. BDL).

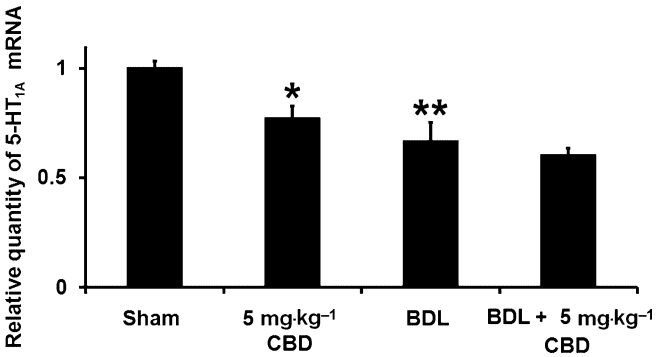

Figure 4 Hippocampal 5-HT$_{1A}$ receptor expression decreased following cannabidiol (CBD) treatment and bile-duct ligation (BDL) and was not restored by CBD. **P* < 0.05 versus sham; ***P* < 0.01 versus sham.

Hepatic function was impaired in BDL and was not normalized by CBD

In order to verify the existence of liver disease and also determine whether the effects of CBD might result from peripheral actions, the levels of the liver enzymes AST and ALT and of bilirubin were measured in the plasma. AST and ALT levels were elevated threefold and fourfold, respectively (Figure 5A) as a result of BDL surgery when tested after 4 weeks (one-way ANOVA for AST: $F_{(3,22)} = 17.04$, $P < 0.0001$; planned comparisons: $P < 0.001$, ALT: $F_{(3,22)} = 20.27$, $P < 0.0001$; planned comparisons: $P < 0.001$). CBD (5 mg·kg^{-1}) did not affect the levels of either sham or BDL animals. Bilirubin levels were elevated 12-fold (Figure 5B) in BDL animals ($F_{(3,22)} = 28.91$, $P <$ 0.0001; planned comparisons: $P < 0.001$) and were not affected by 5 mg·kg^{-1} CBD, either in sham or in BDL animals.

Discussion

It has been known for a long time that liver disease affects brain function. In our previous work, we showed that BDL lowered the expression of the A_{2A} adenosine receptor in the hippocampus, and that CBD improved cognitive and locomotor function through activation of these receptors, thus perhaps compensating for their decrease (Magen *et al*., 2009). In the present study, we aimed to determine whether the cognitive and motor effects of CBD may also be mediated via 5-HT$_{1A}$ receptors, which are metabotropic receptors located both postsynaptic to 5-hydoxytryptaminergic neurones in forebrain regions including the hippocampus, and also on the hydoxytryptaminergic neurones themselves at the level of the soma and dendrites in the mesencephalic and medullary raphe nuclei (Barnes and Sharp, 1999), where they regulate

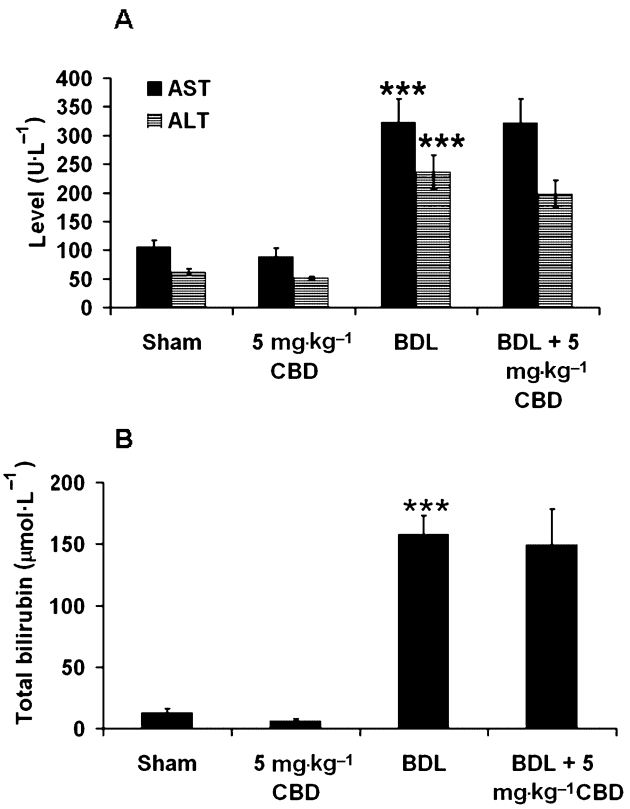

Figure 5 (A) Plasma liver enzymes were elevated in BDL mice but were not restored by CBD. (B) Plasma bilirubin was elevated in BDL mice but was not restored by CBD. ***$P < 0.001$ versus sham. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BDL, bile-duct ligation; CBD, cannabidiol.

5-HT release to, and synthesis in forebrain regions. We also wanted to determine whether their expression is altered in BDL.

Initially, we investigated the effects of the 5-HT_{1A} antagonist, WAY-100635, on the cognitive and motor effects of CBD and found it blocked both the cognitive (Figure 1) and motor (Figure 2) effects of CBD, suggesting the involvement of 5-HT_{1A} receptors in these effects. Following these observations, we aimed to elucidate the role of 5-HT_{1A} receptors in the pathogenesis of BDL and the effect of CBD on their expression. We found a decreased expression of 5-HT_{1A} receptor mRNA in the hippocampus of BDL mice, which was not reversed by CBD. Interestingly enough, 5-HT_{1A} receptor expression was also reduced following CBD treatment in sham mice, but this reduction was accompanied by only a small, non-significant trend of decrease in cognitive ability (Figure 4). This might be explained by the ability of CBD to activate the 5-HT_{1A} receptors and thus counteract this reduction. In contrast, such an activation was absent in BDL mice that were not treated with CBD, and so the cognitive function was impaired. It appears that the down-regulation of 5-HT_{1A} receptors in CBD-treated sham mice opposed the presumed over activation of this receptor by CBD, which explains why cognitive improvement was not observed in these animals (Figure 4), but was observed in CBD-treated BDL mice, in which the 5-HT_{1A} receptor was not down-regulated compared with their controls (vehicle-treated BDL mice). Thus, the therapeutic effect of CBD seems to be specific for BDL and result from an activation of 5-HT_{1A} receptors, which compensates for their down-regulation in the BDL mice. Of course, this interpretation should be made with caution as no signal transduction assays were performed to determine the extent of activation of the 5-HT_{1A} receptors in sham and BDL mice with and without CBD treatment, and the only evidence for their activation is indirect, that is, the reversal of CBD effects by WAY-100635.

The motor improvement by CBD is mediated by 5-HT_{1A} receptors (Figure 2). Similar to the cognitive improvement, the locomotor effect of CBD was also specific to the BDL mice, apparently due to the down-regulation of 5-HT_{1A} receptors in the CBD-treated sham mice, which was not observed in CBD-treated BDL mice. Reduced 5-HT_{1A} expression in the hippocampus has been shown to be associated with reduced motor activity (Schiller *et al.*, 2006).

Previous studies focusing on 5-HT_{1A} receptor density in portacaval-shunted rats (Apelqvist *et al.*, 1998), another model of HE, reported a decrease in two subregions of the hippocampus, CA1 and the dentate gyrus. A non-significant decrease of 5-HT_{1A} receptors in the hippocampus of bile-duct ligated rats has also been reported (Çelik *et al.*, 2005). In contrast, we observed that 5-HT_{1A} receptor expression was significantly decreased in the hippocampus in the BDL model. Thus, the present results shed a new light on the alterations in the 5-hydroxytryptaminergic system following BDL in mice.

In our previous work, we showed that TNFRSF1A, one of the two main receptors mediating the effects of TNF-α that has been found to negatively regulate progenitor proliferation in adult hippocampal neurogenesis (Iosif *et al.*, 2006), increased in the hippocampus of BDL mice and was normalized by CBD (Magen *et al.*, 2009). This effect on TNFRSF1A was blocked by 4-(2-{[5-amino-2-(2-furyl)[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-7-yl]amino}ethyl)phenol (ZM241385), an A_{2A} adenosine receptor antagonist (Magen *et al.*, 2009). WAY-100635 also blocked the effect of CBD on TNFRSF1A in the present study (Figure 3A). The finding that the anti-inflammatory effect of CBD is mediated via the 5-HT_{1A} receptor is, to our knowledge, a novel one, which suggests that cytokine receptor levels may be modulated by 5-HT_{1A}-selective agonists in the treatment of disorders involving a neuroinflammatory response. Moreover, this effect seems to be mediated by the 5-HT_{1A} receptors to a greater extent than by the A_{2A} adenosine receptors, as in our present study we showed that the reversal of the effect of CBD was more pronounced with WAY-100635 than with ZM241385 (Magen *et al.*, 2009). It seems that the anti-inflammatory effect of CBD and the improvement of cognitive function are related to each other, as both were reversed by WAY-100635.

Brain-derived neurotrophic factor is a member of the neurotrophin family, which plays an important role in the survival, maintenance and growth of neurones (Barde *et al.*, 1982; Leibrock *et al.*, 1989). Antisense BDNF oligonucleotide treatment impaired not only acquisition, but also maintenance and/or recall of spatial memory in rats (Mizuno *et al.*, 2003). BDNF depletion has also been reported to impede

terminal differentiation of new granule neurones in the adult hippocampus (Chan *et al.*, 2008). Also, its expression has been found to be down-regulated following lipopolysaccharide-induced neuroinflammation, which is also associated with spatial memory impairments and disrupted neurogenesis (Wu *et al.*, 2007). Therefore, the elevation of BDNF could be a mechanism associating the anti-inflammatory effect of CBD and its effect on cognitive function. However, our previous work demonstrated that the effect of CBD on BDNF expression was not blocked by the A_{2A} adenosine receptor antagonist ZM241385 (Magen *et al.*, 2009), and in our present work, 5-HT_{1A} antagonism did not block the effect of CBD on BDNF expression (Figure 3B). This finding strengthens the idea that the moderation of the inflammatory response by CBD improves cognition not through the elevation of BDNF but through other mechanisms, perhaps by increasing the levels of other neurotrophins like glial cell line-derived neurotrophic factor, or by enhancing neurogenesis, as was reported for TNFRSF1A knockout mice (Iosif *et al.*, 2006). The effects of CBD on BDNF mRNA expression may be mediated by an unknown CBD receptor. One of these potential candidates is the newly discovered G protein-coupled receptor 55 (GPR55) (Ryberg *et al.*, 2007), which should be a target for future investigation.

An intriguing question arising from this study is, whether the effects of CBD might be mediated via a peripheral effect – as cerebral dysfunction occurs downstream to the hepatic dysfunction in HE. We measured liver enzyme and bilirubin levels in the plasma (Figure 5) and found that CBD had no effect on these parameters in the BDL mice, indicating that the cerebral effects of CBD are not related to improved hepatic function, but to a direct action on the brain. Also, the exact mechanism by which CBD regulates gene expression remains to be determined, and should be explored further. One possibility is that CBD inhibits transcription factors, as it has been shown to inhibit the transcription factor NF-kappa B (Esposito *et al.*, 2006), which constitutes the inflammatory pathway.

In summary, we have shown that chronic treatment with CBD improved, via activation of 5-HT_{1A} receptors, cognitive and motor function, and moderated neuroinflammation in a mouse model of HE. This adds to the previously reported effects of CBD mediated by A_{2A} adenosine receptors (Magen *et al.*, 2009). Hence, CBD may represent a treatment for the CNS symptoms secondary to liver disease, along with other drugs that improve liver function, such as CB_2 agonists like HU-308 (Avraham *et al.*, 2008).

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Conflict of interest

The authors state no conflict of interest.

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