Cannabidiol improves brain and liver function in a fulminant hepatic failure-induced model of hepatic encephalopathy in mice

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Short title: CBD moderates fulminant hepatic failure

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Background and purpose: Hepatic encephalopathy is a neuropsychiatric disorder of complex pathogenesis caused by acute or chronic liver failure. We investigated the effects of cannabidiol, a non-psychoactive constituent of *Cannabis sativa* with anti-inflammatory properties that activates the 5-hydroxytryptamine receptor 5-HT_{1A}, on brain and liver functions in a model of hepatic encephalopathy associated with fulminant hepatic failure induced in mice by thioacetamide.

Experimental approach: Female Sabra mice were injected with either saline or thioacetamide and were treated with either vehicle or cannabidiol. Neurological and motor functions were evaluated two and three days, respectively, after induction of hepatic failure, after which brains and livers were removed for histopathological analysis and blood was drawn for analysis of plasma liver enzymes. In a separate group of animals, cognitive function was tested after eight days and brain 5-HT levels were measured twelve days after induction of hepatic failure.

Key results: Neurological and cognitive functions were severely impaired in thioacetamide-treated mice and were restored by cannabidiol. Similarly, decreased motor activity in thioacetamide-treated mice was partially restored by cannabidiol. Increased plasma levels of ammonia, bilirubin, and liver enzymes, as well as enhanced 5-HT levels in thioacetamide-treated mice were normalized following cannabidiol administration. Likewise, astrogliosis in the brains of thioacetamide-treated mice was moderated after cannabidiol treatment.

Conclusions and implications: Cannabidiol restores liver function, normalizes 5-HT levels and improves brain pathology in accordance with normalization of brain function. Therefore, the effects of cannabidiol may result from a combination of its actions in the liver and brain.

Keywords: hepatic encephalopathy; cannabidiol; cognition; liver enzymes; thioacetamide;

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; AUC, area under the curve; CBD, cannabidiol; FHF, fulminant hepatic failure; GFAP, glial fibrillary acidic protein; HE, hepatic encephalopathy; 5-HT, 5-hydroxytryptamine; TAA, thioacetamide; NS, normal saline

Introduction

Hepatic encephalopathy (HE) is a syndrome observed in patients with end-stage liver disease. It is defined as a spectrum of neuropsychiatric abnormalities in patients with liver dysfunction, after exclusion of other known brain diseases, and is characterized by personality changes, intellectual impairments, and a depressed level of consciousness associated with multiple neurotransmitter systems, astrocyte dysfunction and cerebral perfusion (Riggio *et al*., 2005; Avraham et al.,2006; 2008a,2009; Magen *et al*.,2008; Butterworth, 2010). Subtle signs of HE are observed in nearly 70% of patients with cirrhosis and approximately 30% of patients dying of end-stage liver disease experience significant encephalopathy (Ferenci,1995). HE, accompanying the acute onset of severe hepatic dysfunction, is the hallmark of fulminant hepatic failure (FHF) and patients with HE have been reported to have elevated levels of ammonia in their blood (Stahl, 1963). In addition, the infiltration of TNF-α-secreting monocytes into the brain of bile-duct ligated mice, a model of chronic liver disease, has been found 10 days after the ligation, indicating that neuroinflammation is involved in the pathogenesis of HE. This infiltration was shown to be associated with activation of the cerebral endothelium and an increase in the expression of adhesion molecules (Kerfoot *et al*., 2006).

Cannabidiol (CBD) is a non-psychoactive ingredient of *Cannabis sativa* (Izzo *et al*., 2009). Many mechanisms have been suggested for its action, such as agonism of 5-HT_{1A} receptors (Russo *et al*., 2005). It also has a very strong anti-inflammatory activity both *in vivo*, as an anti-arthritic therapeutic (Malfait *et al*., 2000; Durst *et al*.,2007), and *in vitro*, manifested by inhibition of cytokine production in immune cells (Ben Shabat *et al*., 2006). The finding that CBD is devoid of any psychotrophic effects combined with its anti-inflammatory activity makes it a promising tool for treating HE, which is exacerbated by an inflammatory response (Shawcross *et al*., 2004). In the present work, we aimed to explore the effects of CBD in the acute model of HE induced by the hepatotoxin thioacetamide (TAA), focusing on brain function, brain pathology and 5-HT levels, liver function and pathology as possible targets for therapeutic effects of CBD.

Methods

Mice

Female Sabra mice (34-36g), 8 to 10 weeks old, were assigned at random to different groups of 10 mice per cage and were used in all experiments. All cages contained wood-chip bedding and were placed in a temperature-controlled room at 22°C, on a 12 h light/dark cycle (lights on at 07h00min). The mice had free access to water 24 h a day. The food provided was Purina chow and the animals were maintained in the animal facility (SPF unit) of the Hebrew University Hadassah Medical School, Jerusalem. Mice were killed after each treatment by decapitation between 10h00min and 12h00min. Animals were kept at the animal facility in accordance with NIH guidelines and all experiments were approved by the institutional animal use and care committee, No. MD -89.52-4.

Induction of hepatic failure

We adapted the rat model of acute liver failure induced by thioacetamide (TAA) to mice (Zimmermann *et al* ., 1989). The TAA model in mice has been extensively validated previously (Honda et al., 2002; Fernandez-Martinez A et al., 2004; Schnur et al., 2004). TAA was obtained from Sigma–Aldrich (Rehovot, Israel) in powder form and dissolved in sterile normal saline solution (NS); it was injected i.p. as a single dose of 200mg kg^{-1}. Vehicle (NS) was also administered in a separate group of animals that served as controls. Twenty-four hours after injection of TAA all animals (including control) were injected s.c. with 0.5 ml of a solution containing 0.45% NaCl, 5% dextrose and 0.2% KCl in order to prevent hypovolaemia, hypokalaemia and hypoglycaemia. The mice were intermittently exposed to infrared light in order to prevent hypothermia.

Administration of CBD

CBD was extracted from cannabis resin (hashish) and purified as previously reported (Gaoni *et al*.,1971) and was dissolved in a vehicle solution consisting of ethanol, emulphor and saline at a ratio of 1:1:18, respectively, and was injected in a single dose of 5mg kg^{-1} i.p, one day after either NS or TAA treatment. Similarly, the CBD related vehicle (the same mixture without CBD) was administered at the same time points following either NS or TAA treatment. A dose of 5mg kg^{-1} cannabidiol was chosen based on the studies done by Magen *et al*. (2009, 2010) and on preliminary experiments done in our laboratory, which demonstrated that this does produced a maximal effect compared to 1 and 10mg kg^{-1}. Four groups of animals were studied: control naïve animals treated with either CBD or its vehicle, and corresponding TAA-treated animals.

Assessment of neurological function

Neurological function was assessed by a 10 point scale based on reflexes and task performance (Chen *et al*., 1996): exit from a 1 metre in diameter circle in less than 1 min, seeking, walking a straight line, startle reflex, grasping reflex, righting reflex, placing reflex, corneal reflex, maintaining balance on a beam 3, 2 and 1 cm in width, climbing onto a square and a round pole. For each task failed or abnormal reflex reaction a score of 1 was assigned. Thus, a higher score indicates poorer neurological function. The neurological score was assessed one day after induction of hepatic failure by TAA (day 2). The mice were then divided between treatment groups so that all groups had similar baseline neurological scores after TAA induction. The post-treatment neurological score was assessed one day after administration of CBD or vehicle (day 3).

Assessment of activity

The activity test was performed two days after the induction of hepatic failure. Activity of two mice was measured simultaneously for a 5 min period. Two mice were tested together to lower stress to the minimum, as it has been shown that separation of mice induces stress (van Leeuwen *et al*.,1997*;* Hao *et al*.,2001). Activity was assessed in the open field (20x30cm field divided into 12 squares of equal size) as described previously (Fride and Mechoulam., 1993). Locomotor activity was recorded by counting the number of crossings by the mice at 1 min intervals.

Results are presented as the mean number of crossings min^{-1}.

Cognitive function

Cognitive function studies were performed eight days after the induction of hepatic failure. The animals were placed in an eight arm maze, which is a scaled-down version of that developed for rats (Pick and Yanai, 1983,). Mice were deprived of water 2 h prior to the test and a reward of 50µl of water was presented at the end of each arm, in order to motivate them to perform the task. Animals were divided between treatment groups so that all groups had similar baselines neurological scores after TAA induction. The mice were tested (no. of entries) until they made entries into all eight arms or until they completed 24 entries, whichever came first. Hence, the lower the score the better the cognitive function. Food and water were given at the completion of the test. Maze performance was calculated on 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) (Pick and Yanai, 1983,).

Brain histopathology and immunohistochemistry

Two days after the induction of hepatic failure, mice were killed by decapitation and brains were excised and fixed in 4% neutral- buffered paraformaldehyde.

The brain was cut along the midline and separated into 2 pieces containing brain and cerebellum hemispheres. Both sections were embedded en block in paraffin and 6μm saggital sections were adhered to slides. Serial sections were taken in 15 groups of slides (10 slides each, 3 sections per slide) at 100μm intervals. These slides were used for glial fibrillary acidic protein (GFAP) immunohistochemistry (a total of 90 sections), according to standard protocol. Briefly, paraffin sections were deparaffinized and hydrated in xylene and alcohol solutions, rinsed with TBS. Citrate buffer (pH.6) was used for antigen retrieval. The endogenous peroxidase was blocked with H_2O_2 (0,3% in PBS). Sections were then incubated in blocking buffer for 1h. A series of reselected sections were then treated with primary antibody against GFAP (1:2500, DakoCytomation, Denmark), overnight at 4°C, and then with goat anti-rabbit (1:200, Vector Burligame, CA, USA) as secondary antibody. Immunoreactions were visualized with the avidin – biotin complex (Vectastain) and the peroxidase reaction was visualized with diaminobenzidine (DAB) (Vector), as chromogen. Sections were finally counterstained with haematoxylin and examined under light microscope (Zeiss Axioplan 2). Images were captured with a digital camera (NIKON DS-5Mc-L1) mounted on microscope. Astrocytes were evaluated at the hippocampal area of both hemispheres. A total of 5-7 randomly selected visual fields, per hemisphere section, were evaluated. A square with 100 square subdivisions each of 3721 μm^2 as defined by an occular morphometric grid adjusted at the prefrontal lens, was centered at each visual field. The number of GFAP-positive astrocytes per mm^2 was evaluated. Only those cells with an identifiable nucleus, were counted. In addition, in an attempt to evaluate the level of activation of the astrocytes (cell size, extension of cell processes) the number of small square subdivisions with a positive GFAP signal and their % of the total number of square subdivisions counted, was calculated.

Two independent observers who were blinded to sample identity performed all quantitative assessments. In cases where significant discrepancies were obvious between the two observers, the evaluation was repeated by a third one.

Liver histopathology

Two days after the induction of hepatic failure, mice were killed by decapitation and their livers were excised and fixed in 4% neutral- buffered paraformaldehyde.

Liver histopathological analysis and scoring of necrosis (coagulative, centrilobular) were performed as described previously (Avraham et al,2008a).

Serum ammonia, liver enzymes and bilirubin levels

Serum for ALT, AST, bilirubin and ammonia measurements was obtained on day 3 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.

5-HT synthesis

On day 12, mice killed by decapitation and their brains were dissected out for determination of 5-HT levels. The assays for 5-HT were performed by standard alumina extraction, and HPLC with electrochemical detection using dehydroxybenzylamine (DHBA) as an internal standard (Avraham *et al,* 2006).

Experimental design

Experiment 1 On the first day of this experiment, 20 mice were administered with TAA (200mg kg^{-1}) and 20 saline. The following day, neurological evaluation was performed and saline-treated and TAA-treated mice were each assigned to two different sub-groups with approximately equal neurological score, which were administered either saline or CBD (5mg kg^{-1}). On the third day, mice were evaluated for neurological and locomotor function, after which they were killed and their brains and livers were dissected out and fixed with 4% formaldehyde. Blood was drawn and separated for plasma, in which liver enzymes were quantified.

Experiment 2 This was identical to experiment 1 on days 1-3, only the mice were not killed on day 3 but were evaluated for cognitive function using the eight arm maze test, on days 8-12. On day 12, the mice were killed and their livers and brains were dissected out for determination of 5-HT levels.

Statistical analysis

All data are expressed as mean ±SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test.

Results

Neurological score

TAA significantly increased the neurological score of mice compared to the control group (Figure 1; one way ANOVA: $F_{(3,29)}$=43.19, P<0.0001; Bonferroni: P<0.01). Administration of 5mg kg^{-1} CBD to TAA-treated mice improved the neurological score compared to TAA alone (1±0.15; P<0.01). CBD did not affect the score of the control animals.

Activity

TAA decreased the activity level of the mice (Figure 2; ANOVA: $F_{(3,29)}$=64.18, P<0.0001; Bonferroni: P<0.01) and CBD administration significantly increased the activity level in these TAA mice, compared to the untreated TAA mice (P<0.01). CBD did not affect the activity of control animals.

Cognitive function

Cognitive function was significantly impaired following TAA exposure, as reflected by the higher AUC values (Figure 3; ANOVA: $F_{(3,22)}$=7.22, P=0.001; Bonferroni: P<0.05) and this was improved following CBD administration (P<0.01 vs TAA only). CBD did not affect the cognitive function of control animals.

Brain and liver histopathology

Figure 4 shows images of slices from brains of animals from the control group (A), control + CBD group (B), TAA group (C) and TAA + CBD group (D), immunostained for the detection of astrogliosis. Astrogliosis was observed in visual fields studied in TAA animals, as evident both by the increase in the number of GFAP-positive cells mm^{-2} (Figure 4E; ANOVA: $F_{(3,298)}$=26.8, P<0.001; Bonferroni: P<0.001) and by the increased % of GFAP-positive surface (Figure 4F; ANOVA: $F_{(3,298)}$=19.21, P<0.001; Bonferroni: P<0.001). Both parameters were unaffected in CBD-treated controls (Figure 4E, F). However, the number of GFAP-positive cells mm^{-2} in TAA+5mg kg^{-1} CBD treated animals was reduced compared to TAA-treated animals (Figure 4E; Bonferroni: P=0.002). In contrast, CBD had no effect on the % of GFAP-positive surface in TAA animals (Figure 4F). Overall, it seems that TAA administration increased the number of activated astrocytes and CBD significantly reduced this effect. However, astrocytes in both CBD- and vehicle–treated TAA animals did not differ as regards their cellular size or extension of processes.

TAA-treated animals showed the typical TAA-induced liver necrosis lesions that have been described in detail previously (Avraham et al,2008a). The statistical analysis of liver histopathology scores did not reveal significant differences in the extent and severity of necrotic lesions between CBD-treated and untreated mice (data not shown).

5-HT levels

Whole brain 5-HT levels were increased by TAA administration (Figure 5; ANOVA: $F_{(3,17)}$=13.46, P<0.0001; Bonferroni: P<0.01), and CBD partially restored the levels in TAA-treated animals (P<0.01 vs TAA only). CBD did not affect the levels of 5-HT in control animals.

Liver function

The levels of ammonia (Figure 6A), bilirubin (Figure 6B) and the liver enzymes AST (Figure 6C) and ALT (Figure 6D) were increased after TAA administration (ammonia: ANOVA: $F_{(3,26)}$=156.93, P<0.0001; Bonferroni: P<0.01 vs control; bilirubin: ANOVA: $F_{(3,27)}$=34.99, P<0.0001; Bonferroni: P<0.01 vs control; AST: ANOVA: $F_{(3,27)}$=590.84, P<0.0001; Bonferroni: P<0.01 vs control; ALT: ANOVA: $F_{(3,27)}$=314.95, P<0.0001; Bonferroni: P<0.01 vs. control). CBD partially restored all of these indices in TAA-treated animals (P<0.01 vs TAA only for all parameters). CBD did not affect the levels of any of these substances in control animals.

Discussion

Thioacetamide administration induces acute liver failure which leads to CNS changes related to those seen in HE (Zimmerman *et al*, 1989, Avraham *et al*, 2006; 2008a; 2009; Magen *et al,*2008). The hepatotoxicity of TAA is due to the generation of free radicals and oxidative stress (Zimmerman et al, 1989). However, it is not clear whether TAA affects the brain directly or the liver (Albrecht et al ., 1996). In previous studies both a CB_1 antagonist and a CB_2 or TRPV1 agonist have been shown to ameliorate the brain and liver damage that occurs in liver disease and HE (Mallet and Lotersztajn,2008; Avraham *et al*,2006; 2008a,b; 2009). Also cannabidiol, an agonist of the $5\text{-}HT_{1A}$ receptor, was found to ameliorate brain damage in a chronic model of HE induced by bile duct ligation. Hence, we investigated the potential of cannabidiol as a treatment for HE induced by FHF. Our results indicated that it has a neuroprotective role in HE induced by FHF; cannabidiol was found to restore liver function, normalize 5-HT levels and improve the brain pathology in accordance with normalization of brain function. We also showed that cannabidiol affects both central functions : neurological score, motor and cognitive functions, brain 5-HT levels as well as astrogliosis and peripheral functions: reduced liver enzymes, ammonia and bilirubin. Therefore, we conclude that it acts both centrally and peripherally. In addition it has been shown that cannabidiol can cross the BBB and act centrally (for review see Pertwee ,2009). Therefore, its effect may result from a combination of its actions in the liver and the brain. However, to elucidate its mechanism of action future experiments are needed to determine the effects of central administration of cannabidiol .

Previous work from our laboratory has demonstrated an impaired neurological and motor function 3 days, and impaired cognition 12 days after TAA injection to mice (Avraham *et al*, 2006; 2008a; 2009). These results were reproduced in the present study (Figures 1-3). In a more recent study from our laboratory, cognitive and motor deficits were observed 21 days after bile-duct ligation (BDL), a chronic model of liver disease (Magen *et al*, 2009a, b). The different durations of the development of HE symptoms in the two models apparently result from their different characteristics – an acute vs a chronic model of HE. In the latter model, cannabidiol was found to improve cognition and locomotor activity, in accordance with our present data (Magen *et al*, 2009). However, in sharp contrast to the findings reported here no evidence for astrogliosis was found in that study (data not reported); in our acute model induced by TAA we observed astrogliosis after three days (Figure 4C). Those mice with histopathological alterations displayed an increased neurological score and decreased activity level, and 5mg kg^{-1} CBD reversed both the increase in the number of GFAP(+) cells, an index of neuroinflammation (Figure 4E), and the neurological and locomotor impairments (Figure 1 and 2), suggesting a link between neuroinflammation and motor and neurological deficits. Similar results were reported by Jover et al.(2006) who demonstrated a decrease in motor activity in bile-duct ligated rats on a high-protein diet, in association with astrogliosis, and by Cauli *et al*(2009), who reported that treatment with an anti-inflammatory restored the motor activity in HE. In the work of Jover *et al*. (2006), astrogliosis was found only in the bile-duct ligated rats on a high protein diet, but not in the bile-duct ligated rats on a regular diet, similar to our previous findings (Avraham *et al*,2009) This suggests that TAA causes more severe damage to the brain than bile-duct ligation, and that hyperammonaemia is required to worsen the damage in BDL rats to an extent that is equivalent to that observed in TAA mice. The reason for this may be that in chronic liver disease induced by BDL, compensation mechanisms are activated, which moderate the brain damage, while in the acute model induced by TAA, no such mechanisms can come into action because of the severity of the liver insult and the short interval of time between the induction of liver damage and the histopathological examination.

Kerfoot et al. (2006) showed the infiltration of peripheral monocytes into the brain of bile-duct ligated mice ten days after the ligation and suggested that this infiltration may cause the activation of inflammatory cells in the brain. Therefore, it is conceivable that such a mechanism was responsible for the astrogliosis observed in our study, since we found evidence of liver inflammation (data not shown). As evident from the histopathology results, cannabidiol did not appear to affect the development of TAA-induced necrotic lesions in the liver of mice. However, the levels of liver transaminases in the serum of cannabidiol-treated mice were significantly reduced compared to their untreated counterparts, indicating that this substance contributed to a partial restoration of liver function. Recent evidence elucidating the complicated mechanisms involved in the release of hepatocyte cytosolic enzymes such as ALT and AST in the blood may explain the discrepancy between histopathology and serum biochemistry data observed in the present study. Indeed, it is now generally accepted that the release of cytosolic enzymes during both the reversible and irreversible phases of hepatocyte injury and, therefore, their appearance in blood does not necessarily indicate cell death and also that enzyme release during reversible cell damage occurs with an apparent lack of histological evidence of necrosis (Solter, 2005). Following this reasoning, it could be hypothesized that although cannabidiol did not reduce the levels of histologically detectable necrosis, it may have ameliorated the minute reversible hepatocyte damage that causes the so-called "leakage" of cytoplasmic ALT and AST in blood. The interaction between hyperammonaemia and inflammation as a precipitating factor for HE has been discussed in two recent reviews (Shawcross and Jalan ,2005 ;Wright and Jalan ,2007). Further work is required to reveal the exact mechanism/s of the manner by which liver damage is related to dysfunction/damage in the brain, and studies using antagonists of the A_{2A} adenosine receptors, which are potential targets of cannabidiol that may mediate its anti-inflammatory effect (Carrier *et al*, 2006), need to be carried out in order to elucidate the receptors involved in this effect.

Astrogliosis has also been shown to be involved in learning and memory deficits in a mouse model of Alzheimer's disease. In this study, astrogliosis was reduced by caloric restriction, which also reversed the cognitive deficits and increased the expression of neurogenesis in related genes (Wu *et al*, 2008). Further studies, such as expression analysis of such genes using DNA microarray and evaluation of neurogenesis using BrdU staining, needs to be performed in order to explore the mechanisms through which TAA-induced astrogliosis impairs cognition, and through which cannabidiol acts to improve it.

Even though astrogliosis was found a week before cognitive function was observed, and it is not definite whether it was long-lasting, this mechanism seems, in our eyes, to account for the cognitive dysfunction, rather than the increase in 5-HT level (Figure 5). The latter mechanism does not seem to be related to the cognitive dysfunction, even though this increase in 5-HT was reversed by CBD (Figure 5), as 5-HT depletion, not increase, has been shown to cause memory deficits in the eight arm maze (Mazer *et al.*, 1997). On the other hand, there is much evidence that ammonia induces astrocyte swelling which via a number of mechanisms leads to impaired astrocyte/neuronal communication and synaptic plasticity, thereby resulting in a disturbance of oscillatory networks. The latter accounts for the symptoms of hepatic encephalopathy (for review see, Haussinger and Gorg, 2010), among them presumably the cognitive dysfunction.

An increased level of 5-HT in the brain of rats after TAA administration was reported by Yurdaydin *et al.*(1990). In addition, there is indirect evidence that this increase is related to decreased motor activity, as the nonselective 5-HT receptor antagonist methysergide increased motor activity in TAA-injected rats, while the selective 5-HT_2 receptor antagonist seganserin did not (Yurdaydin *et al*, 1996). Likewise, we found that the level of 5-HT was increased following TAA administration and this was restored after cannabidiol treatment (Figure 5). In parallel, motor activity was decreased following TAA injection and increased after cannabidiol treatment, indicating a link between the increase in 5-HT and decrease in motor activity. Hence, it seems that CBD reversed the increased 5-HT level in the brains of TAA mice and thus reversed the decrease in their motor activity. A possible mechanism can be activation of 5-HT_{1A} receptors by CBD (Russo *et al*, 2005), as these receptors have been reported to inhibit 5-HT synthesis (Invernizzi *et al*,1991). We have shown that the effects of cannabidiol in a chronic model of HE, bile duct ligation, are mediated via the 5-HT_{1A} receptors (Magen et al., 2010) and in an earlier study with the same model we demonstrated that the effects of cannabidiol can be also mediated via A_{2A} adenosine receptors (Magen et al., 2009). Thus, the effects of cannabidiol, can be mediated by 5-HT_{1A} or/and A_{2A} adenosine receptors. We think that in the current study the effects of cannabidiol were mediated by the 5-HT_{1A} receptor since activation of the receptor by cannabidiol caused depletion of 5-HT (Figure 5). In our previous studies we showed that cognition is multifactorial and not dependent only on 5-HT levels, therefore there is no direct correlation between cognition and 5-HT levels.

The reversal of astrogliosis was probably related to reduced hepatic toxin formation. Indeed, there is much evidence that ammonia induces astrocyte swelling, which via a number of mechanisms leads to impaired astrocyte/neuronal communication and synaptic plasticity, thereby resulting in a disturbance of oscillatory networks. The

latter accounts for the symptoms of hepatic encephalopathy (for review see, Haussinger and Gorg, 2010), among them presumably the cognitive dysfunction. Neurological and motor functions were improved two and three days, respectively, after induction of hepatic failure at the same time as a partial reversal of the astrogliosis and reduced levels of ammonia, bilirubin and liver enzymes were noticed. It seems that the behavioural effects of cannabidiol are dramatic and occur within 3 days.

In summary, the present study demonstrates the therapeutic effects of CBD in an acute model of HE. It appears that this effect of CBD is multi-factorial and involves cannabinoid (Avraham *et al*, 2006), vanilloid (Avraham *et al*, 2008a; 2009) and 5-HT_{1A} receptors (Magen *et al*,2009b). CBD improves the symptoms of FHF by affecting both brain histopathology and liver function, and thus may serve as therapeutic agent for treating human HE.

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Figure legends

Figure 1 Neurological function, evaluated two days after induction of hepatic failure, was impaired in TAA mice and was restored by CBD. ***P*<0.01 vs control, #*P*<0.01 vs TAA.

Figure 2 Locomotor function, evaluated three days after induction of hepatic failure, was decreased in TAA mice and was restored by CBD. ***P*<0.01 vs control, #*P*<0.01 vs TAA.

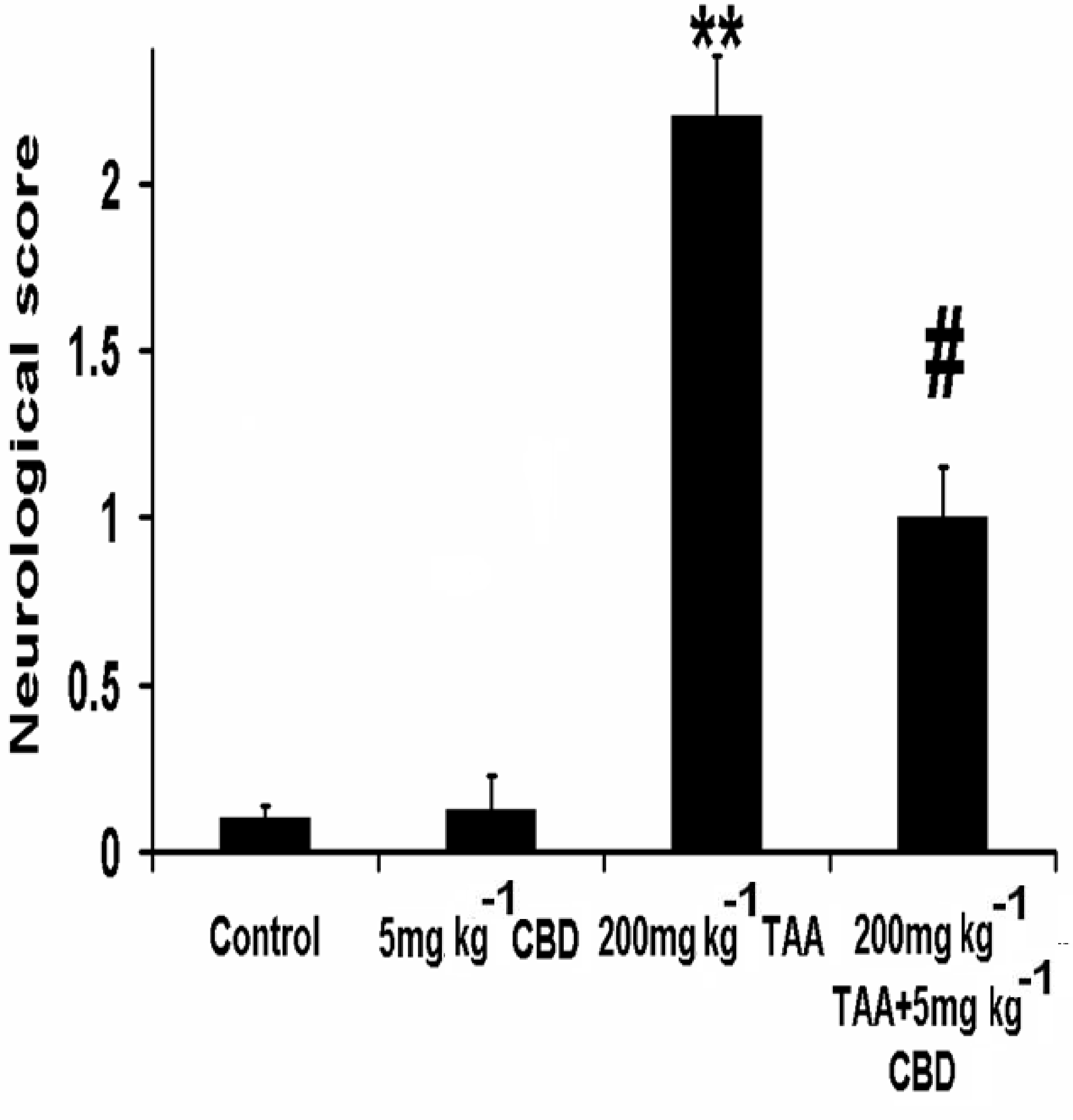
Figure 3 Cognitive function, tested eight days after induction of hepatic failure, was impaired following TAA and was improved by CBD. **P*<0.05 vs control, #*P*<0.01 vs TAA.

Figure 4 GFAP immunohistochemistry indicating the astrocytic reaction throughout the parahippocampal area in naïve controls (A, B) and TAA – treated animals (C,D) following treatment with vehicle (A,C) or CBD (B,D). CBD treatment had no effect on the astrocytic activation of naïve animals. However, in the case of animals with HE, CBD treatment induced significant reduction in the total number of activated astrocytes, although the level of individual cell activation was not impaired. (E) Quantification of GFAP-positive cells mm^{-2}; the number was reduced in TAA mice treated with 5mg kg^{-1} CBD compared to TAA mice treated with vehicle. ****P*<0.001 vs control, #*P*<0.01 vs TAA. (F) Quantification of GFAP-positive surface in μm^2; 5mg kg^{-1} CBD had no effect on the GFAP-positive surface in the brains of TAA-treated mice. ****P*<0.001 vs. control. Scale bars: 100µm.

Figure 5 Brain 5-HT levels, measured twelve days after induction of hepatic failure, were increased in the brains of TAA mice and were restored by CBD.

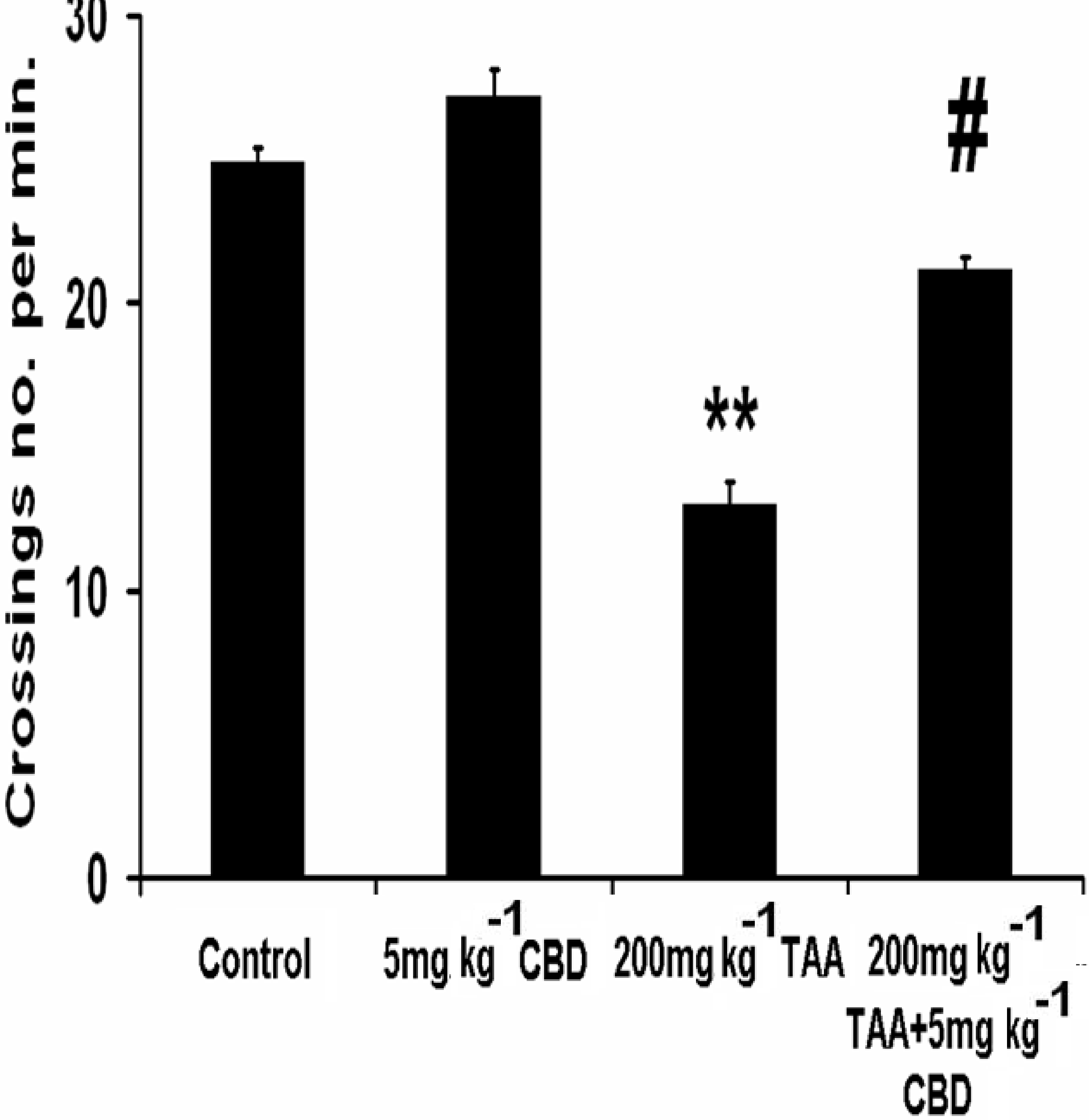
Figure 6 Indices of liver function. The levels of ammonia (A**),** bilirubin (B), AST (C) and ALT (D) were all increased in the plasma of TAA mice and were all reversed by CBD. ***P*<0.01 vs control, #*P*<0.01 vs TAA.

Figure 1



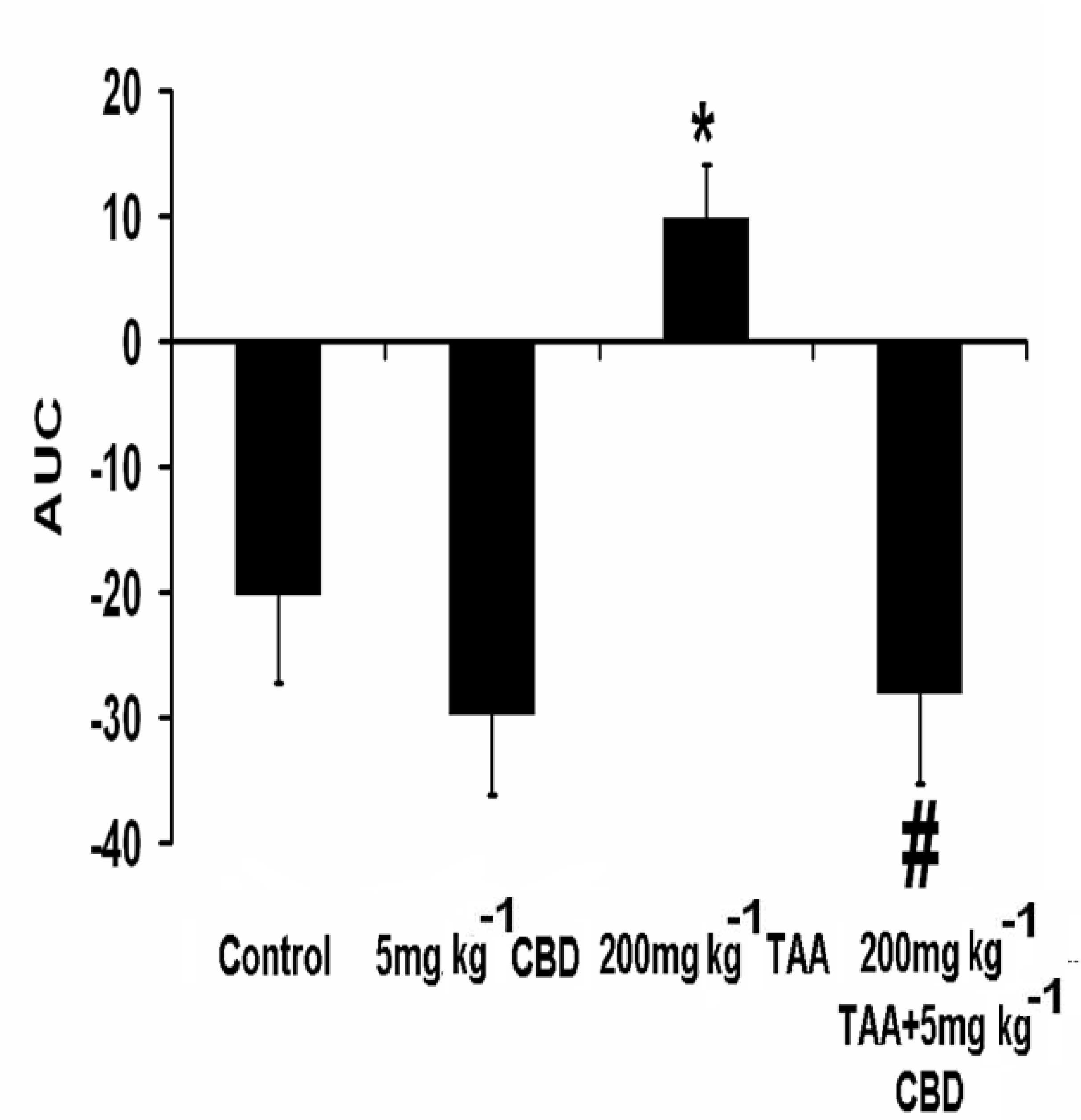
**
#
Neurological score
0
0.5
1
1.5
2
Control
5mg kg-1 CBD
200mg kg-1 TAA
200mg kg-1 TAA+5mg kg-1 CBD

Figure 2



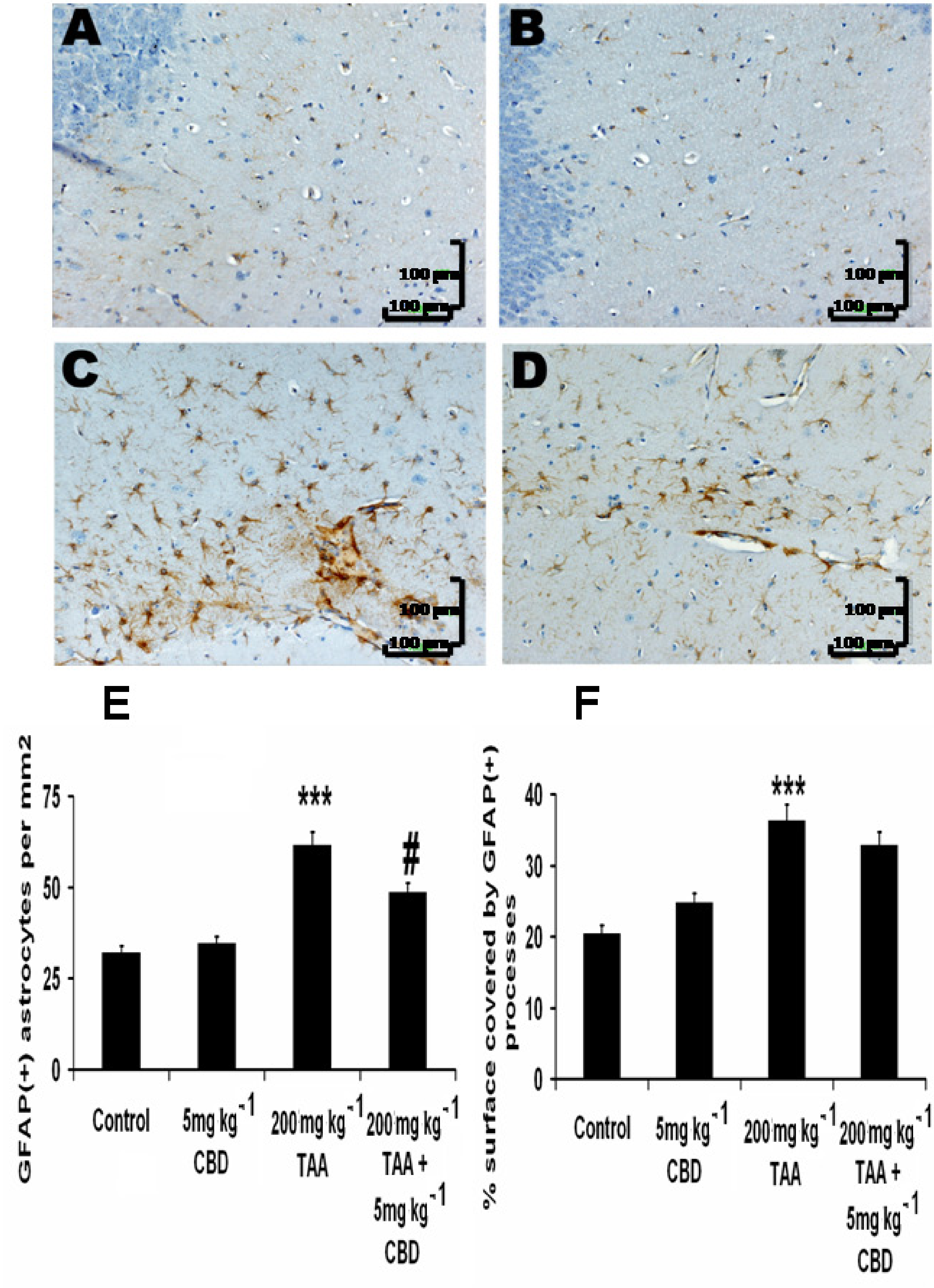
Crossings no. per min.
30
20
10
0
**
#
Control
5mg kg-1 CBD
200mg kg-1 TAA
200mg kg-1 TAA+5mg kg-1 CBD

Figure 3



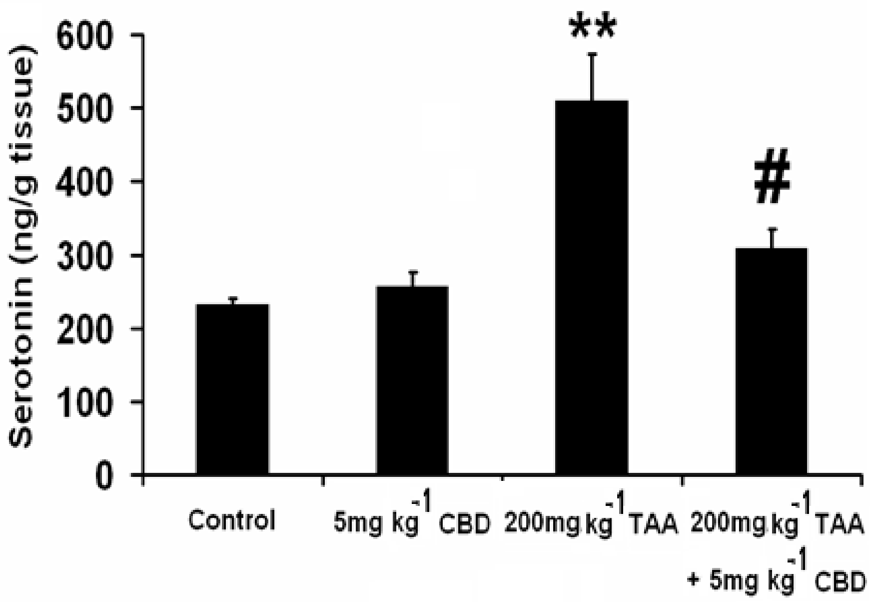
20
10
0
-10
-20
-30
-40
AUC
*
#
Control
5mg kg-1 CBD
200mg kg-1 TAA
200mg kg-1 TAA+5mg kg-1 CBD

Figure 4



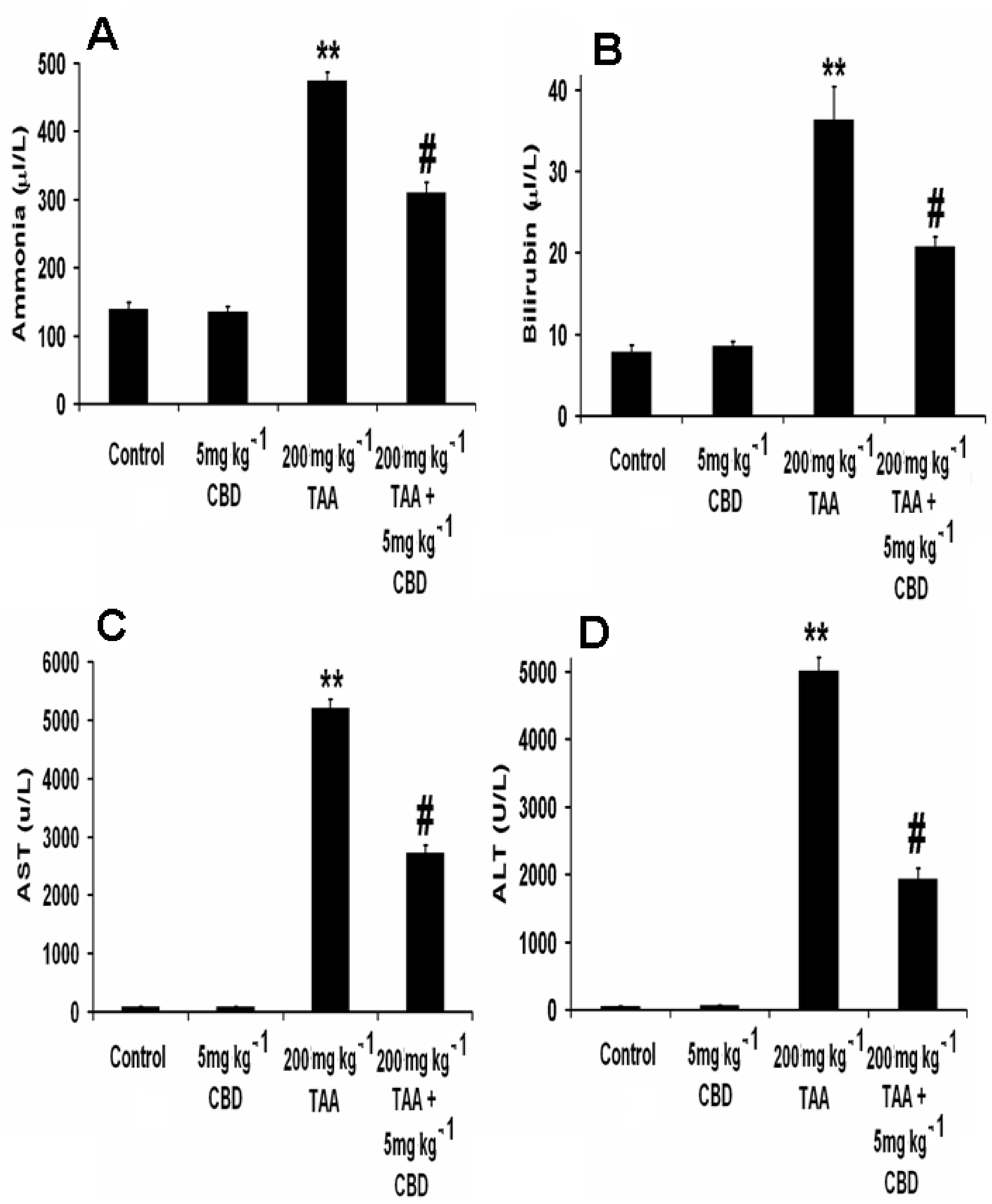
A
B
C
D
100 µm
E
GFAP(+) astrocytes per mm2
75
50
25
0
***
#
Control
5mg kg -1 CBD
200 mg kg -1 TAA
200 mg kg -1 TAA + 5mg kg -1 CBD
F
% surface covered by GFAP(+) processes
40
30
20
10
0
***
Control
5mg kg -1 CBD
200 mg kg -1 TAA
200 mg kg -1 TAA + 5mg kg -1 CBD

Figure 5



Serotonin (ng/g tissue)
600
500
400
300
200
100
0
**
#
Control
5mg kg-1 CBD
200mg kg-1 TAA
200mg kg-1 TAA + 5mg kg-1 CBD

Figure 6



A
**
#
Ammonia (µl/L)
500
400
300
200
100
0
Control
5mg kg -1 CBD
200 mg kg -1 TAA
200 mg kg -1 TAA + 5mg kg -1 CBD
B
**
#
Bilirubin (µl/L)
40
30
20
10
0
Control
5mg kg -1 CBD
200 mg kg -1 TAA
200 mg kg -1 TAA + 5mg kg -1 CBD
C
**
#
AST (u/L)
6000
5000
4000
3000
2000
1000
0
Control
5mg kg -1 CBD
200 mg kg -1 TAA
200 mg kg -1 TAA + 5mg kg -1 CBD
D
**
#
ALT (U/L)
5000
4000
3000
2000
1000
0
Control
5mg kg -1 CBD
200 mg kg -1 TAA
200 mg kg -1 TAA + 5mg kg -1 CBD