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BASIC STUDIES

Role of cannabinoid receptors in alcoholic hepatic injury: steatosis and fibrogenesis are increased in CB_2 receptor-deficient mice and decreased in CB_1 receptor knockouts

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Keywords
alcohol – cannabinoid receptors – fibrogenesis – inflammation – steatosis

Abbreviations
2-AG, 2-arachidonoyl glycerol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CB, cannabinoid receptor; CD, cluster of differentiation; ECM, extracellular matrix; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, hepatic stellate cells; IL-1β, interleukin 1β; MCP-1, monocyte chemotactic protein-1; PCNA, proliferating cell nuclear antigen; PDGFβ-R, platelet-derived growth factor β receptor; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEM, standard error of the mean; α-SMA, α-smooth muscle actin; SREBP-1c, sterol regulatory element binding protein 1c; TNF-α, tumour necrosis factor-α.

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Abstract

Background: Alcohol is a common cause of hepatic liver injury with steatosis and fibrosis. Cannabinoid receptors (CB) modulate steatosis, inflammation and fibrogenesis. To investigate the differences between CB_1 and CB_2 in the hepatic response to chronic alcohol intake, we examined CB knockout mice ($CB_1^{-/-}$, $CB_2^{-/-}$). *Methods:* Eight- to 10-week-old $CB_1^{-/-}$, $CB_2^{-/-}$ and wild-type mice received 16% ethanol for 35 weeks. Animals receiving water served as controls. We analysed triglyceride and hydroxyproline contents in liver homogenates. mRNA levels of CBs, pro-inflammatory cytokines [tumour necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, interleukin (IL)-1β] and profibrotic factors [α-smooth muscle actin (α-SMA), procollagen-Ia, platelet-derived growth factor β receptor (PDGFβ-R)] were analysed by reverse transcription-polymerase chain reaction (RT-PCR). Histology (hemalaun and eosin, oil-red O, CD3, CD45R, CD45, F4/80, Sirius red) characterized hepatic steatosis, inflammation and fibrosis. Activation of lipogenic pathways, activation and proliferation of hepatic stellate cell (HSC) were assessed by western blot [fatty acid synthase (FAS), sterol regulatory element binding protein 1c (SREBP-1c), α-SMA, proliferating cell nuclear antigen (PCNA), cathepsin D]. *Results:* Hepatic mRNA levels of the respective CBs were increased in wild-type animals and in $CB_1^{-/-}$ mice after ethanol intake. Ethanol intake in $CB_2^{-/-}$ mice induced much higher steatosis (SREBP-1c mediated) and inflammation (B-cell predominant infiltrates) compared with wild-type animals and $CB_1^{-/-}$ mice. HSC activation and collagen production were increased in all groups after forced ethanol intake, being most pronounced in $CB_2^{-/-}$ mice and least pronounced in $CB_1^{-/-}$ mice. *Discussion:* The fact that CB_2 receptor knockout mice exhibited the most pronounced liver damage after ethanol challenge indicates a protective role of CB_2 receptor expression in chronic ethanol intake. By contrast, in CB_1 knockouts, the effect of ethanol was attenuated, suggesting aggravation of fibrogenesis and SREBP-1c-mediated steatosis via CB_1 receptor expression after ethanol intake.

Alcohol intake causes steatosis, inflammation, fibrosis and ultimately full-blown cirrhosis. Hepatic stellate cells (HSC) are pivotal in this process (1–5). At activation, HSC transdifferentiate into proliferative and contractile myofibroblasts (5, 6). These activated HSC show increased expression of extracellular matrix proteins (e.g. collagen) and profibrotic mediators. Interestingly, cannabinoid receptors (CB_1, CB_2) are upregulated on HSC together with an increased production of their ligand 2-arachidonoyl glycerol (2-AG) in liver disease (7–11).

It is assumed that upregulation of CB_1 is involved in the development of hepatic fibrosis and steatosis (11–13). In a recent study, the expression of CB_1 receptor was increased in chronic hepatitis C and associated with steatosis in humans (14). By contrast, CB_2 receptor activation mainly protected from liver injury (7, 15). A critical role of CB_1 was shown for diet-induced and

short-term alcohol-induced hepatic steatosis (11, 12). CB_1 receptor stimulation increased lipid accumulation in a recent *in vitro* study (16). This was mediated via the sterol regulatory element binding protein 1c (SREBP-1c) resulting in an upregulation of fatty acid synthase (FAS). In bile-duct ligation and carbon tetrachloride models, CB_1 deficiency or blockade attenuated fibrosis, highlighting its pathogenetic role in fibrosis (13). However, the role of CB_2 with respect to inflammation and steatosis is discussed controversially (7, 15–17). Activated HSC represent both the target and the source of cannabinoids. Activated HSC produce 2-AG, which induces steatosis via paracrine stimulation of the CB_1 receptor on hepatocytes (11). While CB_2 upregulation found in liver damage potentiated inflammation and steatosis in the presence of obesity or in hepatocytes *in vitro* (16, 17), it also induced apoptosis of activated HSC (7), which might be protective.

Cannabis use aggravates liver damage in humans (14, 18, 19). This is of special interest because it is frequently associated with alcohol intake, steatosis and chronic hepatitis C infection (14, 18, 19). Despite many studies on cannabinoids and liver injury, the role of both receptors in the context of hepatic damage following chronic alcohol intake remains to be further clarified. Therefore, we evaluated the sequelae of long-term ethanol drinking in CB_1 and CB_2 knockout mice, with a special emphasis on steatosis, inflammation and fibrosis.

Materials and methods

Animals

For our experiments, we used 39 female mice with the C57BL/6J background: 15 wild-type, 11 $CB_1^{-/-}$ and 13 $CB_2^{-/-}$ mice. Female mice were chosen in order to facilitate forced ethanol intake as female mice show higher ethanol preference and consumption (20). Eight- to 10-week-old mice were moved to single cages and kept there for 35 weeks. They received water or a 16% ethanol solution (16%) as described below. All mice received normal chow *ad libitum*. This study was approved by the local committee for animal studies (LANUV 8.87–50.10.35.08.324).

Forced ethanol intake

After habituation in a single-cage environment for 1 week, the mice received ethanol as their only fluid source as described previously (21). Because alcohol-naïve mice do not tolerate high concentrations of ethanol very well, the mice were gradually adapted to increasing ethanol concentrations as follows: days 1–3: 4% ethanol, days 4–10: 8% ethanol, from day 11 on: 16% ethanol. Ethanol consumption (in g/kg), body weight, liver weight and daily food consumption of the animals were recorded at weekly intervals (Fig. 1A–C; Table 1).

Tissue collection

After 35 weeks of ethanol or water intake, the animals were sacrificed by cervical dislocation. The liver was removed and cut into fragments. Liver samples were either snap-frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ or fixed in formaldehyde (4%).

Biochemical analysis

Serum was obtained from the animals receiving ethanol. The levels of aminotransferases [alanine aminotransferase (ALT), aspartate aminotransferase (AST)] as well as ethanol were investigated using standard methods.

Quantitative reverse transcription-polymerase chain reaction

RNA isolation, reverse transcription with 0.5 μg total RNA and detection by reverse transcription-polymerase chain reaction (RT-PCR) were performed as described previously (22). The primers and probes for RT-PCR were obtained as a ready-to-use mix [CB_1 receptor, CB_2 receptor, tumour necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, interleukin (IL)-1β, α-smooth muscle actin (α-SMA), procollagen Ia, platelet-derived growth factor β receptor (PDGFβ-R), F4/80 from Applied Biosystems, Foster City, CA, USA]. 18S rRNA served as an endogenous control (primers and probes ready-to-use mix by Applied Biosystems). RT-PCR (ABI 7300 sequence detector) and PCR reaction (2xTaqMan-PCR-mastermix, Applied Biosystems) were performed as described previously (22). For each of the genes, a validation experiment was performed. The efficiencies of RT-PCR for the target gene and the endogenous control were approximately equal. $-\Delta C_t$ expresses the difference between the number of cycles (C_t) of the target genes and the endogenous control. For a better understanding, we normalized all $-\Delta C_t$ values to those of the wild type. The results of liver samples were expressed as $2^{-\Delta\Delta C_t}$, and express the x-fold increase of gene expression compared with the control group.

Hepatic triglyceride content

Hepatic triglyceride content was determined in analogue segments (50 mg) of snap-frozen livers using standard methods. The results are calculated to μg/g of wet liver tissue (Fig. 2C).

Histological staining

Hemalaun and eosin staining (HE staining) for histological evaluation of paraffin-embedded liver sections (3–4 μm) was performed using standard methods. For the quantification of inflammation, two independent blinded pathologists evaluated the staining using the standard histology activity index as described (23).

Immunohistochemistry evaluated the cells involved in inflammation. Immunochemical staining for CD3 (T lymphocytes), F4/80 (macrophages), CD45 (leucocytes), CD45R (B lymphocytes) and Ki67 was performed on 4-μm-thick paraffin-embedded sections by use of the

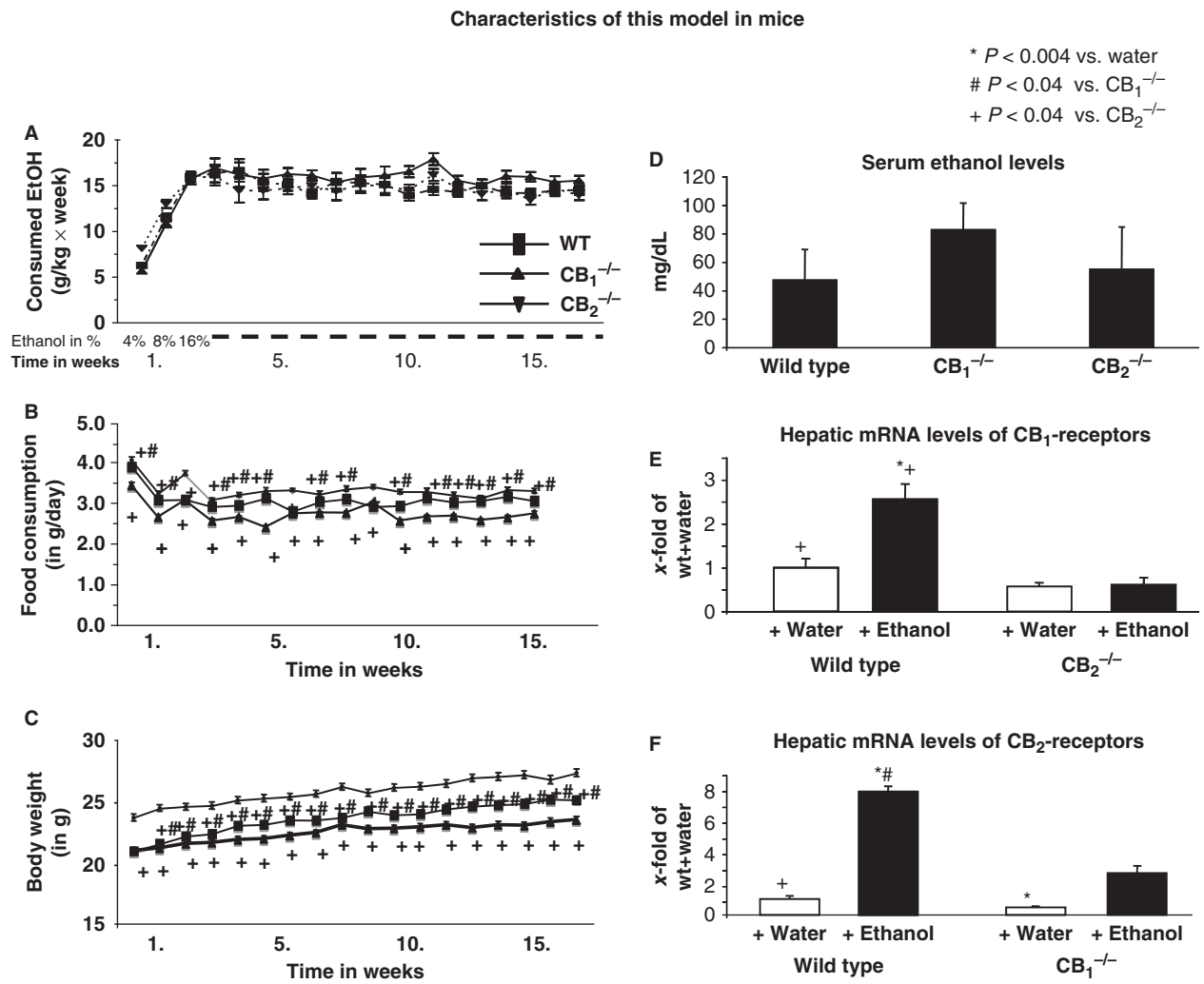

Fig. 1. Food consumption, ethanol intake, serum ethanol levels and hepatic CB receptor expression: weekly ethanol intake (A), food consumption (B) and body weight (C) are shown for a period of 17 weeks. These parameters remained stable for 35 weeks (data not shown). Ethanol concentration was 4% on days 1–3, 8% on days 4–10 and 16% from day 11 onwards. Mortality during ethanol drinking was < 5% and did not differ between groups. Serum ethanol levels did not differ significantly between groups (D). Hepatic mRNA levels of CB_1 (E) and CB_2 receptor (F) were determined by quantitative RT-PCR and corrected to 18S rRNA as the housekeeping gene and are expressed as x-fold increase compared with wild-type controls. While receptors were upregulated in wild-type mice by long-term ethanol intake, the respective receptor increased only in $CB_1^{-/-}$ mice. CB, cannabinoid receptor; RT-PCR, reverse transcription-polymerase chain reaction.

Table 1. Liver function tests, liver weight and liver/body weight ratio of the different strains after ethanol intake

	Wild type	$CB_1^{-/-}$	$CB_2^{-/-}$
ALT (U/l)	17.0 ± 1.4	19.4 ± 3.1	17.4 ± 1.1
Reference range 12–78 U/l			
AST (U/l)	58.0 ± 3.8^{+}·#	77.2 ± 11.1^{+}	46.6 ± 2.9
Reference range 15–37 U/l			
Liver weight (g)	1.06 ± 0.09	0.88 ± 0.07^{+}	1.3 ± 0.07
Liver/body weight ratio (%)	4.8 ± 0.4#	3.7 ± 0.2^{+}	5.3 ± 0.2

Data are means ± standard errors.

#$P < 0.05$ vs. $CB_1^{-/-}$.

$^{+}P < 0.05$ vs. $CB_2^{-/-}$.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CB, cannabinoid receptor.

peroxidase-conjugated avidin–biotin method. Primary antibodies included the CD3 monoclonal antibody (1:100, LabVision, Fremont, CA, USA), the F4/80 monoclonal antibody (1:100, BMA Biomedicals, Augst, Switzerland), the CD45 monoclonal antibody (1:300, BD Pharmingen, San Diego, CA, USA), the CD45R monoclonal antibody (1:50, BD Pharmingen) and the Ki67 antibody (Dako, Glostrup, Denmark) in a dilution of 1:25.

For the detection of fat accumulation in the liver, 10-μm-thick sections from snap-frozen liver samples were prepared using a cryostat and fixed in 10% formalin. After the washing procedure, the sections were stained with oil-red O (3%), counterstained with hemalaun and analysed by light microscopy.

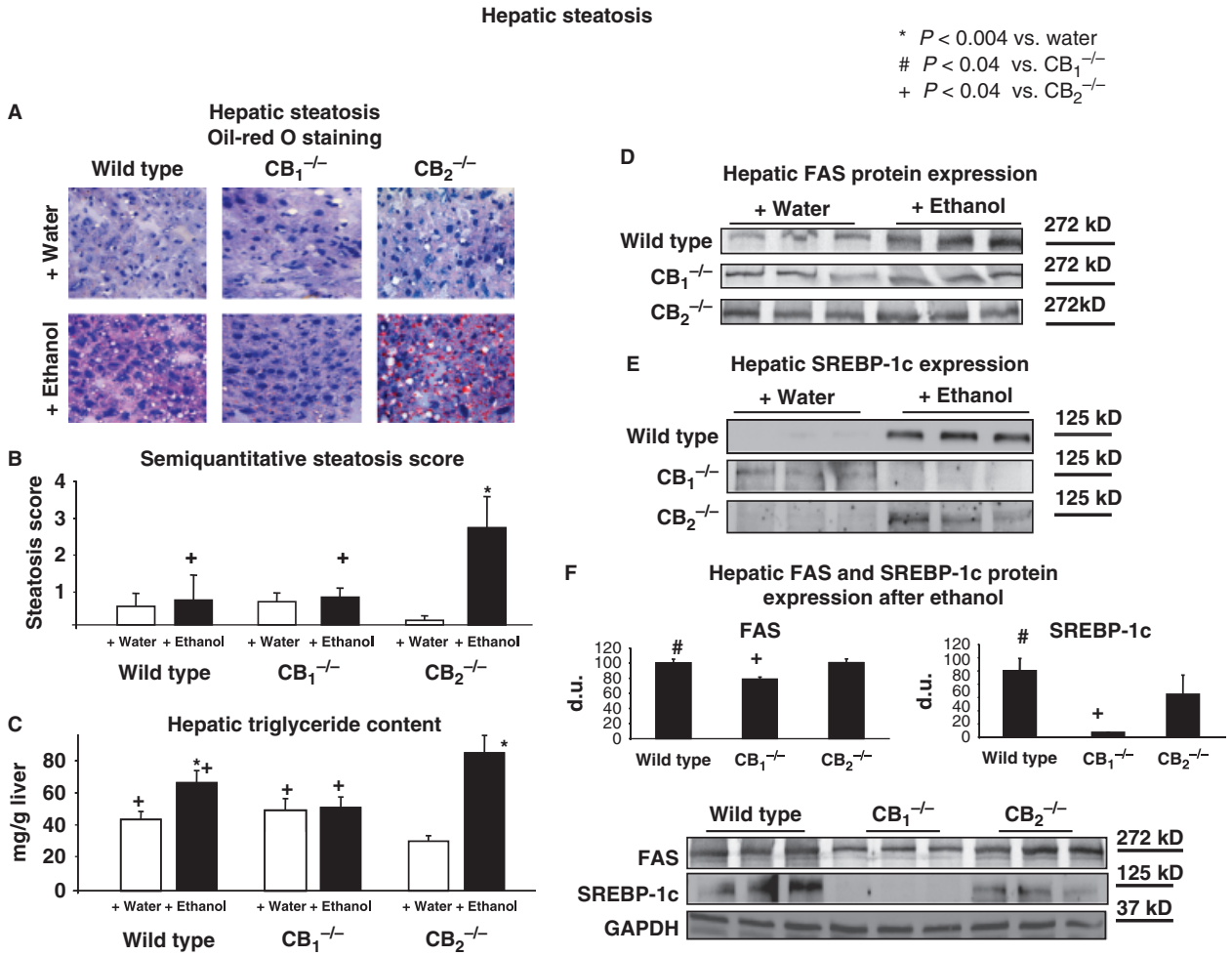

Fig. 2. Effect of CB receptor deficiency on hepatic steatosis after alcohol intake: Effect of forced ethanol intake (16%) on hepatic fat accumulation assessed histologically by oil-red O staining (A and B) and by determination of hepatic triglyceride content (C) in wild-type, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice compared with their respective controls. Representative sections of oil-red O staining are shown in A (original magnification × 400). Hepatic protein expression of FAS (D), as well as SREBP-1c (E) in wild-type, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice before and after forced ethanol intake is compared. In (F), hepatic protein expression of FAS and SREBP-1c between wild-type, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice after forced ethanol intake is compared. Representative western blots and densitometric quantifications are shown in means ± SEM relative densitometric units (d.u.) compared with wild-type mice after chronic ethanol intake set to 100 d.u. GAPDH western blots are shown as endogenous controls. In $CB_2^{-/-}$ mice, long-term ethanol intake resulted in more severe histological and biochemical steatosis via induction of the lipogenic pathway.
CB, cannabinoid receptor; FAS, fatty acid synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; SEM, standard error of the mean; SREBP-1c, sterol regulatory element binding protein 1c.

A scoring system was used to describe the proportion (1–4, 5–25, 26–50 and 51–100%) of positively stained red droplets of the total area. This was accomplished by randomly selecting 10 high-power fields on each slide at × 400 magnification. To enable statistical analysis, integer values were assigned to the proportion of area stained (0–4).

To detect collagen fibres, paraffin-embedded liver sections were stained in 0.1% Sirius red F3B in saturated picric acid (Chroma, Münster, Germany) as described previously (24). For the morphometric analysis of Sirius red-stained specimen, at least 10 mm^2 of liver tissue was analysed by means of computational analysis (Histoquant, 3DHistech, Budapest, Hungary). Large bile ducts and vessels were excluded. The principle of computational analysis has been described elsewhere (25).

Western blotting

Samples of snap-frozen livers were homogenized in a buffer containing 25 mM Tris/HCl, 5 mM ethylenediamine tetra-acetic acid, 10 µm phenylmethanesulphonyl fluoride, 1 mM benzamidine and 10 µg/ml leupeptin. Samples were diluted with sample buffer as described previously (22). Protein determination of the homogenates was performed with the DC Assay kit (Biorad, Munich, Germany). Samples (20 µg of protein/lane) were subjected to sodium dodecyl sulphate polyacrylamide gel

electrophoresis (10% gels), and proteins were blotted on nitrocellulose membranes. Ponceau-S staining was performed to ensure equal protein loading, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an endogenous control. The membranes were blocked, incubated with the primary antibody for α-SMA (Abcam plc, Cambridge, UK), FAS (C20G5, Cell Signaling, Boston, MA, USA), GAPDH, cathepsin D, PCNA and SREBP-1c (sc-25778, sc-6487, sc-56 and sc-13551, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Thereafter, the membranes were incubated with the corresponding secondary peroxidase-coupled antibody (Calbiochem, San Diego, CA, USA). Blots were developed with enhanced chemiluminescence (Amersham, Buckinghamshire, UK). The intensities of the digitally detected bands were evaluated densitometrically using Chemi-Smart (PeqLab, Biotechnologies, Erlangen, Germany).

Hepatic hydroxyproline content

Hepatic hydroxyproline was determined photometrically in liver hydrolysates as described previously (22, 26). Analogue segments (100 mg) of snap-frozen livers were hydrolysed in HCl (6 N) at 110 °C for 16 h and filtered. Aliquots of 50 µl from each sample were successively incubated with chloramine T (2.5 mM) for 5 min and Ehrlich's reagent (410 mM) for 30 min at 60 °C. Absorption was measured in duplicate at 558 nm. Hydroxyproline concentration was determined using a standard curve. Results are expressed as µg/g of wet liver tissue.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). ANOVA was used for comparison between groups. *P*-values < 0.05 were considered statistically significant.

Results

General characteristics, intake and serum levels of ethanol

The weekly ethanol intake was identical in all groups examined throughout the forced-intake procedure (Fig. 1A). Mortality was $< 5\%$ and did not differ between groups. We found a significant strain difference in body weight. $CB_2^{-/-}$ animals had significantly higher body weight and $CB_1^{-/-}$ mice had significantly lower body weight compared with wild-type mice (Fig. 1C). Similarly, $CB_1^{-/-}$ mice showed the lowest liver weight and liver/body weight ratio (Table 1). These differences are in accordance with their daily food consumption (Fig. 1B). Of note, within each genotype (wild-type, $CB_1^{-/-}$ and $CB_2^{-/-}$), ethanol intake did not influence body weight when compared with the corresponding water controls (data not shown). Ethanol serum levels did not differ significantly between the groups (Fig. 1D).

Hepatic mRNA levels of CB_1 and CB_2 receptors

The forced ethanol intake induced a marked increase of CB_1 and CB_2 receptor mRNA levels in the liver of wild-type animals (Fig. 1E and F). Ethanol intake upregulated the expression of the CB_2 receptor in the liver of $CB_1^{-/-}$ mice (Fig. 1F). Yet, the hepatic mRNA levels of the CB_1 receptor in $CB_2^{-/-}$ animals remained unchanged (Fig. 1E), probably – as recently shown – because of the lack of CB_2-receptor agonism (16).

Liver function tests

Serum ALT levels remained within the reference range (12–8 U/l) during ethanol intake in all groups (Table 1), while AST levels (reference range: 15–7 U/l) increased in all strains (Table 1). Interestingly, $CB_1^{-/-}$ mice showed the highest serum AST levels, followed by wild-type animals (Table 1).

Hepatic steatosis

Hepatic steatosis was investigated by triglyceride content and oil-red O staining. The highest hepatic triglyceride content and histological steatosis was found in $CB_2^{-/-}$ group after 35 weeks of ethanol intake (Fig. 2A–C). Similarly, chronic ethanol intake in wild-type mice led to an increase in the hepatic triglyceride content (Fig. 2C). In contrast, we found no increase in hepatic triglycerides following the forced-intake procedure in $CB_1^{-/-}$ mice (Fig. 2C).

Interestingly, in water controls, $CB_2^{-/-}$ mice showed a significantly lower hepatic triglyceride content than wild-type and $CB_1^{-/-}$ mice (Fig. 2C), although no significance was reached in the histological analysis (Fig. 2B).

Ethanol increased the hepatic expression of FAS and SREBP-1c in wild-type animals (Fig. 2D–F). $CB_2^{-/-}$ mice showed an excessive hepatic expression of SREBP-1c, but only a discrete increase in FAS expression after ethanol intake (Fig. 2D and E). These results confirm previous findings indicating that CB_1-dependent alcoholic steatosis is mediated mainly by SREBP-1c. The absence of CB_1 receptors protected the liver from the development of alcoholic steatosis (Fig. 2A–C), as well as from induction of lipogenic SREBP-1c/FAS pathway (Fig. 2D–F). However, wild-type animals showed a tendency towards higher hepatic SREBP-1c expression than $CB_2^{-/-}$ mice, even though a significance was not reached (Fig. 2F).

Hepatic inflammation

Histological evaluation of liver sections revealed that inflammation was significantly increased in $CB_2^{-/-}$ mice after long-term ethanol intake (Fig. 3A and B). The other groups showed no significant histological inflammation after ethanol intake for 35 weeks (Fig. 3A and B). Immunohistochemical staining (Fig. 3A) revealed mixed cell infiltrate including macrophages (F4/80-positive cells) and many leucocytes (CD45-positive cells). These leucocytes are mostly B lymphocytes as shown by

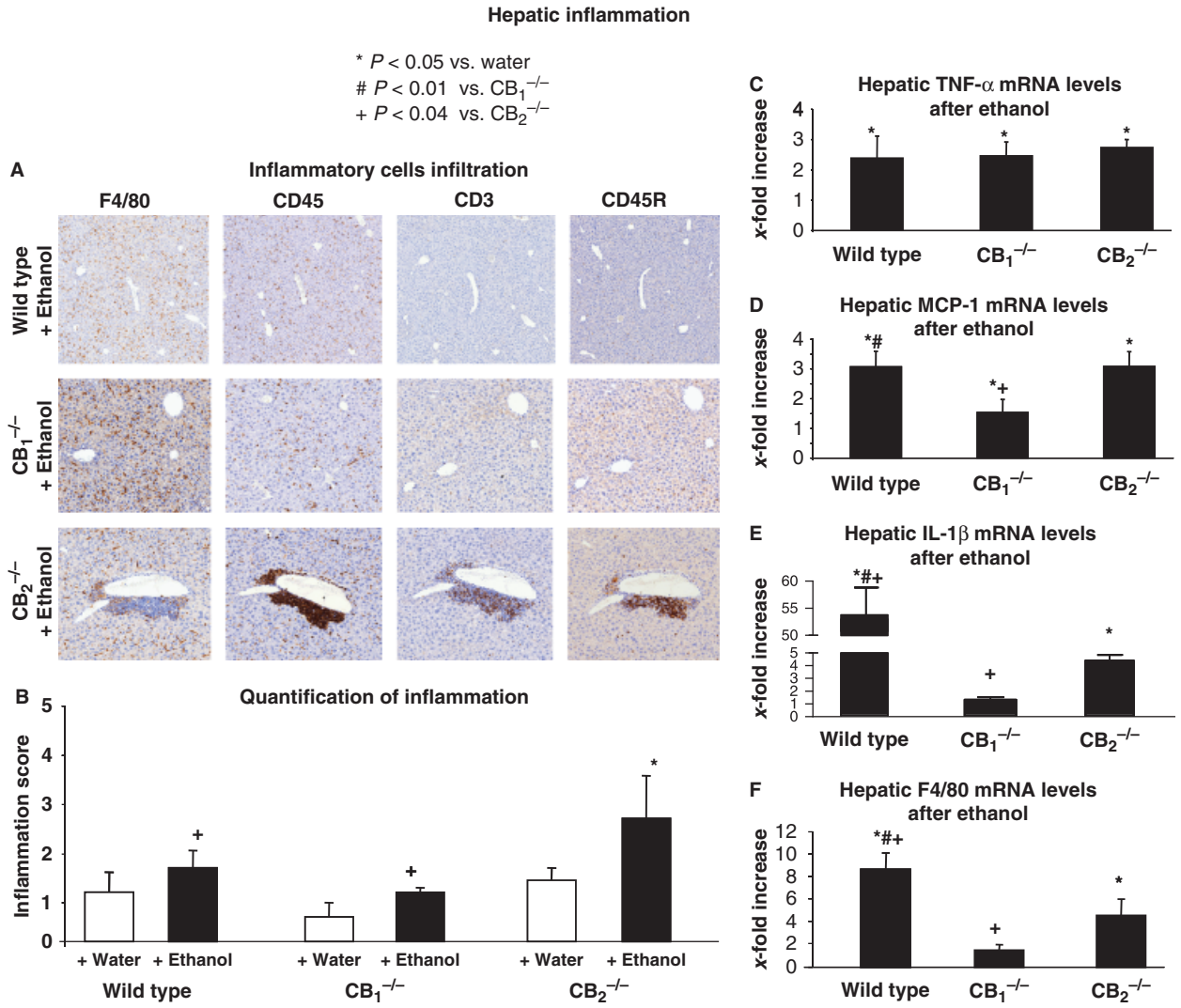

Fig. 3. Effect of CB receptor deficiency on hepatic inflammation after ethanol intake. Effect of forced ethanol intake (16%) on hepatic inflammation in wild-type, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice compared with their respective controls. The quantification of hemalaun and eosin and immunohistochemical staining (B) and representative sections of immunohistochemical liver staining with F4/80, CD45, CD3 and CD45R (A) (original magnification × 100) are shown. Hepatic mRNA levels of TNF-α (C), MCP-1 (D), IL-1β (E) and F4/80 (F) were determined by quantitative RT-PCR and corrected to 18S rRNA as the housekeeping gene and are expressed as x-fold increase compared with respective controls. Long-term ethanol intake induced mixed cell inflammation especially in $CB_2^{-/-}$ mice. CB, cannabinoid receptor; IL, interleukin; MCP, monocyte chemotactic protein; RT-PCR, reverse transcription-polymerase chain reaction; TNF, tumour necrosis factor.

CD45R-positive staining, and to a lower extent T lymphocytes (CD3-positive cells).

In all groups, ethanol intake equally increased the hepatic expression of the pro-inflammatory cytokine TNF-α (Fig. 3C). While the hepatic mRNA levels of MCP-1 increased as well, this was more pronounced in wild-type and $CB_2^{-/-}$ animals than that in $CB_1^{-/-}$ mice (Fig. 3D). A massive increase in hepatic mRNA levels of IL-1β occurred in wild-type mice after ethanol challenge (53 times higher), whereas these levels were only 4.4 times higher in $CB_2^{-/-}$ animals (Fig. 3E). This was paralleled by a more pronounced increase in F4/80 mRNA levels in wild-type animals (nine times higher) than in $CB_2^{-/-}$ animals (five times higher) after ethanol intake (Fig. 3F). Nevertheless, $CB_2^{-/-}$ showed the most severe inflammation (Fig. 3A and B). Interestingly, the absence of the CB_1 receptor prevented the hepatic upregulation of IL-1β and F4/80 (Fig. 3E and F).

Hepatic stellate cell activation and collagen production

Hepatic α-SMA expression is a marker for HSC activation. After forced ethanol intake, α-SMA mRNA and protein expression was significantly increased in all groups (Fig. 4A and C). Interestingly, $CB_2^{-/-}$ mice showed the highest α-SMA mRNA and protein expression after

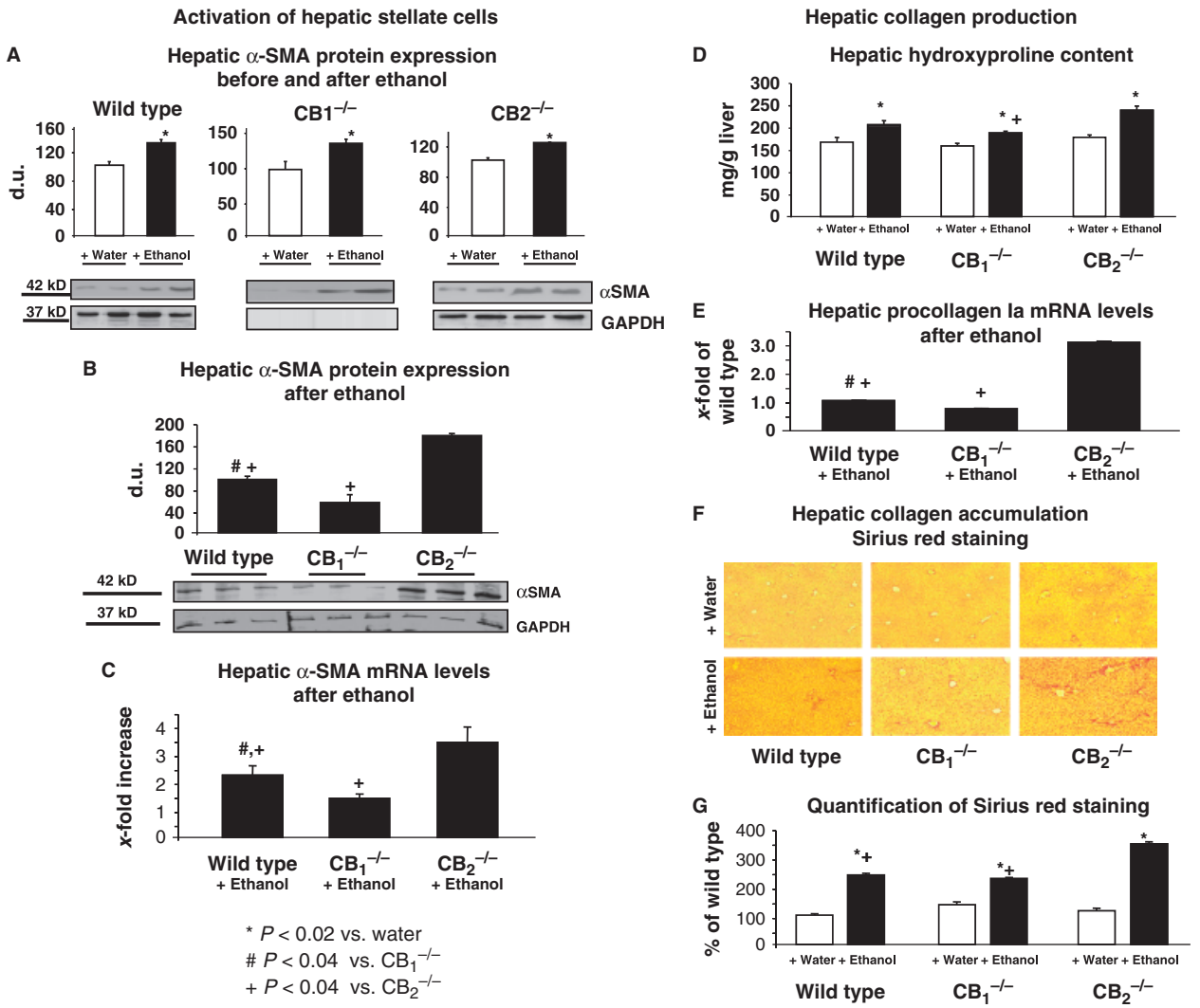

Fig. 4. Effect of CB receptor deficiency on hepatic stellate cell activation and fibrosis after ethanol intake. Hepatic α-SMA expression is a marker for the activation of hepatic stellate cells after liver injury. Hepatic protein expression of α-SMA in wild-type, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice before and after forced ethanol intake is compared (A). In (B), hepatic protein expression of α-SMA between wild-type, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice after forced ethanol intake is compared. Representative western blots and densitometric quantifications are shown in means ± SEM relative densitometric units (d.u.) compared with wild-type mice after chronic ethanol intake set to 100 d.u. Glyceraldehyde 3-phosphate dehydrogenase western blots are shown as endogenous controls. Hepatic mRNA levels of α-SMA (C) were determined by quantitative RT-PCR and corrected to 18S rRNA as the housekeeping gene and are expressed as x-fold increase compared with the respective controls. Effect of forced ethanol intake on hydroxyproline formation as a marker of extracellular matrix accumulation in the liver: Effect of forced ethanol intake (16%) on hepatic hydroxyproline content (D) in wild-type, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice are compared with their respective controls. Hepatic mRNA levels of procollagen Ia (E) were determined by quantitative RT-PCR and corrected to 18S rRNA as the housekeeping gene and are expressed as x-fold increase compared with the respective controls. (F) The representative liver sections of Sirius red staining and (G) morphometric quantification (original magnification × 100). Long-term ethanol intake increased the activation of hepatic stellate cells together with fibrosis predominantly in $CB_2^{-/-}$ mice. CB, cannabinoid receptor; RT-PCR, reverse transcription-polymerase chain reaction; SEM, standard error of the mean; α-SMA, α-smooth muscle actin.

ethanol challenge (Fig. 4B and C). In $CB_1^{-/-}$ mice, α-SMA mRNA and protein expression were significantly blunted compared with wild-type mice (Fig. 4B and C).

Hepatic collagen deposition was investigated by mRNA levels of procollagen Ia, hydroxyproline content of the liver as the main amino acid of collagen and by Sirius red staining. Similar to α-SMA expression, forced ethanol intake significantly increased the hepatic hydroxyproline content in all groups (Fig. 4D). $CB_2^{-/-}$ mice showed a significantly greater amount of hydroxyproline accumulation after ethanol intake than $CB_1^{-/-}$ mice (Fig. 4D). The hydroxyproline content of wild-type mice after ethanol intake was higher than that in $CB_1^{-/-}$ mice and lower than that in $CB_2^{-/-}$ mice, but this did not differ

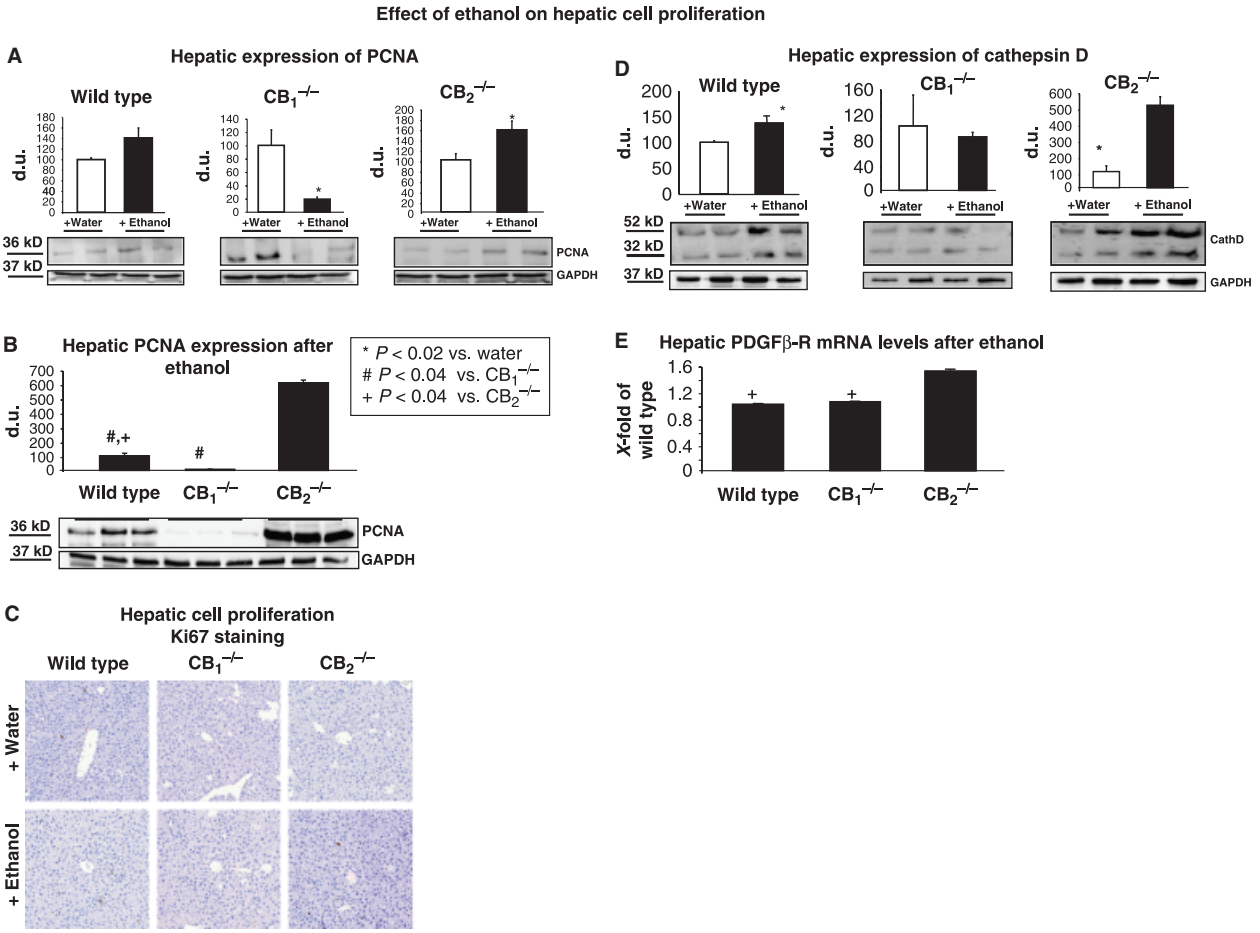

Fig. 5. Effect of CB receptor deficiency on hepatic cell proliferation after ethanol intake. Increased expression of PCNA is a marker for cell proliferation. Hepatic protein expression of PCNA is compared in wild-type, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice before and after forced ethanol intake (A). In (B), hepatic protein expression of PCNA is compared between wild-type, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice after forced ethanol intake. (C) The representative liver sections of Ki67 immunohistochemical staining as a cell proliferation marker (original magnification × 100). Increased expressions of cathepsin D and the PDGFβ receptor are markers for hepatic stellate cells proliferation after their activation. Hepatic protein expression of cathepsin D in wild-type, $CB_1^{-/-}$ and $CB_2^{-/-}$ mice with and without forced ethanol intake (D). Representative western blots and densitometric quantifications are shown. All results are means ± SEM relative densitometric units (d.u.) compared with the respective controls (100 d.u.). Glyceraldehyde 3-phosphate dehydrogenase western blots are shown as endogenous controls. Hepatic mRNA levels of the PDGFβ-receptor (E) were determined by quantitative RT-PCR and corrected to 18S rRNA as the housekeeping gene and are expressed as x-fold increase compared with the wild type. Long-term ethanol intake induced the proliferation of hepatic cells, and especially of hepatic stellate cells predominantly in $CB_2^{-/-}$ mice. CB, cannabinoid receptor; PDGF, platelet-derived growth factor; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcription-polymerase chain reaction; SEM, standard error of the mean.

significantly compared with either group (Fig. 4D). These results were confirmed by the increase of procollagen Ia mRNA levels in $CB_2^{-/-}$ mice when compared with $CB_1^{-/-}$ and wild-type animals after forced ethanol intake (Fig. 4E). Representative sections of Sirius red staining of $CB_2^{-/-}$ mice after forced ethanol intake showed a mild liver fibrosis, while $CB_1^{-/-}$ mice had almost no hepatic fibrosis. This was also the case in the morphometric quantification of the staining (Fig. 4F and G).

Hepatic cell proliferation

Hepatic cell proliferation was investigated using PCNA protein expression as a marker for cell replication (Fig. 5A and B). After forced ethanol intake, $CB_2^{-/-}$ mice showed a significant increase in the hepatic protein expression of PCNA, while wild-type animals showed only a tendency towards increased proliferation, without reaching significance (Fig. 5A). By contrast, ethanol intake reduced proliferation measured by PCNA protein expression in the livers of $CB_1^{-/-}$ mice (Fig. 5A). After forced ethanol intake, the absence of the CB_2 receptor induced the highest level of hepatic proliferation, while the lack of the CB_1 receptor decreased hepatic proliferation (Fig. 5B). The observed proliferation did not include parenchymal cells (below 1%) as shown clearly by Ki67 stainings of the liver sections (Fig. 5C).

Two different studies show that increased hepatic expression of cathepsin D reflects the proliferation of activated HSC (24, 27). $CB_2^{-/-}$ mice subjected to the forced ethanol intake procedure showed an increased (four- to five-fold) hepatic expression of cathepsin D (Fig. 5D). In contrast, wild-type mice developed only a slight increase in cathepsin D expression (Fig. 5D), and in $CB_1^{-/-}$ mice, cathepsin D expression remained unaffected (Fig. 5D). We investigated hepatic PDFGβ receptor mRNA levels in these mice as a marker for HSC activation and possibly proliferation (Fig. 5E). After forced ethanol intake, $CB_2^{-/-}$ mice showed the highest levels of hepatic PDFG receptor mRNA (Fig. 5E).

Discussion

This study demonstrates that chronic ethanol intake for 35 weeks upregulates hepatic CBs in wild-type mice, one possible mechanism for the induction of lipogenic, pro-inflammatory and profibrotic pathways in the livers of these animals. Further, our study dissects the role of CB_1 and CB_2 receptors in alcohol-induced hepatic injury. We could show that the absence of the CB_2 receptor aggravates hepatic injury with regard to steatosis, inflammation and fibrosis, whereas the deficiency of the CB_1 receptor protects these mice from liver damage after long-term ethanol intake.

Cannabinoid system in liver injury

Alcoholic and other liver injuries promote hepatic upregulation of CB_1 receptors (7, 11, 13, 14), as well as upregulation of its ligand, 2-AG (7, 11). Similarly, CB_2 receptors are upregulated in alcoholic (7) and non-alcoholic liver injury (17, 28). *In vitro*, CB_2 receptor agonists increased mRNA levels of the CB_1 receptor in hepatocytes (16). These data suggest an involvement of CBs in hepatic injury. Our study demonstrates that ethanol induces hepatic upregulation of CB_1 and CB_2 receptors in wild-type animals (Fig. 1E and F). Long-term ethanol intake in CB_1 receptor knockout mice also increased the hepatic expression of the CB_2 receptor. In contrast, in the absence of the CB_2 receptor, hepatic mRNA levels of the CB_1 receptor remained unchanged (Fig. 1E and F). This is in line with the finding that increased CB_1 receptor expression is linked to CB_2 receptor stimulation via an unknown mechanism (16).

Role of cannabinoid receptors in alcohol-induced hepatic steatosis

Using an *in vitro* model of fatty liver disease, a recent study showed that CB_1 receptor stimulation increased lipid accumulation in hepatocytes (16), confirming the critical role of the CB_1 receptor in hepatic steatosis (12, 29). Moreover, increased hepatic CB_1 receptor expression is found in chronic hepatitis C infection, and is associated with hepatic steatosis in humans (14). Alcohol-induced hepatic steatosis is also attributed to paracrine stimulation of the CB_1 receptor on hepatocytes by 2-AG derived from activated HSC (11). In our study, ethanol intake induced an upregulation of hepatic lipogenic pathways and hepatic triglyceride content in wild-type mice, together with an increase in CB_1 receptor levels. Vice versa, our study showed that ethanol intake for 35 weeks induced almost no hepatic steatosis in CB_1 receptor-deficient mice, as shown by unchanged hepatic triglyceride levels. These mice were protected because hepatic expression of lipogenic mediators as SREBP-1c/FAS remained constant (Fig. 2). In these animals, liver weight and liver/body weight ratio were the lowest because of the absence of steatosis (Table 1).

In contrast to the well-defined pathogenic role of the CB_1 receptor, the role of the CB_2 receptor in hepatic injury and steatosis is still controversially discussed (7, 15–17). In models of fibrosis and alcoholic cirrhosis, CB_2 receptors might be beneficial (7, 15–17). However, $CB_2^{-/-}$ mice showed less hepatic steatosis in a model of non-alcoholic fatty liver disease (17). Furthermore, CB_2 receptor stimulation increased lipid accumulation *in vitro* (16), and hepatic steatosis *in vivo* (17), using models of non-alcoholic fatty liver. Our model of long-term ethanol intake induced lipogenic pathways (SREBP-1c) in $CB_2^{-/-}$ mice, which, in histology, showed the most pronounced steatosis and the highest hepatic triglyceride levels compared with the other groups (Fig. 3). Because CB_2 receptor stimulation induces diet-dependent hepatic steatosis, the observed steatosis in our $CB_2^{-/-}$ mice was probably induced by ethanol and not by diet. This assumption is supported by our findings in $CB_2^{-/-}$ mice receiving water that had the highest food consumption and the lowest hepatic triglyceride levels (Fig. 3C) – confirming previous findings (16, 17). Thus, ethanol induced most severe steatosis in $CB_2^{-/-}$ mice, probably partly CB_1 receptor-mediated (the SREBP-1c/FAS pathway) and partly because of other mechanisms following the absence of the CB_2 receptor in these mice. The findings of Figure 2F, where wild-type animals showed a tendency towards higher hepatic SREBP-1c expression than $CB_2^{-/-}$ mice, support the last hypothesis, i.e. that SREBP-1c/FAS pathway-independent mechanisms are involved (30).

Role of cannabinoid receptors in hepatic inflammation induced by ethanol intake

Ethanol increased pro-inflammatory cytokine mRNA levels in wild-type mice without obvious histological inflammation. Thus, increased macrophage infiltration could not be shown immunohistochemically, despite high hepatic IL-1β and F4/80 levels (Fig. 3). These findings might reflect a macrophage activation – but without increase of the cell population – by ethanol and/or ethanol-induced IL-1β expression in hepatocytes as an alternative source (31, 32). Possibly, CB_2 receptor upregulation protects from inflammation in these animals. In $CB_1^{-/-}$ mice, partly because of upregulation of hepatic CB_2 receptor levels, ethanol induced less increase in

hepatic MCP-1 mRNA levels and no increase in hepatic IL-1β mRNA levels. In CB_2-deficient mice, the increase in hepatic fat accumulation (Fig. 2) was accompanied by an increased hepatic inflammation, despite the fact that the levels of pro-inflammatory cytokines were similar to those of wild-type animals (Fig. 3). These results underscore the anti-inflammatory effect of the CB_2 receptor (7, 33–35). The present study shows clearly that alcohol intake in the absence of CB_2 receptors leads to inflammation consisting in mixed cell infiltration with the predominance of B lymphocytes (Fig. 3A).

Further, we speculate that CB_1 was one mediator for liver damage in these animals. Our results suggest that in alcoholic liver disease, inflammation mediated via MCP-1 and IL-1β is partially CB_1 receptor-dependent, and that CB_2 receptor stimulation might counteract this effect. However, $CB_1^{-/-}$ mice showed higher levels of ALT, possibly because of a direct hepatotoxic effect of ethanol or acetaldehyde, despite the fact that these mice showed the lowest liver weight or liver/body weight ratio (Table 1) compared with wild type and $CB_2^{-/-}$, without subsequent steatosis, inflammation or fibrosis (Fig. 4A and B).

Role of cannabinoid receptors in stellate cell activation and hepatic fibrogenesis

Previous studies have already shown that CB_1 plays a crucial role in fibrogenesis in different models of hepatic fibrosis (bile-duct ligation, chronic thioacetamide, carbon tetrachloride intoxication) (14), while CB_2 receptor stimulation reduced fibrosis and liver injury (7, 15, 36). The current study investigated for the first time the effect of chronic ethanol intake on the activation of HSC and fibrogenesis. We could reveal a slight activation of HSC and accumulation of collagen in all groups after ethanol intake (Fig. 4). Of note, CB_1 knockout attenuated α-SMA expression, as a marker for HSC activation, after ethanol intake when compared with wild-type and $CB_2^{-/-}$ mice (Fig. 4A–C). Ethanol intake for 35 weeks resulted in more proliferation of hepatic cells (Fig. 5A and B), without an increase of parenchymal cell proliferation in the livers of CB_2-deficient mice, as shown by Ki67 stainings (Fig. 5C). The increased hepatic non-parenchymal cellular proliferation might, at least partially, mirror the proliferation of activated HSC, as suggested by the increased cathepsin D and PDGF receptor expression in CB_2-deficient mice (Fig. 5D and E). These findings explain the highest increase in hepatic collagen production in CB_2-deficient mice (Fig. 4D–F). By contrast, in $CB_1^{-/-}$ mice, alcohol induced less cellular proliferation and less HSC activation and proliferation, as well as less increase in collagen production (Fig. 4D–F).

Here, our data suggest that CB_1 receptor stimulation not only promotes hepatic steatosis (11) but also fibrogenesis in the model of chronic alcoholic liver injury. Our data thus reveal that CB_1 receptor knockout protects from ethanol-induced liver injury, while CB_2 receptor deficiency aggravates the ethanol effect.

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