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RESEARCH PAPER

# Differential hepatoprotective role of the cannabinoid $CB_1$ and $CB_2$ receptors in paracetamol-induced liver injury

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**Background and Purpose:** Protective mechanisms of the endogenous cannabinoid system against drug-induced liver injury (DILI) are actively being investigated regarding the differential regulatory role of the cannabinoid $CB_1$ and $CB_2$ receptors in liver fibrogenesis and inflammation.

**Experimental Approach:** The 2-arachidonoylglycerol (2-AG)-related signalling receptors and enzymatic machinery, and inflammatory/fibrogenic factors were investigated in the liver of a mouse model of hepatotoxicity induced by acute and repeated overdoses (750 $mg \cdot kg^{-1} \cdot day^{-1}$) of paracetamol (acetaminophen), previously treated with selective $CB_1$ (ACEA) and $CB_2$ (JWH015) agonists (10 $mg \cdot kg^{-1}$), or lacking $CB_1$ and $CB_2$ receptors.

**Key Results:** Acute paracetamol increased the expression of $CB_2$, ABHD6 and COX-2, while repeated paracetamol increased that of $CB_1$ and COX-2 and decreased that of DAGLβ. Both acute paracetamol and repeated paracetamol decreased the liver content of acylglycerols (2-AG, 2-LG and 2-OG). Human liver samples from a patient suffering APAP hepatotoxicity confirmed $CB_1$ and $CB_2$ increments. Acute paracetamol-exposed $CB_2$ KO mice had higher expression of the fibrogenic αSMA and the cytokine IL-6 and lower apoptotic cleaved caspase 3. $CB_1$ deficiency enhanced the repeated APAP-induced increases in αSMA and cleaved caspase 3 and blocked those of CYP2E1, TNF-α, the chemokine CCL2 and the circulating γ-glutamyltransferase (γGT). Although JWH015 reduced the expression of αSMA and TNF-α in acute paracetamol, ACEA increased the expression of cleaved caspase 3 and CCL2 in repeated paracetamol.

**Conclusion and Implications:** The differential role of $CB_1$ versus $CB_2$ receptors on inflammatory/fibrogenic factors related to paracetamol-induced hepatotoxicity should be considered for designing alternative therapies against DILI.

**Abbreviations:** 2-AG, 2-arachidonoylglycerol; 2-LG, 2-linoleoylglycerol; 2-OG, 2-oleoylglycerol; *Abhd6*, α/β-hydrolase domain-containing 6 or 2-arachidonoylglycerol hydrolase; AGs, acylglycerols; AILI, paracetamol-induced liver injury; ALT, alanine aminotransferase; APAP, paracetamol or acetaminophen; AST, aspartate aminotransferase; *Cnr1*, cannabinoid receptor 1 gene; *Cnr2*, cannabinoid receptor 2 gene; *Cox-2* (Ptgs2), PG-endoperoxide synthase 2; *Cyp2e1*, cytochrome P450 2E1 gene; *Dagla*, DAG lipase α gene; *Daglβ*, DAG lipase β gene; DILI, drug-induced liver injury; *Faah*, fatty acid amide hydrolase; MAGL/*Mgll*, monoacylglycerol lipase/ gene; *Nape-pld*, *N*-acylphosphatidylethanolamine PLD; *αSma/Acta2*, α-smooth muscle actin; γGT, γ-glutamyltransferase.

  

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## 1 | INTRODUCTION

Paracetamol (acetaminophen in the U.S.A.; APAP) is a common drug used for its analgesic and antipyretic actions. Only a minimal amount of the paracetamol that is consumed as a therapeutic doses (approximately 4 g·day$^{-1}$) and converted to **N-acetyl-4-benzoquinoneimine** (napqi) by the cytochrome P450 and is in fact detoxified by conjugation with glutathione (GSH). An acute overdose or repeated doses of paracetamol can lead to drug-induced liver injury (DILI) as a result of the liver accumulation of *N*-acetyl-4-benzoquinoneimine, a reactive metabolite that induces hepatotoxicity (Jollow et al., 1973). Practically, paracetamol is the only drug that causes dose-dependent liver injury (Andrade, Robles, Ulzurrun, & Lucena, 2009). Moreover, paracetamol-induced liver injury (AILI) is a common cause of acute liver failure characterized by elevated levels of serum aminotransferases, low levels of serum bilirubin and often renal insufficiency (Davern, 2012).

Despite being poorly understood, the pathogenesis of drug-induced liver injury is thought to involve the endogenous **cannabinoid system**. Cumulated evidences point to the endogenous cannabinoid system as a key player in the pathophysiology of hepatic diseases such as fatty liver, hepatitis, fibrosis and cirrhosis (Alswat, 2013; Caraceni, Domenicali, Giannone, & Bernardi, 2009). The endogenous cannabinoid system is a lipid signalling system involved in the modulation of central and peripheral pathological conditions involving immune and inflammatory processes, neuroprotective mechanisms, pain, energy balance and cell proliferation. The endogenous cannabinoids most studied are **arachidonoyl ethanolamide** (anandamide, AEA) and **2-arachidonoylglycerol** (2-AG). Anandamide is a non-selective, partial cannabinoid agonist that also binds to **TRPV1** and **GPR55** receptors (Di Marzo, De Petrocellis, Fezza, Ligresti, & Bisogno, 2002; Ryberg et al., 2007). 2-AG is also a non-selective cannabinoid agonist and a potent GPR55 agonist that was found in the liver at concentrations 1,000-fold higher than that of anandamide (Artmann et al., 2008; Ryberg et al., 2007). Hepatocytes and non-parenchymal cells in the liver are able to produce anandamide and 2-AG, which are in turn degraded locally by specific degrading enzymes fatty acid amide hydrolase (**FAAH**) and monoacylglycerol lipase (**MAGL**) respectively. Normal liver expresses high levels of FAAH and MAGL but low or even absent levels of **$CB_1$** and **$CB_2$** receptors. In contrast, liver injury up-regulates $CB_1$ and $CB_2$ receptors (Alswat, 2013; Caraceni et al., 2009; Jeong et al., 2008), leading to the hypothesis that the endogenous cannaboid system might serve as a therapeutic target for hepatic diseases.

### What is already known

- Cannabinoid receptors play a role in liver fibrogenesis and inflammation.

### What this study adds

- Acute and repeated oral overdose of paracetamol altered the liver 2-AG-related signalling system.
- $CB_1$ and $CB_2$ receptors exert differential hepatoprotective functions in paracetamol-induced hepatotoxicity.

### What is the clinical significance

- Pharmacotherapies involving the 2-AG/$CB_2$-related signalling system should be considered for treating paracetamol-induced liver injury.

Experimental evidence suggests the potential antifibrogenic properties of $CB_2$ stimulation, while $CB_1$ activation has been proposed as a profibrogenic mechanism (Teixeira-Clerc et al., 2006, 2010). Thus, $CB_1$ receptor activation promotes steatogenic and fibrogenic mechanisms likely related to enhanced paracrine effects of 2-AG produced by hepatic stellate cells (Jeong et al., 2008). On the contrary, $CB_2$ receptors might play a potential role on healing from liver steatosis and fat inflammatory responses associated with insulin resistance (Aguado et al., 2010; Mendez-Sanchez et al., 2007). Supporting this role, the pharmacological activation of $CB_2$ receptors (e.g. **JWH133**) accelerates liver regeneration and decreases fibrosis likely related to reduced hepatic collagen content (Muñoz-Luque et al., 2008; Teixeira-Clerc et al., 2010). In addition, both $CB_1$ and $CB_2$ receptors seem to have a potential impact on the inflammatory response associated with liver injury (Agudo et al., 2010; Gary-Bobo et al., 2007; Mendez-Sanchez et al., 2007). Clinical evidence of dysregulated endogenous cannaboid system (e.g. higher concentrations of 2-AG) was found in patients with hepatic steatosis and obese subjects prone to develop fatty acid liver disease and metabolic syndrome (Blüher et al., 2006; Côté et al., 2007; Westerbacka et al., 2010).

Previous studies showed that the pharmacological actions of paracetamol are partially mediated through interactions with the endogenous cannaboid system (Ayoub et al., 2011; Högestätt et al., 2005; Jóźwiak-Bebenista & Nowak, 2014; Mallat, Teixeira-

Clerc, Deveaux, & Lotersztajn, 2007; Ottani, Leone, Sandrini, Ferrari, & Bertolini, 2006). In the present study, we hypothesize that the endogenous cannaboid signalling through $CB_1$ and $CB_2$ receptors might play a differential modulatory role in the development of fibrosis and inflammation of liver injury associated with paracetamol overdose. To this aim, we described the evolution of the 2-AG-related signalling receptors and enzymatic machinery (mRNA expression of *Cnr1*, *Cnr2*, *Dagla*, *Daglb*, *Mgll*, *Ptgs2* and *Abhd6*) and 2-AG content in the liver of two mouse models of hepatotoxicity induced by the acute and repeated administration of paracetamol. After evaluating the impact of paracetamol-induced hepatotoxicity on the endogenous cannaboid system, we applied the most appropriate model of paracetamol-induced liver injury to analyse the apoptotic **caspase 3** and inflammatory (**TNF-α**, **IL-6** and **CCL2**) and fibrogenic (αSMA and **COL3A1**) factors in the liver of mice lacking either cannabinoid $CB_1$ or $CB_2$ receptors and mice previously treated with the selective $CB_1$ receptor agonist arachidonyl-2-chloroethylamide (**ACEA**) and the selective $CB_2$ receptor agonist **JWH015**.

## 2 | METHODS

### 2.1 | Ethics statement

The protocols for animal care and use were approved by the Ethics and Research Committee at the Regional University Hospital of Málaga and University of Málaga (Ref. No. 24-2015-A). All experimental animal procedures were carried out in strict accordance with the European Communities Directive 86/609/ECC (24 November 1986) and Spanish legislation (BOE 252/34367-91, 2005) regulating animal research. All efforts were made to minimize animal suffering and to reduce the number of animals used. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*.

Liver samples (biopsies) were obtained after a written inform consent from all the patients and donor's family, as requested by specific legal and clinical guidelines of University Hospitals of Málaga. Research procedures were approved and supervised by the Ethics and Research Committee at the University Hospitals of Málaga (Ref. No. PI-0337-2012) and were conducted according to the principles expressed in the Declaration of Helsinki.

### 2.2 | Animal models

Male Crl:CD1 (ICR, RRID:MGI:5652673) mice (Charles Rivers Laboratories, Barcelona, Spain) and male C57BL/6J wild-type (WT, RRID: MGI:5658887), $CB_1$ knockout ($CB_1$ KO, *Cnr1*$^{-/-}$) and $CB_2$ knockout ($CB_2$ KO, *Cnr2*$^{-/-}$) mice (Diez-Alarcia et al., 2016), weighing 25–30 g and ageing 3–4 months, were used in this study. They were housed in standard conditions (Animal House Service, University of Málaga) at 20 ± 2°C room temperature, 40 ± 5% relative humidity and a 12-h light/dark cycle with dawn/dusk effect. Water and standard rodent chow (Prolab RMH 2500, 2.9 kcal·g$^{-1}$) were available ad libitum. The animals were daily handled for 10 min and habituated to oral gavage procedure for 1 week before experimentation in order to minimize stress effects.

### 2.3 | Experimental design and treatment with paracetamol, ACEA and JWH015

Paracetamol (Cat. No. A7085, Sigma-Aldrich, St. Louis, MO, USA) was administered orally (gavage) at doses of 750 mg·kg$^{-1}$ (10 ml·kg$^{-1}$), as it was previously described (Rivera et al., 2017). The potent and highly selective $CB_1$ receptor agonist arachidonyl-2′-chloroethylamide (ACEA; Cat. No. 1319, Tocris) and the potent and selective $CB_2$ receptor agonist JWH015 (Cat. No. 1341, Tocris) were dissolved in a vehicle containing 33% (v/v) DMSO in sterile 0.9% NaCl solution, just before each experiment, and were injected intraperitoneally at doses of 10 mg·kg$^{-1}$ in a final volume of 1 ml·kg$^{-1}$ of body weight.

#### 2.3.1 | Experimental Design 1

To analyse the effect of acute paracetamol, we generated four experimental groups ($n$ = 8 per group): an administration of vehicle (vehicle group) and one administration of paracetamol and killed 6 h later (paracetamolx1 6h group), 24 h later (paracetamolx1 24h group) or 48 h later (paracetamolx1 48h group) (Figure 1a). $CB_2$ receptor KO and corresponding WT mice were exposed to an acute administration of vehicle or paracetamol and killed 24 h later ($n$ = 6 per group). An additional batch of WT mice were administered with either vehicle or JWH015, 1 h before the acute paracetamol administration and killed 24 h later ($n$ = 7 per group).

#### 2.3.2 | Experimental Design 2

To analyse the effect of repeated paracetamol, we generated three experimental groups ($n$ = 6 per group):- a repeated administration of vehicle for 4 days (vehicle group), a repeated administration of paracetamol for 3 days (paracetamolx3 group) and a repeated administration of paracetamol for 4 days (paracetamolx4 group) (Figure 1b). For analysing the putative recovery effect due to the cessation of the paracetamol administration, we generated two additional experimental groups ($n$ = 6 per group), a repeated administration of paracetamol for 4 days and killed 6 days later (paracetamolx4 6d group) and a repeated administration of paracetamol for 4 days and killed 15 days later (paracetamolx4 15d group). $CB_1$ receptor KO and corresponding WT mice were exposed to a repeated administration of vehicle or paracetamol for 3 days and killed 24 h later ($n$ = 6 per group). An additional group of WT mice were administered with either vehicle or ACEA, 1 h before each administration of repeated paracetamol, for three consecutive days and killed 24 h later ($n$ = 8 per group).

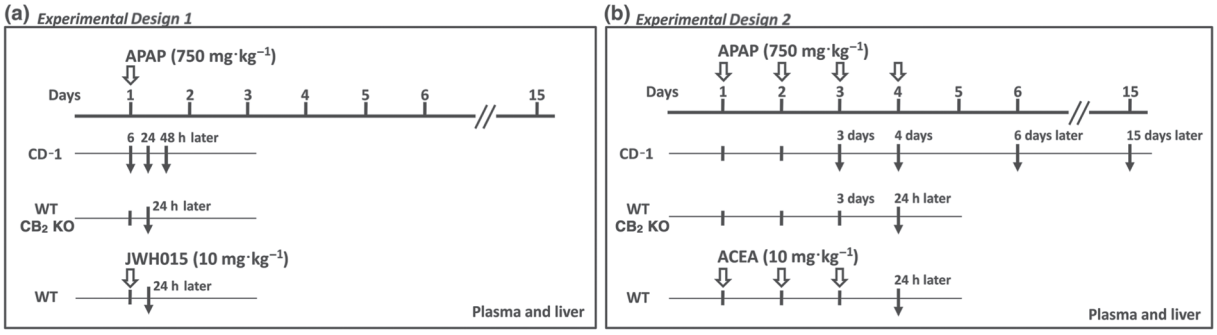


**FIGURE 1** Schematic timeline of (a) Experimental Design 1 and (b) Experimental Design 2, showing mouse models (CD-1 background and $CB_1$ receptor KO, $CB_2$ KO and respective WT with C57BL/6J background), doses of paracetamol (APAP), JWH015 and ACEA, and timing of administration and resting

Animals were fasted for 12 h before kill to avoid food-induced changes in liver genes (Rivera et al., 2017).

## 2.4 | Sample collection

Previous to kill, all animals were anaesthetized (sodium pentobarbital, 50 mg·kg$^{-1}$ of body weight, i.p.) in a room separate from the other experimental animals. Blood samples were collected transcardially into tubes containing K3EDTA and centrifuged (1,600 *g*) for 10 min at 4°C. The plasma was stored at −80°C for biochemical analysis. A portion of the liver was dissected, snap frozen in liquid nitrogen and kept at −80°C for LC/MS–MS and mRNA expression analyses. Animals were then fixed transcardially with 4% formaldehyde in 0.1-M phosphate buffer and other portions of the liver were collected for histological and immunohistochemical analyses.

## 2.5 | Biochemical analysis

The circulating hepatic enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyl transpeptidase (γGT) were analysed using commercial kits (Refs. DF43A, DF41A and DF45A, respectively, Flex® reagent cartridge, Dimension, Siemens Healthcare GmbH, Erlangen, Germany) according to the manufacturer's instructions in a Siemens Dimension Vista 500 Lab System (Siemens Healthcare GmbH). In all cases, a calibration curve and internal controls were included in each assay.

## 2.6 | Quantification of acylglycerols in the liver

Acylglycerol (AG) species are produced by the action of a family of diglycerol lipases (DAGLs), acting on common precursors and degraded by the same enzyme MAGL. Thus, alterations in the concentrations of AGs point to a robust effect on its signalling turnover. To analyse this, we measured the concentration of the acylglycerols 2-AG, 2-linoleoylglycerol (2-LG) and 2-oleoylglycerol (2-OG) in liver samples as described previously (Pastor, Farré, Fitó, Fernandez-Aranda, & de la Torre, 2014), with minimal modifications in the extraction processes from liver tissue. Determinations were made by using an LC/MS–MS system.

## 2.7 | RNA isolation and RT-qPCR analysis

We performed real-time qPCR (TaqMan, Life Technologies) in mouse liver as described previously (Rivera et al., 2017). Real-time qPCR was performed using a CFX96 Touch TM Real-Time PCR Detection System (Bio-Rad). Primers used were obtained based on TaqMan Gene Expression Assays and the FAM™ dye label format (Thermo Fisher) (see Table S1 and Figure S1 for additional information). Absolute values from each sample were normalized to the reference gene $\beta_2$-microglubulin. The relative quantification was calculated using the ΔΔCt method and normalized to the control group (*Y* axis represents 'fold mean of the control values').

## 2.8 | Western blot analysis

Protein levels from mouse liver were measured as previously described (Crespillo et al., 2011). The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology*. For protein detection, appropriate combination of primary antibodies: β-actin (1:1,000, mouse monoclonal antibody, Sigma, Cat. No. A5316, RRID:AB_476743), $CB_1$ (1:150, rabbit polyclonal antibody, Abcam, Cat. No. ab23703, RRID:AB_447623), $CB_2$ (1:200, rabbit monoclonal antibody, Abcam, Cat. No. ab3561, RRID:AB_303908), DAGLα (1:100, rabbit polyclonal antibody, developed by our group), DAGLβ (1:100, rabbit polyclonal antibody, developed by our group), MAGL (1:200, rabbit polyclonal antibody, Cayman, Cat. No. 100035, RRID:AB_10079049), αSMA (1:200, rabbit polyclonal antibody, Abcam, Cat. No. ab5694, RRID:AB_2223021), caspase 3 (1:500, rabbit polyclonal antibody, Cell Signaling Technology, Cat. No. 9662, RRID:AB_331439) and cleaved caspase 3 (1:300, rabbit polyclonal antibody, Cell Signaling Technology, Cat. No. 9661, RRID:AB_2341188) and HRP-conjugated anti-rabbit or anti-mouse IgG (H + L) secondary antibodies (1:10,000, Promega, Cat. No. W4011, RRID:AB_430833 and

W4021, RRID:AB_430834, respectively) were used. Please see Table S2 for additional information. Stripping/reprobing steps were used when necessary. After extensive washing in TBS-T, the membranes were incubated for 1 min with the Western Blotting Luminol Reagent Kit (Santa Cruz Biotechnology, Santa Cruz, CA) and the specific protein bands were visualized and quantified by chemiluminescence using an imaging AutoChemi UVP BioImaging System (LTF Labortechnik, Wasserburg/Bodensee, Germany). Western blots showed that each primary antibody detected a protein of the expected molecular size. The protein intensity was quantified with the image processing software ImageJ (Rasband, W.S., ImageJ, U.S., NIH, http://imagej.nih.gov/ij, 1997–2012). Normalization was performed using reference proteins of the same membrane. The results were expressed as the protein/β-actin ratio or phosphorylated/total protein ratio and normalized to the control group (*Y* axis represents 'fold mean of the control values').

## 2.9 | Human subjects

Human liver biopsies were retrospectively selected from 27 patients with drug-induced liver injury and from eight brain-dead, heart-beating, non-diabetic, non-obese, adult matched controls, all diagnosed by clinical, biochemical and histopathological criteria. Liver samples were retrieved from the tissue bank of Pathology Service at the University Regional Hospital and University Virgen de la Victoria Hospital of Málaga. The control group consisted of liver samples selected from healthy donors. We confirmed histopathologically the absence of macroscopic and microscopic alterations. Regarding the aetiology of the drug-induced liver injury patients analysed, we could only select a unique case of acute hepatic failure, due to an abusive consumption of paracetamol over time, without other relevant co-morbidities. At the moment of emergency admission, the patient (male, 40 years old, several suicide attempts) showed low BP (70/40), drowsiness, peripheral cyanosis, conjunctival icterus, right upper quadrant abdominal pain and altered circulating levels of AST (>4,600 $U{\cdot}L^{-1}$), creatinine (4.6 $mg{\cdot}dl^{-1}$), leukocytosis (39,000,000 $U{\cdot}L^{-1}$), among others. The paracetamol concentration in serum measured at admission of the patient to the emergency room (over 4 h after ingestion) was 220 $\mu g{\cdot}ml^{-1}$, indicating possible liver toxicity (Rumack–Matthew monogram). The clinicians proceeded with *N*-acetylcysteine treatment. The post-mortem analysis of the liver (2 days after emergency admission) indicated extensive necrosis, congestion, infiltration, haemorrhage and ischaemia, all signs compatible with a hepatotoxic aetiology.

## 2.10 | Histology and immunohistochemistry

Mouse and human liver samples were embedded in paraffin and then cut into 5-μm-thick sections using a paraffin microtome. Deparaffinized and rehydrated sections were stained with haematoxylin–eosin (H-E) for histological assessment of tissue injury. Adjacent sections were incubated in the following diluted primary antibody for 48 h at 4°C: rabbit anti-CB1 (1:25, Abcam, Cat. No. ab23703) and rabbit anti-$CB_2$ (1:25, Abcam, Cat. No. ab3561). See also Table S2 for additional information and Figure S1 for positive and negative controls. Then, sections were incubated in a 1:500 dilution of a biotinylated donkey anti-rabbit IgG secondary antibody (Amersham ECL, Cat No. RPN1004) for 1 h. Sections were then incubated in dark for 1 h in a 1:2,000 dilution of ExtrAvidin peroxidase (Sigma). Immunolabelling was revealed by exposing to 0.05% diaminobenzidine (DAB; Sigma), 0.05% nickel ammonium sulphate and 0.03% $H_2O_2$ in 0.1-M PBS (pH 7.4). Sections were dehydrated in ethanol, cleared in xylene and coverslipped with Eukitt mounting medium (Kindler GmbH and Co., Freiburg, Germany). For annexin V detection, we used the FITC Annexin V Apoptosis Detection Kit I according to the manufacturer's instructions (BD Biosciences, Madrid, Spain, Cat. No. 556547). Digital high-resolution microphotographs of the liver section were taken with 10× objective under the same conditions of light and brightness/contrast with an Olympus BX41 microscope equipped with an Olympus DP70 digital camera (Olympus Europa GmbH, Hamburg, Germany).

## 2.11 | Data analysis and statistics

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology. Experiments were designed to generate groups of equal size. All data are represented as mean ± SEM (per experimental group. Group size is the number of independent values and was selected based on an SD less than 20% of variance. Statistical analysis was undertaken for studies where each group size was of, at least, $n = 5$. No outliers were removed from the data. For blinding, operators and analysts were different persons. Levene's normality test was used to assess the equality of variances. Statistical analysis was performed using one- and two-way ANOVAs, being treatment (vehicle vs. paracetamol), genotype (WT vs. KO) and drug (vehicle vs. JWH015 or ACEA) as factors, followed by subsequent multiple comparisons between groups by using Tukey's adjustments or simple effect analysis in cases of no interaction. The analysis of two single groups was performed using Student's unpaired *t*-test. Correlation analyses were also performed through Pearson's correlation coefficient (*r*). *P* value <0.05 was considered significant.

## 2.12 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos, et al., 2019; Alexander, Fabbro, et al., 2019).

## 3 | RESULTS

### 3.1 | Acute paracetamol modified the mRNA expression of *Cnr2*, *Dagla*, *Abhd6* and *Ptgs2* and the concentrations of 2-AG, 2-LG and 2-OG in a time-dependent manner

CD-1 mice were treated with an acute oral overdose of paracetamol (750 mg·kg$^{-1}$) and killed 6, 24 and 48 h later (Figure 2a). Our results indicated the main effects on the mRNA expression was that there were significant increases in *Cnr2*, *Dagla*, *Abhd6* and *Ptgs2* after 24 and/or 48 h of paracetamol administration (Figure 2b–d). The acute administration of paracetamol did not change the mRNA expression of *Cnr1*, *Dagla* and *Mgll* (Figure 2b–d). Paracetamol also increased the protein expression of cleaved caspase 3 (Figure 2e). Further the main effects on the liver concentrations of 2-AG, 2-LG and 2-OG were found to be significantly decreased in mice killed at 6, 24 and 48 h after the paracetamol administration (Figure 2f).

In the liver of mice exposed to acute paracetamol, the mRNA expression of *Cnr2*, *Dagla* and *Daglb* displayed positive correlations with the fibrogenic *αSma*. *Cnr2* and *Dagla*, and also correlated positively with the apoptotic *Caspase 3* (Casp3;Table 1). No additional correlations were found.

### 3.2 | $CB_2$ receptor activation alleviates acute paracetamol-induced liver injury

Because $CB_2$ receptor deficiency aggravates acute paracetamol-induced hepatotoxicity, we evaluated the effect of the selective $CB_2$ receptor agonist JWH015 on inflammation and fibrogenesis 24 h after paracetamol administration (Figure 4a). Histopathologically, liver injury induced by acute paracetamol (sinusoidal dilatation, lymphocyte infiltration and ballooning parenchyma) was not found when mice were pretreated with JWH015 (Figure 4b). Effects of drug (vehicle vs. JWH015) on circulating aspartate aminotransferase indicate reductions associated with JWH015 (Figure 4c).

Significant interactions between treatment (vehicle and paracetamol) and drug (vehicle and JWH015) were found in the mRNA expression of *Faah*, *Cyp2e*, *αSma*, *Col3a1*, *Caspase3* and *Tnfα*. These results indicate that the effect of JWH015 on these inflammatory and fibrogenic factors depends on paracetamol-induced hepatotoxicity. JWH015 decreased the mRNA expression of *Faah*, *αSma*, *Caspase3* and *Tnfα* in acute paracetamol-exposed mice (JWH015–paracetamol mice) compared with the corresponding vehicle–paracetamol mice (Figure 4d–f). Lower mRNA expression of *Faah*, *αSma*, *Col3a1*, *Tnfα* and *Il6* was also observed in the liver of JWH015–paracetamol mice compared with JWH015–vehicle mice. Effects of treatment (vehicle vs. paracetamol) on mRNA expression of *Faah*, *Cyp2e1*, *Col3a1*, *Caspase3*, *Mcp1* and *Il6* were found. Effects of drug (vehicle vs. JWH015) on mRNA expression of *Faah* and *Mcp1* indicate reductions associated with JWH015. We found a drug effect on the protein expression of cleaved caspase 3, having a significant increase in the protein expression of cleaved caspase 3 specifically observed in the liver of paracetamol mice treated with JWH015 (Figure 4g).

### 3.3 | $CB_2$ receptor deficiency aggravates acute paracetamol-induced liver injury

Because acute paracetamol increased $CB_2$ receptor mRNA expression in the liver, we evaluated the effect of $CB_2$ receptor deficiency on inflammation and fibrogenesis 24 h after paracetamol administration (Figure 3a). We observed a differential effect of acute paracetamol on the liver histopathology in WT and $CB_2$ receptor KO mice (Figure 3b). The liver of vehicle groups (WT and $CB_2$ receptor KO) exhibited a normal lobular architecture with well-defined sinusoids and normal hepatic parenchyma. Acute paracetamol induced a moderate sinusoidal dilatation, lymphocyte infiltration and ballooning parenchyma that turned into necrotic areas in the liver of $CB_2$ receptor KO mice. (Figure 3b). Histopathology was accompanied by changes in the circulating levels of hepatic transaminases (Figure 3c). Significant interaction between genotype and treatment was found in circulating aspartate aminotransferase, with a significant increase in $CB_2$ receptor KO mice exposed to acute paracetamol compared with vehicle– $CB_2$ receptor KO mice and paracetamol–WT mice (Figure 3c). Significant effects of genotype and treatment on circulating aspartate aminotransferase were observed.

Significant effects of genotype on mRNA expression of *Faah*, *Cyp2e1* and *Caspase3* were found. Although no interaction between genotype and treatment was observed, single-effect analysis indicated that $CB_2$ receptor deficiency increased the mRNA expression of *Faah* and *Cyp2e1* compared with WT mice (Figure 3d). These increments in $CB_2$ receptor KO mice were not altered by acute paracetamol (Figure 3d). Acute paracetamol specifically increased the mRNA expression of the fibrogenic *αSma*, the apoptotic *Caspase3* and the inflammatory *Il6* in the liver of $CB_2$ receptor KO mice compared with vehicle–$CB_2$ receptor KO mice and paracetamol–WT mice (Figure 3e,f). No significant differences, but a tendency to increase, in the mRNA expression of *Col3a1*, *Mcp1* and *Tnfα* were found in $CB_2$ receptor KO mice exposed to acute paracetamol. Additionally, a significant effect of genotype on protein expression of αSMA and interaction between factors in protein expression of cleaved caspase 3 were also found. The increased protein expression of cleaved caspase 3 specifically observed in the liver of vehicle–$CB_2$ receptor KO mice, compared with vehicle–WT mice, was blocked in $CB_2$ receptor KO exposed to acute paracetamol (Figure 3g).

### 3.4 | $CB_2$ receptor activation alleviates acute paracetamol-induced liver injury

Because $CB_2$ receptor deficiency aggravates acute paracetamol-induced hepatotoxicity, we evaluated the effect of the selective $CB_2$ receptor agonist JWH015 on inflammation and fibrogenesis 24 h after paracetamol administration (Figure 4a). Histopathologically, liver injury

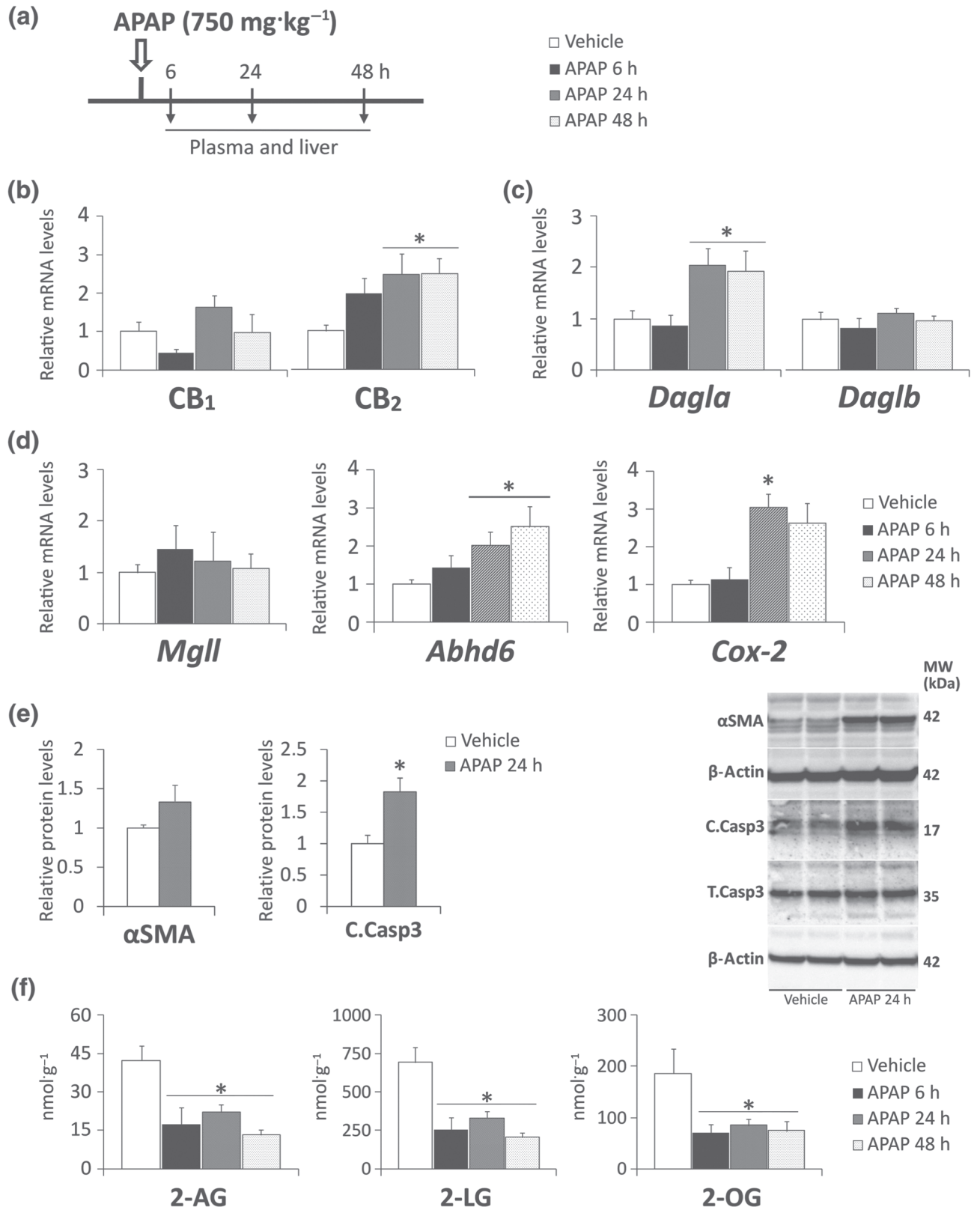


**FIGURE 2** Effects of the acute oral overdose of paracetamol (APAP) (750 mg·kg$^{-1}$) on the 2-AG-related signalling and enzymatic machinery in the mouse liver after 6, 24 and 48 h of administration. (a) Timeline of the Experimental Design 1. (b–d) Quantification of the mRNA expression of *Cnr1*, *Cnr2*, *Dagla*, *Daglb*, *Mgll*, *Abhd6* and *Ptgs-2*. (e) Quantification of the protein expression of αSMA and cleaved caspase 3. MWs are indicated. (f) Quantification of the hepatic concentration of the acylglycerols 2-AG, 2-LG and 2-OG. Histograms represent the mean ± SEM ($n$ = 8; $n$ = 6 in e). Tukey's test or unpaired Student's $t$-test: $^{*}P < 0.05$ versus vehicle mice. See Figures S4 and S5 for additional information

**TABLE 1** Correlation of components of the endogenous cannabinoid signalling system and acylglycerols with fibrogenic and inflammatory factors in the liver of mice exposed to an acute administration of acetaminophen (750 mg·kg$^{-1}$)

| | *αSma* | *Col3a1* | *Tnfα* | *Il6* | *Mcp1* | *Caspase3* |
|---|---|---|---|---|---|---|
| *Cnr1* | ns | ns | ns | ns | ns | ns |
| *Cnr2* | $r$ = 0.499<br>$P$ < 0.05 | ns | ns | ns | ns | $r$ = 0.847<br>$P$ < 0.001 |
| *Dagla* | $r$ = 0.590<br>$P$ < 0.05 | ns | ns | ns | ns | ns |
| *Daglb* | $r$ = 0.594<br>$P$ < 0.01 | ns | ns | ns | ns | $r$ = 0.827<br>$P$ < 0.001 |
| *Mgll* | ns | ns | ns | ns | ns | ns |
| *Abhd6* | ns | ns | ns | ns | ns | ns |
| *Ptsg2* | ns | ns | ns | ns | ns | ns |
| 2-AG | ns | ns | ns | ns | ns | ns |
| 2-OG | ns | ns | ns | ns | ns | ns |
| 2-LG | ns | ns | ns | ns | ns | ns |

induced by acute paracetamol (sinusoidal dilatation, lymphocyte infiltration and ballooning parenchyma) was not found when mice were pretreated with JWH015 (Figure 4b). Effects of drug (vehicle vs. JWH015) on circulating aspartate aminotransferase indicate reductions associated with JWH015 (Figure 4c).

Significant interactions between treatment (vehicle and paracetamol) and drug (vehicle and JWH015) were found in the mRNA expression of *Faah* *Cyp2e1*, *αSma*, *Col3a1*, *Caspase3* and *Tnfα*. These results indicate that the effect of JWH015 on these inflammatory and fibrogenic factors depends on paracetamol-induced hepatotoxicity. JWH015 decreased the mRNA expression of *Faah*, *αSma*, *Caspase3* and *Tnfα* in acute paracetamol-exposed mice (JWH015–paracetamol mice) compared with the corresponding vehicle–paracetamol mice (Figure 4d–f). Lower mRNA expression of *Faah*, *αSma*, *Col3a1*, *Tnfα* and *Il6* was also observed in the liver of JWH015–paracetamol mice compared with JWH015–vehicle mice. Significant effects of treatment (vehicle vs. paracetamol) on mRNA expression of *Faah*, , *Col3a1*, *Caspase3*, *Mcp1* and *Il6* were found. Effects of drug (vehicle vs. JWH015) on mRNA expression of *Faah* and *Mcp1* indicate reductions associated with JWH015. We found a drug effect on the protein expression of cleaved caspase 3, having a significant increase in the protein expression of cleaved caspase 3 specifically observed in the liver of paracetamol mice treated with JWH015 (Figure 4g).

### 3.5 | Repeated paracetamol modified the mRNA expression of *Cnr1*, *Daglb*, *Mgll* and *Ptgs2* and the concentrations of 2-AG, 2-LG and 2-OG in a resting time-dependent manner

Paracetamol was administered for 3 and 4 days (750 mg·kg$^{-1}$·day$^{-1}$) and mice were then killed 6 h, 6 days and 15 days later (resting period for recovery; Figure 5a). Our results indicated the main effects were on the mRNA expression of *Cnr1*, *Daglb*, *Mgll* and *Ptgs2*. A 4-day administration of paracetamol significantly increased the mRNA expression of *Cnr1* and *Ptgs2* and decreased the mRNA expression of *Daglb* and *Mgll* in the liver of mice killed 6 h and 6 days later compared with vehicle mice and 15-day resting mice (Figure 5b–f). Our results also indicated that there was a main effects on the protein expression *Dagla*, *Daglb* and *Mgll*, showing decreases in the liver of mice exposed to repeated paracetamol for 3 and/or 4 days compared with vehicle mice and 15-day resting mice (Figure 5d,e). Compared to vehicle mice, changes in mRNA expression were partially normalized after a resting period of 15 days (Figure 5c–f). No changes were observed in *Cnr2* expression in any of the repeated paracetamol groups (Figure 5c). A 4-day administration of paracetamol also increased the protein expression of αSMA (Figure 5g). The apoptotic annexin V is highly expressed in the hepatocytes of CD-1 mice repeatedly exposed to paracetamol (Figure S2).

The main effects on the hepatic concentrations of 2-AG, 2-LG and 2-OG were found to be decreases after the repeated administration of paracetamol for 3 and/or 4 days in mice killed 6 h and 6 days later compared with vehicle mice and 15-day resting mice (Figure 5h). These decreases were abolished after a resting period of 15 days (Figure 5h).

The mRNA expression of *Cnr1*, *Dagla* and *Cox-2* displayed positive correlations with the mRNA expression of *αSma*, *Col3a1* and *Tnfα* (Table 2). Positive correlations were also found between *Cnr2* and *αSma*, between *Dagla* and *Caspase3* and between *Ptgs2* and *Mcp1*. In contrast, negative correlations were found when *Mgll* and *Tnfα* were compared. Liver concentrations of 2-AG, 2-LG and 2-OG also displayed negative correlations with *Caspase3*. Only 2-LG had a negative correlation with *Col3a1* (Table 2).

### 3.6 | $CB_1$ receptor deficiency aggravates repeated paracetamol-induced liver injury

Because repeated paracetamol induced specific alterations in *Cnr1* mRNA expression in the liver, we evaluated the effect of *Cnr1* deficiency on inflammation and fibrogenesis after the repeated administration of paracetamol for three consecutive days (Figure 6a). Histopathology indicated that the repeated administration of paracetamol augmented liver injury characterized by a destruction of liver parenchyma architecture, extensive pericentral hepatic necrosis, haemorrhage and inflammation (lymphocyte infiltration) (Figure 6b). Increased necrosis, infiltration and haemorrhage were specifically observed in the hepatic parenchyma of the $CB_1$ receptor KO mice exposed to paracetamol for 3 days (Figure 6b).

Histopathology was accompanied by changes in circulating transaminases (Figure 6c). Significant interactions between genotype and treatment were found in the plasma levels of γ-glutamyl transpeptidase and alanine aminotransferase, showing an increase in circulating γ-glutamyl transpeptidase in WT mice exposed to repeated paracetamol compared with vehicle–WT mice and paracetamol–$CB_1$ KO mice (Figure 6c), as well as an increase in circulating alanine

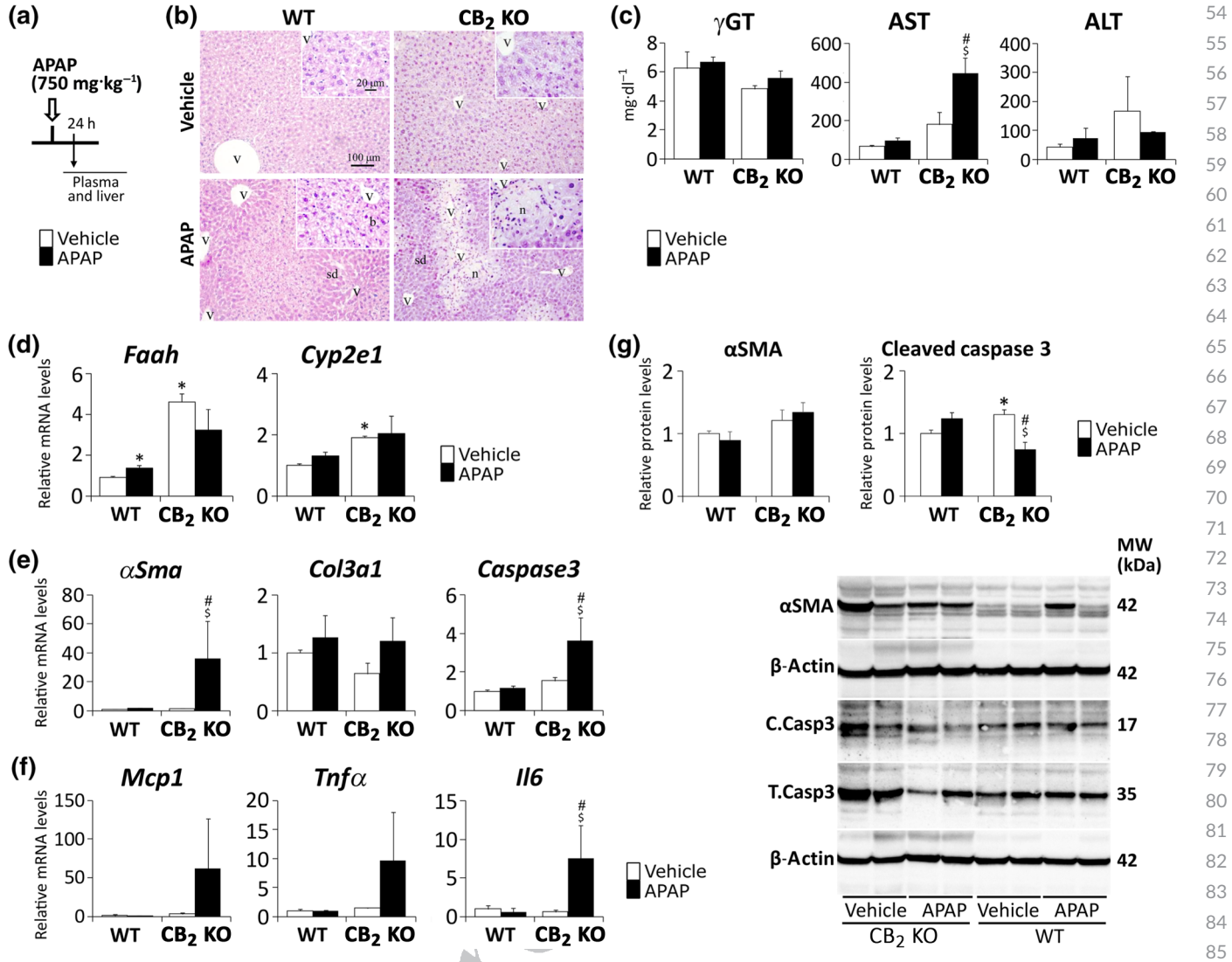


**FIGURE 3** Effects of the acute oral overdose of paracetamol (APAP; 750 mg·kg$^{-1}$) on histopathology; the expression of hepatic injury-related factors including fibrogenic, apoptotic and chemo-attractive/cytokine factors; and plasma levels of liver transaminases in WT and $CB_2$ receptor KO mice after 24 h of administration. (a) Timeline of the Experimental Design 1. (b) Representative photomicrographs showing high magnification views of the liver sections stained by H-E (groups: vehicle–WT, APAP–WT, vehicle– $CB_2$ receptor KO and APAP– $CB_2$ receptor KO). b, ballooning; n, necrosis; sd, sinusoidal dilatation; v, blood vessel. (c) Quantification of the circulating levels of γGT, AST and ALT. (d–f) Quantification of the mRNA expression of *Faah*, *Cyp2e1*, *αSma*, *Col3a1*, *Caspase3*, *Mcp1*, *Tnfα* and *Il6*. (g) Quantification of the protein expression of αSMA and cleaved caspase 3. MWs are indicated. Histograms represent the mean ± SEM ($n$ = 6). Tukey's test: $^{*}P < 0.05$ versus vehicle–WT mice; $^{\#}P < 0.05$ versus vehicle– $CB_2$ receptor KO mice; $^{\$}P < 0.05$ versus APAP–WT mice. See Figures S4 and S5 for additional information

aminotransferase in $CB_1$ receptor KO mice exposed to repeated paracetamol compared with vehicle–$CB_1$ receptor KO mice and paracetamol–WT mice (Figure 6c). Although no additional interaction between genotype and treatment was observed, single-effect analysis indicated increased circulating levels of aspartate aminotransferase in $CB_1$ receptor KO mice exposed to repeated paracetamol compared with vehicle– $CB_1$ receptor KO mice and paracetamol–WT mice (Figure 6c). We found effects of genotype on the plasma levels of γ-glutamyl transpeptidase, aspartate aminotransferase and alanine aminotransferase. Significant effects of paracetamol were detected on the plasma levels of γ-glutamyl transpeptidase, aspartate aminotransferase and alanine aminotransferase.

Significant interactions between genotype and treatment were found in the mRNA expression of *Faah*, *Cyp2e1* and *Il6*, showing decreases in WT mice exposed to repeated paracetamol compared with vehicle–WT mice, as well as increases in $CB_1$ receptor KO mice exposed to repeated paracetamol compared with vehicle–$CB_1$ receptor KO mice and paracetamol–WT mice (Figure 6d–f). Although no additional interaction between genotype and treatment was observed, single-effect analysis indicated increased mRNA expression of *Col3a1*, *Mcp1* and *Tnfα* in the liver of WT mice exposed to paracetamol for 3 days compared with vehicle–WT mice (Figure 6e,f). $CB_1$ receptor KO mice exposed to repeated paracetamol had significant increased mRNA expression of *αSma*, *Col3a1* and *Caspase3*

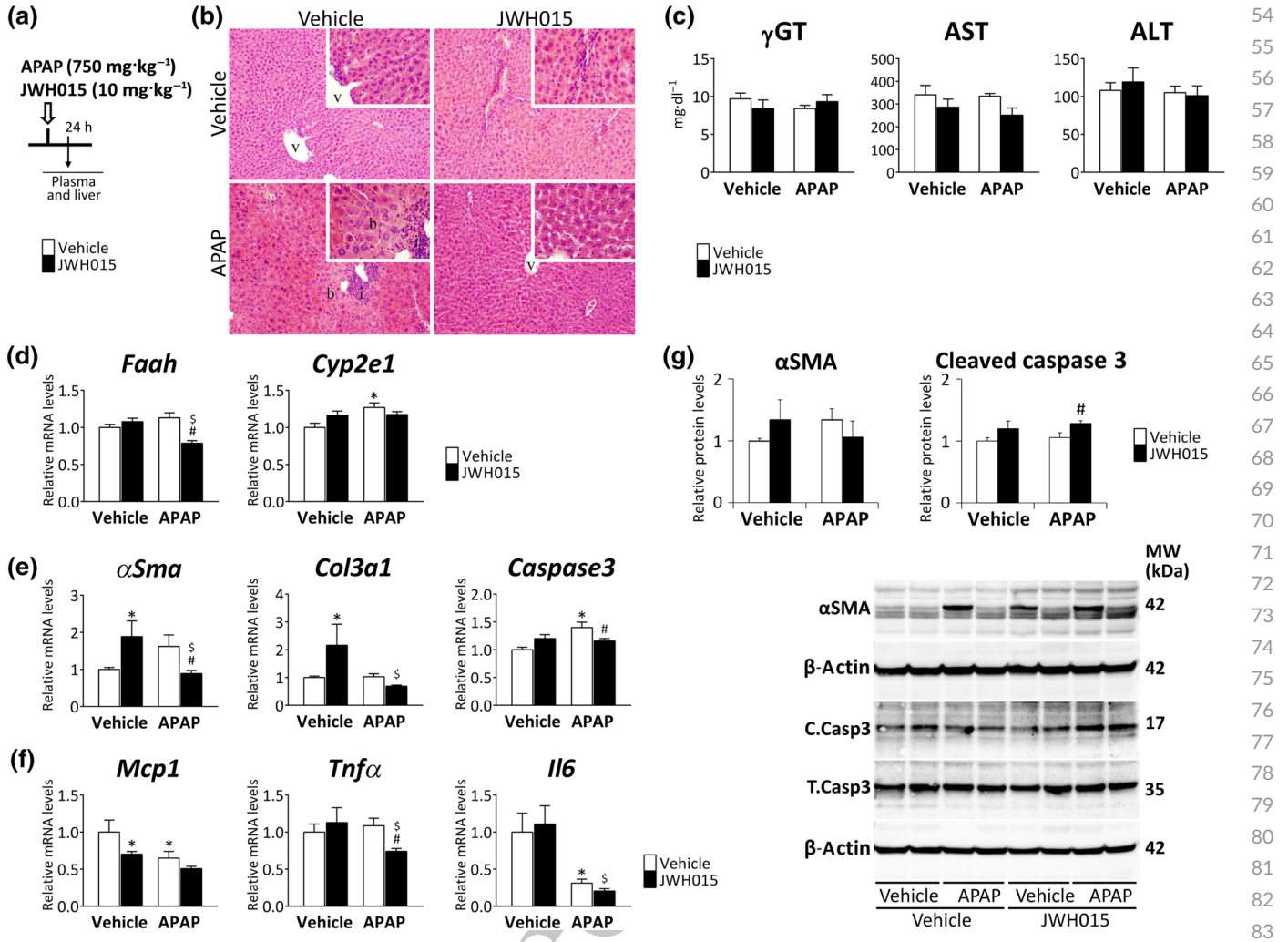


**FIGURE 4** Effects of the acute oral overdose of paracetamol (APAP; 750 mg·kg$^{-1}$) on histopathology; the expression of hepatic injury-related factors including fibrogenic, apoptotic and chemo-attractive/cytokine factors; and plasma levels of liver transaminases in WT mice previously treated with JWH015 (10 mg·kg$^{-1}$). (a) Timeline of the Experimental Design 1. (b) Representative photomicrographs showing high magnification views of the liver sections stained by H-E (groups: vehicle–WT, APAP–WT, vehicle–JWH015 and APAP–JWH015). b, ballooning; i, infiltration; v, blood vessel. (c) Quantification of the circulating levels of γGT, AST and ALT. (d–f) Quantification of the mRNA expression of *Faah*, *Cyp2e1*, *αSma*, *Col3a1*, *Caspase3*, *Mcp1*, *Tnfα* and *Il6*. (g) Quantification of the protein expression of αSMA and cleaved caspase 3. MWs are indicated. Histograms represent the mean ± SEM ($n$ = 7; $n$ = 6 in g). Tukey's test: $^{*}P < 0.05$ versus vehicle–WT mice; $^{\#}P < 0.05$ versus vehicle–*CB2* ko mice; $^{\$}P < 0.05$ versus APAP–WT mice. See Figures S4 and S5 for additional information

compared with vehicle– $CB_1$ receptor KO mice and paracetamol–WT mice (Figure 6e). We also found significant effects of genotype on the mRNA expression of *Faah*, *Cyp2e*, *αSma* and *Caspase3*. Significant effects of paracetamol on the mRNA expression of *Cyp2e1*, *αSma*, *Col3a1*, *Tnfα*, *Mcp1* and *Caspase3* were also observed. Additionally, effects of paracetamol on protein expression of αSMA were found. Additionally, genotype effect on cleaved caspase 3 was also observed. Increased αSMA was observed in the liver of both WT and $CB_1$ receptor KO mice exposed to repeated paracetamol compared with respective control mice (Figure 6g). Increased cleaved caspase 3 was specifically found in $CB_1$ receptor KO mice exposed to repeated paracetamol compared with paracetamol–WT mice (Figure 6g).

### 3.7 | $CB_1$ receptor activation exacerbates repeated paracetamol-induced liver injury

Because $CB_1$ receptor deficiency aggravates repeated paracetamol-induced hepatotoxicity, we evaluated the effect of the selective $CB_1$ receptor agonist ACEA on inflammation and fibrogenesis after the repeated administration of paracetamol for three consecutive days and killed 24 h later (Figure 7a). Histopathologically, liver injury induced by repeated paracetamol (destruction of parenchyma architecture, extensive necrosis, haemorrhage and inflammation) remains when mice were pretreated with ACEA (Figure 7b). Despite no differences in circulating γ-glutamyltransferase, effects of paracetamol on the remaining transaminases were found likely to be due to an overall

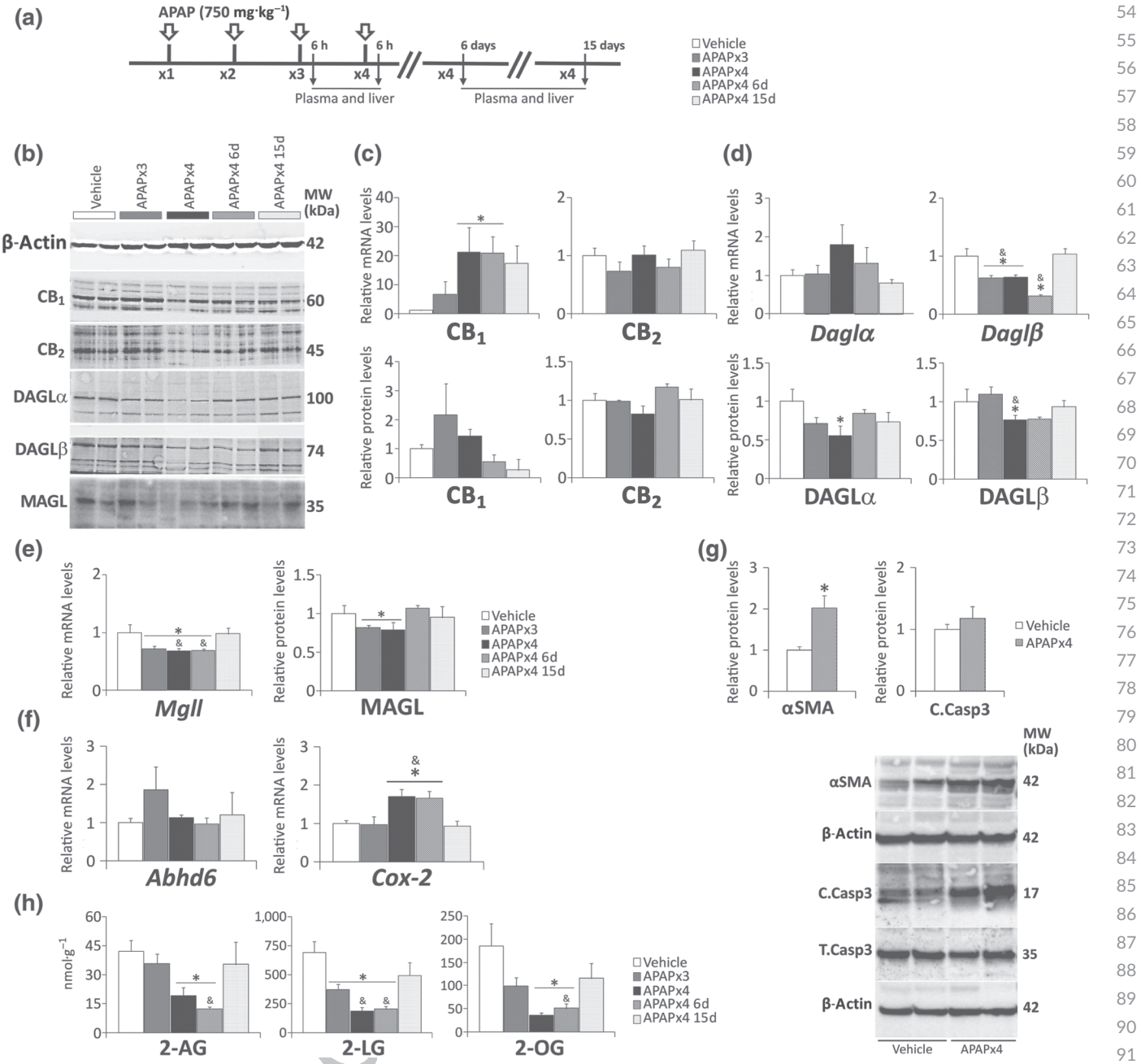


**FIGURE 5** Effects of the repeated oral overdose of paracetamol (APAP; 750 mg·kg$^{-1}$·day$^{-1}$) for 3 and 4 days on the 2-AG-related signalling and enzymatic machinery in the mouse liver after 6 h (APAPx3 and APAPx4), 6 days (APAPx4 6d) and 15 days (APAPx4 15d) of the last administration. (a) Timeline of the Experimental Design 2. (b) Representative immunoblots. (c–f) Quantification of the mRNA and protein levels of *Cnr1*/$CB_1$, *Cnr2*/$CB_2$, *Dagla*/DAGLα, *Daglb*/DAGLβ, *Mgll*/MAGL, *Abhd6* and *Ptsg2*. (g) Quantification of the protein expression of αSMA and cleaved caspase 3. MWs are indicated. (h) Quantification of the hepatic concentration of 2-AG, 2-LG and 2-OG. Histograms represent the mean ± SEM ($n$ = 6; $n$ = 5 in b). Tukey's test: $^{*}P < 0.05$ versus vehicle mice; $^{\&}P < 0.05$ versus APAPx4 15d. See Figures S4–S8 for additional information

decrease in aspartate aminotransferase levels and an overall increase in alanine aminotransferase levels in the plasma of the mice exposed to repeated paracetamol (Figure 7c).

Effects of treatment (vehicle vs. paracetamol) on mRNA expression of *Cyp2e1*, *αSma*, *Col3a1*, *Caspase3*, *Mcp1* and *Tnfα* were found. Although no interaction between treatment and drug was observed, single-effect analysis indicated that ACEA increased the mRNA expression of *Caspase3* and *Mcp1* and decreased that of *Col3a1* in the liver of repeated paracetamol-exposed mice (ACEA–paracetamol mice) compared with the corresponding vehicle–paracetamol mice (Figure 7e,f). Additionally, effect of treatment (vehicle vs. paracetamol) on protein expression of αSMA and cleaved caspase 3 was

**TABLE 2** Correlation of components of the endogenous cannabinoid signalling system and acylglycerols with fibrogenic and inflammatory factors in the liver of mice exposed to repeated administration of acetaminophen (750 mg·kg$^{-1}$·day$^{-1}$) for 4 days

| | *αSma* | *Col3a1* | *Tnfα* | *Il6* | *Mcp1* | *Caspase3* |
|---|---|---|---|---|---|---|
| *Cnr1* | $r$ = 0.618<br>$P$ < 0.001 | $r$ = 0.484<br>$P$ < 0.01 | $r$ = 0.449<br>$P$ < 0.01 | ns | ns | ns |
| *Cnr2* | $r$ = 0.335<br>$P$ < 0.05 | ns | ns | ns | ns | ns |
| *Dagla* | $r$ = 0.750<br>$P$ < 0.001 | $r$ = 0.482<br>$P$ < 0.01 | $r$ = 0.479<br>$P$ < 0.001 | ns | ns | $r$ = 0.707<br>$P$ < 0.001 |
| *Daglb* | ns | ns | ns | ns | ns | ns |
| *Mgll* | ns | ns | $r$ = −0.396<br>$P$ < 0.05 | ns | ns | ns |
| *Abhd6* | ns | ns | ns | ns | ns | ns |
| *Ptgs2* | $r$ = 0.336<br>$P$ < 0.05 | $r$ = 0.616<br>$P$ < 0.001 | $r$ = 0.468<br>$P$ < 0.01 | ns | $r$ = 0.375<br>$P$ < 0.05 | ns |
| 2-AG | ns | ns | ns | ns | ns | $r$ = −0.418<br>$P$ < 0.05 |
| 2-LG | ns | $r$ = −0.355<br>$P$ < 0.05 | ns | ns | ns | $r$ = −0.423<br>$P$ < 0.05 |
| 2-OG | ns | ns | ns | ns | ns | $r$ = −0.353<br>$P$ < 0.05 |

respectively found. We also found interaction between factors in the protein expression of cleaved caspase 3. Increased expression of cleaved caspase 3 was specifically observed in the liver of the paracetamol mice treated with ACEA compared with the corresponding vehicle–paracetamol and ACEA–vehicle mice (Figure 7g).

### 3.8 | Overdoses of paracetamol in mouse and human increased the immunohistochemical expression of the $CB_1$ and $CB_2$ receptors in the liver

Immunohistochemical analyses of $CB_1$ and $CB_2$ receptors in the liver of a subject diagnosed with hepatotoxicity induced by paracetamol overdoses (Figure 8a,b,e,f) are consistent with the increased expression of $CB_1$ and $CB_2$ in the liver of mice exposed to the repeated administration of paracetamol (Figure 8c,d,g,h) and supported the results previously obtained from mRNA analysis.

## 4 | DISCUSSION

The present study demonstrated that acute and repeated oral overdose of paracetamol (750 mg·kg$^{-1}$·day$^{-1}$) altered the liver expression of relevant components of the 2-AG-related signalling system including the cannabinoid $CB_1$ and $CB_2$ receptors and the enzymatic machinery that synthesizes (DAGLα and DAGLβ) and degrades (MAGL, COX-2 and ABHD6) 2-AG. Based on the differential expression of $CB_1$ and $CB_2$ receptors when acute and repeated administration of paracetamol were compared, we performed two additional experiments: The acute overdose study was done in mice lacking the $CB_2$ receptors and mice previously treated with the $CB_2$ agonist JWH015, whereas the repeated administration of paracetamol was addressed in mice lacking $CB_1$ receptors and mice treated with the $CB_1$ agonist ACEA. Results suggest that both cannabinoid receptors exert differential hepatoprotective roles involving fibrogenesis and inflammation in the context of paracetamol-induced liver injury that were dependent on the timing of injury and the number of doses. This information might help to understand the type of endogenous cannabinoid-based intervention that might be addressed as a therapeutic strategy against acute single dose-associated liver injury or chronic multiple dose-associated liver lesion.

$CB_1$ and $CB_2$ receptors and the main enzymatic machinery of 2-AG were differently expressed depending on the acute or repeated administration of paracetamol. Specifically, acute paracetamol increased the expression of *Cnr2*, *Dagla*, *Abhd6* and *Ptgs2*, while repeated paracetamol increased the expression of *Cnr1* and decreased the expression of *Daglb* and *Mgll* in the liver. However, both acute paracetamol and repeated paracetamol lowered the hepatic contents of three acylglycerols, 2-AG, 2-LG and 2-OG, suggesting a decreased generation or an enhanced degradation of acylglycerols. Without actual data on the activity of DAGL isoforms, MAGL, ABHD6 and COX-2, we cannot identify the main source for this variation that clearly suggests a deficit in liver endogenous cannabinoid signalling affected by paracetamol-induced toxicity. Regarding the time response (6, 24 and 48 h) of the effect of acute paracetamol on the

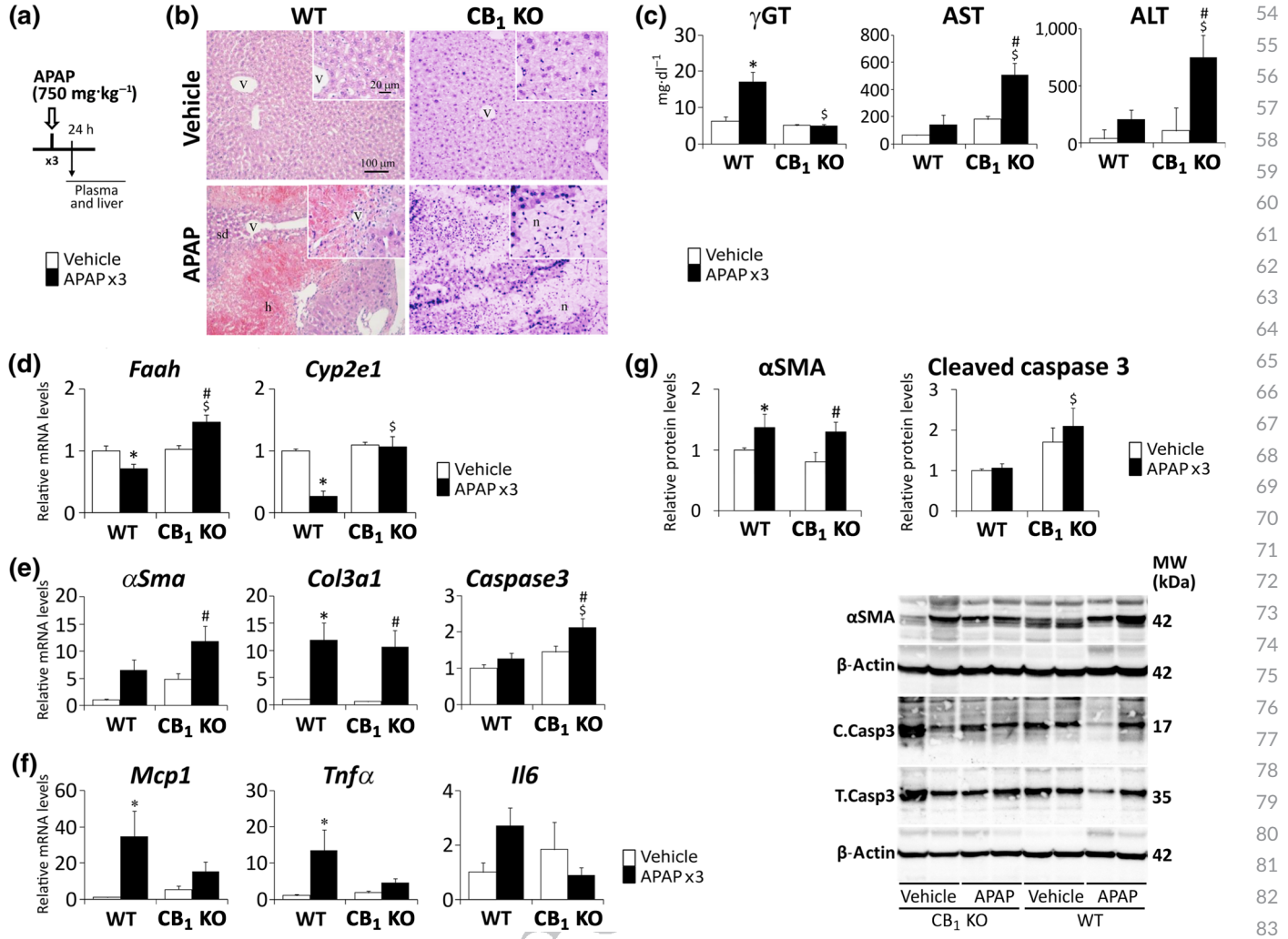


**FIGURE 6** Effects of the repeated oral overdose of paracetamol (APAP; 750 mg·kg$^{-1}$·day$^{-1}$) for 3 days on histopathology; the expression of hepatic injury-related factors including fibrogenic, apoptotic and chemo-attractive/cytokine factors; and plasma levels of liver transaminases in WT and $CB_2$ receptor KO mice after 24 h of last administration. (a) Timeline of the Experimental Design 2. (b) Representative photomicrographs showing high magnification views of the liver sections stained by H-E (groups: vehicle–WT, APAP–WT, vehicle– $CB_1$ KO and APAP– $CB_1$ KO ). h, haemorrhage; n, necrosis; sd, sinusoidal dilatation; v, blood vessel. (c) Quantification of the circulating levels of γ-glutamyl transpeptidase (γGT), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). (d–f) Quantification of the mRNA expression of *Faah*, *Cyp2e1*, *αSma*, *Col3a1*, *Caspase3*, *Mcp1*, *Tnfα* and *Il6*. (g) Quantification of the protein expression of αSMA and cleaved caspase 3. MWs are indicated. Histograms represent the mean ± SEM ($n = 6$). Tukey's test or single-effect analysis: $^{*}P < 0.05$ versus vehicle–WT mice; $^{\#}P < 0.05$ versus vehicle– $CB_1$ receptor KO mice; $^{\$}P < 0.05$ versus APAP–WT mice. See Figures S4 and S5 for additional information

endogenous cannabinoid system, we suggest that the increased expression of *Abhd6* and *Ptgs2* could be the main enzymatic mechanism that results in the down-regulated levels of acylglycerols. On the other hand, the lower expression of *Daglb* and *Mgll* and the increased expression of *Ptgs2* induced by the repeated paracetamol may result in a lower turnover of acylglycerols. Further studies are necessary to clarify whether this change is associated with a paracrine down-regulation in hepatic myofibroblasts (Siegmund, Wojtalla, Schlosser, Zimmer, & Singer, 2013).

2-AG represents a natural defence mechanism against inflammation (Mounsey et al., 2015; Turcotte, Chouinard, Lefebvre, & Flamand, 2015). Accordingly, we observed that the decreased concentrations of acylglycerols including 2-AG after repeated paracetamol in WT mice were accompanied by increased expression of the inflammatory chemoattractant/cytokines *Mcp1*, *Tnfα* and *Il6* and the fibrogenic *αSma* and *Col3a1*. In this context, the expression of *Cnr1*, *Dagla* and *Ptgs2* correlated positively with *Tnfα*, *αSma* and *Col3a1*. These results are consistent with those data previously described by Mai et al. (2015). *Col3a1* has been previously shown to correlate positively with the expression of the endogenous cannabinoid biosynthetic enzymes *Dagla* and *Nape-pld* in human and mouse models of fibrotic liver (Mai et al., 2015). However, the negative correlation between *Col3a1* and *Mgll* described previously in a murine model of carbon tetrachloride-induced liver injury was not reproduced in our mouse model of paracetamol-induced liver injury (Mai et al., 2015). It was also described that 2-AG can mediate cell death in hepatic stellate

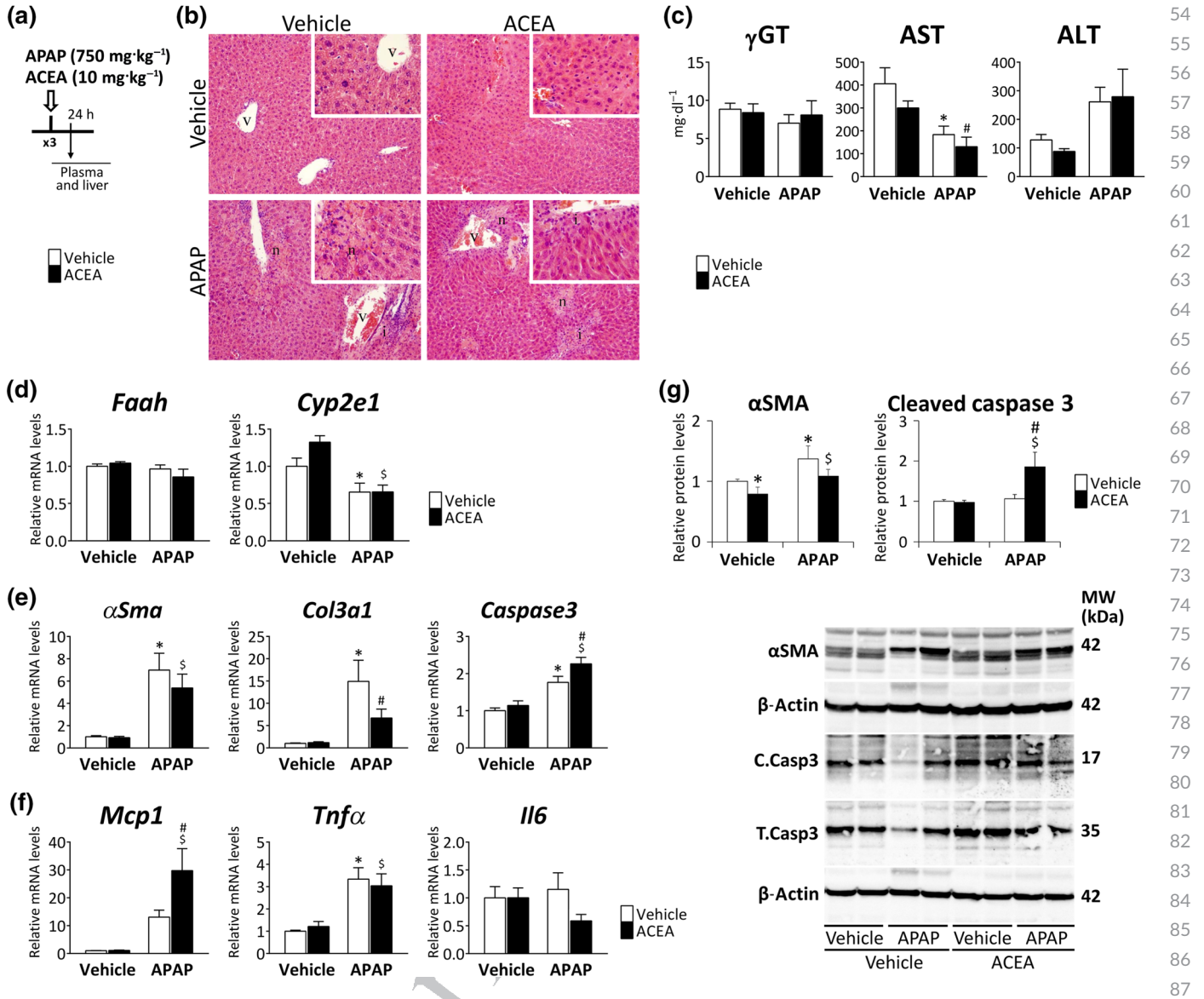


**FIGURE 7** Effects of the repeated oral overdose of paracetamol (APAP; 750 mg·kg$^{-1}$·day$^{-1}$) for 3 days on histopathology; the expression of hepatic injury-related factors including fibrogenic, apoptotic and chemo-attractive/cytokine factors; and plasma levels of liver transaminases in WT mice previously treated with ACEA (10 mg·kg$^{-1}$) for 3 days. (a) Timeline of the Experimental Design 2. (b) Representative photomicrographs showing high magnification views of the liver sections stained by H-E (groups: vehicle–WT, APAP–WT, vehicle–ACEA and APAP–ACEA). i, infiltration; n, necrosis; v, blood vessel. (c) Quantification of the circulating levels of γGT, AST and ALT. (d–f) Quantification of the mRNA expression of *Faah*, *Cyp2e1*, *αSma*, *Col3a1*, *Caspase3*, *Mcp1*, *Tnfα* and *Il6*. (g) Quantification of the protein expression of αSMA and cleaved caspase 3. MWs are indicated. Histograms represent the mean ± SEM ($n$ = 8; $n$ = 6 in g). Tukey's test: $^{*}P < 0.05$ versus vehicle–WT mice; $^{\#}P < 0.05$ versus vehicle–*CB1* ko mice; $^{\$}P < 0.05$ versus APAP–WT mice. See Figures S4 and S5 for additional information

cells, a mechanism that attenuates the fibrogenic response (Siegmund et al., 2013). Interestingly, the putative hepatoprotective actions of acylglycerols that can be diminished in paracetamol-induced liver injury are consistent with the negative correlations between *Tnfα* and *Mgll* and between the apoptotic factor *Caspase3* and the liver contents of acylglycerols. In accordance with these results, positive correlations between *Caspase3* and the 2-AG biosynthesis machinery (*Dagla* and *Daglb*) were obtained after acute or repeated paracetamol. We also identified a positive correlation between *Ptgs2* and the chemokine *Mcp1* after repeated paracetamol, suggesting a chemoattractive mechanism of inflammatory cells driven by prostaglandin precursors. These results support a key mechanism of 2-AG biosynthetic/degradation machinery and cannabinoid signalling that limits hepatic inflammation, fibrosis and cell death induced by paracetamol. In this regard, the identification of the mechanism that mediates the endogenous cannabinoid-mediated anti-inflammatory response in the liver is very relevant. Whereas $CB_1$ receptor antagonism has been identified as an antifibrogenic therapy in non-alcoholic steatohepatitis (Iyer et al., 2017; Teixera et al., 2006), $CB_2$ receptors seem to be protective (Guillot et al., 2014). Thus, it is crucial to understand how the anti-inflammatory/antifibrogenic properties of enhanced 2-AG availability engage both $CB_1$ and $CB_2$ receptors (Habib et al., 2018).

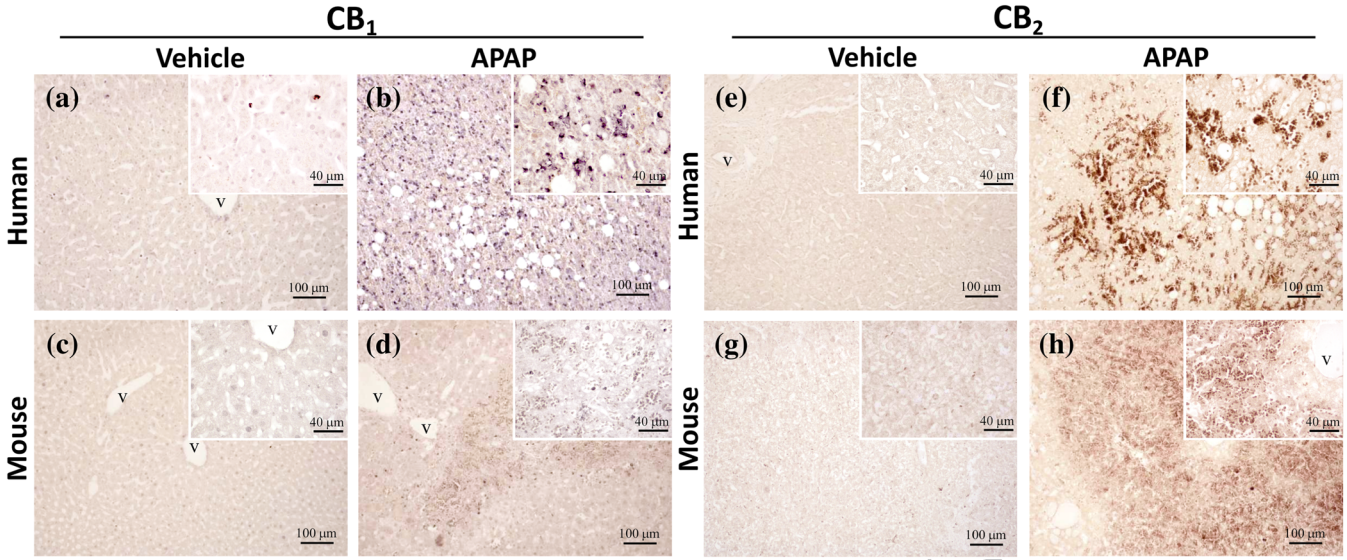


**FIGURE 8** Representative high-magnification photomicrographs showing the immunohistochemical expression of the (a–d) $CB_1$ and (e–h) $CB_2$ receptors in liver sections of a patient with an acute hepatic failure caused by a prolonged consumption of acetaminophen compared with healthy controls and liver sections of mice exposed to oral overdoses of APAP (750 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$) for 4 days. Scale bars are included. v: blood vessel

Because $CB_1$ and $CB_2$ receptors were differentially expressed in the liver after paracetamol overdose, we evaluated the differential role of $CB_1$ and $CB_2$ receptors in fibrogenesis and inflammation associated with paracetamol-induced liver injury using genetically modified animals and mice treated with the selective $CB_1$ and $CB_2$ agonists ACEA and JWH015 respectively. To this aim, we firstly analysed the effects of the acute and repeated administration of paracetamol on the liver of $CB_2$ receptor KO and $CB_1$ receptor KO mice respectively. Acute paracetamol produced a higher histopathological damage (e.g. increased necrosis) in the hepatic parenchyma of $CB_2$ receptor KO mice, in accordance with the protective role for $CB_2$ receptors (Guillot et al., 2014). The hepatic damage in $CB_2$ receptor KO mice was accompanied by a deregulation in fibrogenic (*αSma*), inflammatory (*Il6*) and apoptotic (*Caspase3*)-related factors and with a threefold increase in circulating transaminase aspartate aminotransferase. According to these results and those previous studies with cultured lymphocytes treated with the $CB_2$ agonist JWH133 (Guillot et al., 2014), we found that the treatment of JWH015, 1 h before the acute paracetamol administration, blocked the paracetamol-induced lymphocytic infiltration in liver parenchyma that was accompanied by a reduced expression of *αSma* and *Tnfα* and increased cleaved caspase 3. Similar to $CB_2$ receptor deficiency, the hepatic damage induced by repeated paracetamol for 3 days in the $CB_1$ receptor KO mice was also more severe compared with WT mice, suggesting that $CB_1$ receptor is also relevant for the defence against paracetamol-induced liver injury. Large necrotic areas, infiltration and haemorrhage in the hepatic parenchyma of the repeated paracetamol-exposed $CB_1$ receptor KO mice were accompanied by a deregulation of *αSma*, *Col3a1* and cleaved caspase 3 and with a threefold increase in circulating transaminases aspartate aminotransferase and alanine aminotransferase. However, its role is not as simple as to promote inflammation and fibrosis. Although $CB_1$ receptor deficiency normalized *Mcp1* and *Tnfα* (and a tendency in *Il6*) and circulating γ-glutamyl transpeptidase in the repeated paracetamol-exposed mice, the treatment with ACEA aggravated the repeated paracetamol-induced increase in cleaved caspase 3 and *Mcp1*. Moreover, liver damage induced by repeated paracetamol (destruction of parenchyma architecture, extensive necrosis, haemorrhage and inflammation) remains after pretreatment with ACEA. These results suggest that both $CB_1$ and $CB_2$ receptors participate in the defence against paracetamol-induced liver injury in a different manner, although they might have different actions on liver inflammatory in a context of paracetamol-induced hepatotoxicity. This is consistent with a role for $CB_1$ receptors in activating bone marrow-derived macrophages that contributes to the inflammatory response in liver injury (Mai et al., 2015).

Our results indicate that the roles of cannabinoid receptors on defence against paracetamol-induced liver injury are more complex than initially thought. Previous studies support facing roles of $CB_1$ and $CB_2$ receptors in alcoholic and metabolic steatosis, liver injury, regeneration, and hepatic fibrogenesis and inflammation (Alswat, 2013; Dibba et al., 2018; Mallat, Teixeira-Clerc, Deveaux, Manin, & Lotersztajn, 2011). $CB_1$ activation enhances fibrogenesis, whereas *Cnr1* deletion is associated with an amelioration of liver fibrosis and steatosis (Jeong et al., 2008; Teixeira-Clerc et al., 2006). Conversely, $CB_2$ receptor stimulation has antifibrogenic and anti-inflammatory properties, whereas *Cnr2* deletion results in increased deposition of collagen, liver steatosis and inflammation (Horváth et al., 2012; Louvet et al., 2011). The opposite roles of both cannabinoid receptors in fibrogenesis and inflammation are likely related to their differential up-regulation in response to acute or chronic liver injury. Specifically, the profibrogenic properties of $CB_1$ receptors, expressed mainly in hepatocytes and hepatic myofibroblasts, have been described in the

liver of cirrhotic patients and mouse models undergoing bile duct ligation, chronic exposure to carbon tetrachloride or thioacetamide and non-alcoholic steatohepatitis elicited by prolonged fat feeding (DeLeve, Wang, Kanel, Atkinson, & McCuskey, 2008; Mallat, Teixeira-Clerc, & Lotersztajn, 2013; Teixeira-Clerc et al., 2006). The antifibrogenic role of the pharmacological blockade of $CB_1$ receptors (e.g. rimonabant) was associated with proapoptotic effects of the hepatic myofibroblasts (Teixeira-Clerc et al., 2006). In our mouse model of repeated paracetamol-induced liver injury, $CB_1$ receptor deficiency exhibited profibrogenic and proapoptotic properties but blocked the repeated paracetamol-related increase of chemoattractive and inflammatory factors. Whether the inactivation of $CB_1$ receptors displays hepatoprotective effects against paracetamol-induced liver injury, as anticipated from their anti-inflammatory effects, deserves further pharmacological studies.

On the other hand, $CB_2$ receptors are mainly expressed in hepatic immune cells and hepatic myofibroblasts, but not in hepatocytes, during liver injury (Mallat et al., 2011; Mallat et al., 2013). Activation of $CB_2$ receptors in Kupffer cells reduced the paracrine release of the proinflammatory cytokines TNF-α and IL-1β in a context of alcoholic liver disease (Louvet et al., 2011). Moreover, $CB_2$ agonists and MAGL inhibitors enhancing 2-AG-mediated $CB_2$ receptor signalling in hepatic stellate cells and hepatocytes protect against apoptosis in liver injury models (Cao et al., 2013; Jeong et al., 2008; Teixeira-Clerc et al., 2010). In addition, liver regeneration by hepatocyte proliferation was facilitated by $CB_2$ signalling via a paracrine IL-6-dependent pathway in hepatic myofibroblasts (Teixeira-Clerc et al., 2010). The hepatoprotective role of $CB_2$ signalling is consistent with the profibrogenic, proapoptotic and proinflammatory properties associated with $CB_2$ deficiency in our mouse model of acute paracetamol-induced liver injury (Dibba et al., 2018; Mallat et al., 2013). The recovery of the liver levels of 2-AG after a resting period of 15 days may facilitate the hepatoprotective actions of $CB_2$ signalling.

Our results should be critically evaluated regarding the clinical implication of cannabis use in liver diseases. Recent clinical studies suggest a protective effect of cannabis consumption in alcoholic liver disease and non-alcoholic fatty liver disease, including liver steatosis, resulting in a lower prevalence in cannabis users (Adejumo et al., 2017, 2018; Vázquez-Bourgon et al., 2019). However, the use of cannabis as a risk or protective factor in chronic liver diseases, such as hepatitis C virus infection, remains inconclusive (Wijarnpreecha, Panjawatanan, & Ungprasert, 2018). Considerable research and clinical interest in constituents of cannabis have opened up potential application in the symptom management of liver diseases (Goyal, Rahman, Perisetti, Shah, & Chhabra, 2018).

In conclusion, we demonstrate for the first time that $CB_1$ and $CB_2$ receptors are also implicated in the regulation of fibrogenesis and inflammation in response to liver injury induced by acute and repeated overdoses of paracetamol. Although previous evidences indicate that blocking the $CB_1$ receptor (**rimonabant**, **AM6545** and JD5037) and $CB_2$ receptor activation (JWH015, JWH133 and HU-910) often display hepatoprotective functions involving antifibrogenic and anti-inflammatory effects in several models of liver injury (Dibba et al., 2018; Mallat et al., 2013), this might not be completely true in the case of paracetamol-induced liver injury. Future research is warranted to develop pharmacotherapies involving the 2-AG-related signalling and enzymatic machinery as a means of treating the hepatotoxicity induced by paracetamol.

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## AUTHOR CONTRIBUTIONS

Study design: P.R., F.R.d.F., J.D. and J.S. Study conduct: P.R. and J.S. Data collection: A.V., E.A., A.P., A.J.L.-G., J.D., L.S.-M., D.M.-V., A.B. and R.d.l.T. Data analysis and integrity: P.R. and J.S. Data interpretation: P.R., M.I.L., A.S., F.J.P., F.R.d.F. and J.S. Drafting the manuscript: P.R. and J.S. Revising the manuscript: P.R., J.D. and J.S. Approving the final version of the manuscript: all authors.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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