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Cannabinoïd receptor-1 signaling in hepatocytes and stellate cells does not contribute to NAFLD

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Abstract

The endocannabinoid system regulates appetite and energy expenditure and inhibitors of the cannabinoid receptor-1 (CB-1) induce weight loss with improvement in components of the metabolic syndrome. While CB-1 blockage in brain is responsible for weight loss, many of the metabolic benefits associated with CB-1 blockade have been attributed to inhibition of CB-1 signaling in the periphery. As a result, there has been interest in developing a peripherally restricted CB-1 inhibitor for the treatment of nonalcoholic fatty liver disease (NAFLD) that would lack the unwanted centrally mediated side effects. Here, we produced mice that lacked CB-1 receptors in hepatocytes or stellate cells to determine if CB-1 signaling contributes to the development of NAFLD or liver fibrosis. Deletion of CB-1 receptors in hepatocytes did not alter the development of NAFLD in mice fed a high sucrose high fat diet or high fat diet (HFD). Similarly, deletion of CB-1 deletion specifically in stellate cells also did not prevent the development of NAFLD in mice fed the HFD nor did it protect mice for carbon tetrachloride (CCl₄)-induced fibrosis. Combined, these studies do not support a direct role for hepatocyte or stellate cell CB-1 signaling in the development of NAFLD or liver fibrosis.
Introduction

NAFLD is the most common condition affecting the liver in the U.S. owing to its association with obesity and the metabolic syndrome (1). The largest study to date using magnetic resonance spectroscopy to quantify liver triglyceride (TG) content showed that ~33% of individuals have hepatic steatosis (2). NAFLD encompasses a continuum of histological findings that starts with steatosis, which can progress to nonalcoholic steatohepatitis (NASH), characterized by inflammation and cell death, and eventually cirrhosis (3). Given the large number of individuals afflicted with this condition, there is a clear need to develop effective and safe therapies to treat NAFLD.

Over the past two decades, significant progress has been made in defining how and why fat accumulates in liver. NAFLD develops as a result of insulin resistance, which is most often associated with obesity (4, 5). Recent studies suggest that up to 38% of the fatty acids in TGs in liver are derived from de novo lipogenesis, while ~15% come from diet and the remaining from free fatty acids in the blood (6). The genes required for de novo lipogenesis are all regulated by the sterol regulatory element-binding transcription factor-1c (SREBP-1c), which is activated by insulin and is induced in animal models and humans with fatty liver (7, 8), and ChREBP, which is activated by glucose (9, 10). Blocking the lipogenic pathway through the inhibition of sterol regulatory element-binding proteins, acetyl-CoA carboxylase, the first committed enzyme in fatty acid synthesis pathway, or diacylglycerol acyltransferase 2, the enzyme that catalyzes the final step in TG synthesis, markedly reduces hepatic steatosis in animal models of NAFLD (11-13) and in humans (11, 12, 14, 15).
A second way to reduce liver lipogenesis is to improve insulin resistance through weight loss (1). To date, weight loss either through dietary restriction, bariatric surgery, or possibly GLP-1 agonism has been the most reliable intervention to consistently improve NAFLD histologically (16, 17) (18, 19). Based on these studies, it is likely that a therapeutic agent that induces an 8-10% weight loss would likely have a positive impact on NAFLD progression.

CB-1 has a well-defined role in regulating appetite and energy expenditure through its actions in brain (20, 21). Rimonabant, a centrally acting inverse agonist of CB-1, resulted in significant weight loss and improved measures of the metabolic syndrome in humans (22, 23). Unfortunately, the drug had to be withdrawn from the market due to central side effects and before its efficacy in NAFLD was fully assessed (24). More recent investigations have suggested that the beneficial effects of CB-1 antagonism on NAFLD is mediated through peripheral CB-1 receptors (25). Mice that lacked CB-1 in hepatocytes and fed a HFD had similar caloric intake and weight gain as wild-type mice but had lower hepatic TGs, reduced measures of insulin resistance, and lower rates of hepatic lipogenesis (26, 27). It was suggested that signaling through the CB-1 led to increased SREBP-1c levels and increased hepatic lipogenesis. Liu et al. (28) reported that the CB-1 receptors in liver were necessary and sufficient for the development of diet-induced hepatic insulin resistance and that CB-1 signaling in hepatocytes improved glycemic control via increased energy expenditure (29). If true, it is possible that a peripherally restricted antagonist of CB-1 could be developed that would lack the deleterious neuropsychiatric side effects but still be effective for the treatment of NAFLD (30).
To further interrogate mechanisms by which CB-1 signaling might regulate lipogenesis and the development of NAFLD, we independently generated mice that lack CB-1 receptors in hepatocytes or hepatic stellate cells (HSCs). Here, we show that the deletion of CB-1 receptors did not prevent the development of NAFLD in mice fed a high sucrose diet (HSD) or HFD. Similarly, the deletion of CB-1 specifically in stellate cells did not alter the severity of NAFLD in response to a HFD nor did it protect mice for CCl₄-induced fibrosis.

**Results**

*Metabolic consequences of CB-1 deletion in hepatocytes.* Conditional CB-1 knockout mice (*Cnr1^{fl/fl}* ) were generated using CRISPR/Cas9 genome editing to insert one *loxP* site upstream and another *loxP* site downstream of exon 2 of cannabinoid receptor 1 (*Cnr1*) allele (Supplemental Figure 1A). *Cnr1^{fl/fl}* mice were bred with mice expressing Cre recombinase under the control of mouse zona pellucida 3 promoter (*ZP3-Cre*) (31) to generate a germ line deletion of *Cnr1* (*Cnr1^{Δ/Δ}* ) mice. *Cnr1* mRNA was undetectable in multiple tissues of *Cnr1^{Δ/Δ}* mice, which confirmed that the *loxP* sites were functional and that Cre recombinase successfully deleted CB-1 expression (32). We further assessed brain, which is enriched in CB-1 expression (33). *Cnr1^{fl/fl}* mice were crossed with *SF1-Cre* mice (34) to delete CB-1 from SF1-expressing neurons (*Cnr1^{SF1-KO}* ). RNA *in situ* hybridization showed a significant reduction of Cnr1 mRNA levels in the ventromedial hypothalamus of *Cnr1^{SF1-KO}* mice compared with that in *Cnr1^{fl/fl}* mice (32).
The Cnr1*fl/fl* mice (Supplemental Figure 1B) were next bred to transgenic mice expressing Cre-recombinase under the control of hepatocyte-specific albumin promotor (*Alb-Cre*) to produce the hepatocyte-specific *Cnr1* knockout mice (*Hep-Cnr1*−/−). Real-time qPCR was used to quantify the expression of CB-1 mRNA in tissues of *Cnr1*fl/fl* and *Hep-Cnr1*−/− mice (Supplemental Figure 1C). CB-1 expression in whole liver RNA extracts was very low compared to the expression in hypothalamus, white adipose tissue, brown adipose tissue, and muscle.

Inasmuch as previous studies have suggested that caloric excess upregulated CB-1 expression and induced SREBP-1c in hepatocytes (27), we first investigated whether hepatic CB-1 mRNA levels changed in livers of mice fed a HSD or HFD. HSD feeding for 17 weeks resulted in obesity, hepatocyte damage, and liver steatosis as evidenced by a marked increase in body weight, plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, liver histology, and liver TG levels in wild-type mice (Figure 1, A-C and F and Figure 2, A and B). No significant differences in response to the HSD were observed between control and *Hep-Cnr1*−/− mice (Figure 1). CB-1 mRNA levels were not induced by the HSD in control livers (Figure 1D); however, the expected induction of SREBP-1 protein as well as a significant increase in the level of mRNAs coding genes involved in lipogenesis was observed (Figure 2, C, D, and E). Concurrently, 17-week HSD feeding resulted in elevated mRNA levels of genes associated with fibrosis such as collagen type 1 alpha 1 (*Col1A1*) and alpha smooth muscle actin (αSMA), but there was no evidence of fibrosis as determined by picrosirius red (PSR) staining of liver sections and collagen quantification (Figure 1, E, and F). Taken together, these data suggest that CB-1 expression is extremely low in liver and that *Cnr1* deletion in hepatocytes did not prevent the hepatocellular metabolic dysfunction induced by the HSD.
We next determined whether CB-1 in hepatocytes has a role in regulating glucose metabolism and insulin sensitivity. *Hep-Cnr1* −/− and control mice were fed chow or the HSD for 16 weeks. In both cohorts of mice, the HSD led to similarly elevated fasting blood glucose levels and there was no difference in glucose tolerance (Figure 3, A and B) or insulin sensitivity (Figure 3, C and D). To test whether hepatocyte CB-1 deletion altered insulin resistance, *Hep-Cnr1* −/− and control mice were euthanized after 17-week challenge of the HSD. Hepatocyte CB-1 deletion did not alter plasma glucose and insulin levels (Figure 3, E and F). These studies demonstrated that hepatocyte-specific deletion of CB-1 did not prevent glucose intolerance and insulin resistance induced by the HSD.

To determine whether hepatocyte-specific deletion of CB-1 could alter the pathologic features of HFD-induced hepatic steatosis, *Hep-Cnr1* −/− and control mice were fed chow or a HFD for 12 weeks before euthanasia. CB-1 deletion did not alter body weights (Supplemental Figure 2, A and B), plasma ALT, AST levels (Supplemental Figure 2C), glucose tolerance (Supplemental Figure 3, A and B), insulin sensitivity (Supplemental Figure 3, C and D), liver TGs and cholesterol concentrations (Supplemental Figure 3, E and F) or liver histology (Supplemental Figure 2F). There were also no discernable differences of CB-1 mRNA levels in response to the HFD between control and *Hep-Cnr1* −/− mice, but similar induction of mRNAs coding genes involved in fibrogenesis and lipogenesis was observed (Supplemental Figure 2D and Supplemental Figure 3G). PSR staining of liver sections and collagen quantification did not reveal evidence of significant fibrosis induced by HFD (Supplemental Figure 2, E and F). These studies demonstrated
that deletion of CB-1 does not change hepatic steatosis or the metabolic disorders induced by a HFD.

To determine whether hepatocyte-specific deletion of CB-1 could reverse the pathologic features of diet-induced hepatic steatosis, Cnr1fl/fl mice were fed a HFD for 8 weeks and then injected with an adeno-associated virus expressing Cre recombinase (AAV-Cre) to delete CB-1 in hepatocytes or a control (AAV-GFP) virus. Mice were maintained on the HFD for an additional 8 weeks before euthanasia. CB-1 deletion did not alter body weights (Supplemental Figure 4A), plasma ALT, AST levels (Supplemental Figure 4B), glucose tolerance (Supplemental Figure 4C), insulin sensitivity (Supplemental Figure 4D) or liver TGs and cholesterol concentrations (Supplemental Figure 5A-B). There were also no significant differences in liver histology (Supplemental Figure 5D). These studies showed that an inducible deletion of CB-1 does not reverse hepatic steatosis or alter the HFD-induced pathological features.

Single-cell sequencing of hepatocytes and HSCs reveals low Cnr1 expression in livers of mice fed a chow or HSD. To determine if a small population of cells in liver express CB-1 at high levels either before and/or after HSD feeding, we performed single cell RNA-sequencing of livers from wild-type mice fed chow or a HSD for 17 weeks and analyzed CB-1 expression. Previous studies showed that hepatocytes are sensitive to flow sorting (35); therefore, we optimized a hepatocyte isolation protocol without flow cytometry to harvest the greatest number of viable hepatocytes for sequencing. A total of 32,020 cell transcriptomes were obtained from two pairs of mice (21,891 chow, 10,129 HSD). The single-cell transcriptomes of each group were aggregated and analyzed
with Seurat R pipeline (36, 37). Hepatocytes had a higher ratio of mitochondrial genome transcripts than other resident liver cells likely due to lysis during isolation (35). Nevertheless, our approach resulted in significantly more viable hepatocytes than previously published methods (Supplemental Figure 6, A and B). A total of 17,530 cells were plotted in chow-fed group (Figure 4A) and 8,256 cells in HSD-fed group (Figure 4B), respectively, with a uniform manifold approximation and projection (UMAP) (38).

A panel of genes were used to confirm the identity of each cluster based on cell type-specific transcriptomes previously published (35, 39, 40). Hepatocyte clusters revealed an abundant expression of Alb (data not shown), which could be further divided into groups of spatially heterogeneous cells based on zonated gene expression profiles along the portal-central axis of liver lobule using the signature genes: cytochrome P450 family 2 subfamily F member 2 (Cyp2f2) (periportal region), cytochrome P450 family 2 subfamily E member 1 (Cyp2e1) and , cytochrome P450 family 1 subfamily A member 2 (Cyp1a2) (midzonal/pericentral region) (Figure 4, C and D) (41, 42). Collagen type 3 alpha 1 (Col3a1) (data not shown) marked the HSC cluster, which can be characterized into two general subpopulations of quiescent and activated HSCs. lecithin retinol acyltransferase (Lrat) was highly expressed in quiescent HSCs, whereas Coll1a1 enrichment represented a more activated state of HSCs in both groups (Figure 4, C and D) (43). HSD feeding led to HSC activation confirmed by an induction of actin alpha 2 (Acta2) mRNA expression in HSC compared with chow-fed mice (Figure 4D). Non-parenchymal liver cells like cholangiocytes, endothelial cells, Kupffer cells and mixed immune cell types (data not shown) were identified using Keratin 19 (Krt19), kinase insert domain receptor (Kdr), adhesion G protein-coupled receptor E1 (Adgre1) and protein tyrosine phosphatase receptor type C (Ptprc), respectively.
(Supplemental Figure 6, C and D). Next, we queried whether CB-1 is differentially distributed or enriched in a particular cell type. Clustering analysis displayed rare Cnr1 expression in hepatocytes and HSCs, as well as other non-parenchymal liver cells of mice fed a chow or the HSD (Figure 4, C and D). Together, transcriptomes of hepatocytes and HSCs in mice fed a chow or HSD verifies that Cnr1 expression is extremely low in hepatocytes and HSCs, and its expression was not induced in a subpopulation of cells in the liver by the HSD.

**CB-1 deletion in HSCs.** Previous studies have suggested that CB-1 expression was induced in cirrhotic livers of humans, and that CB-1 antagonists suppress the progression of liver fibrosis in mice (44). To determine if CB-1 expression in HSCs contributed to the development of fibrosis, we generated mice that lacked CB-1 in HSCs by breeding Cnr1fl/fl mice with mice expressing Cre recombinase under the control of a tetracycline-responsive promoter element (TRE-Cre). Offspring with both targeted alleles and the Cre transgene were bred with mice expressing reverse tetracycline-controlled transactivator protein (rtTA) driven by Lrat (45) promoter. Cnr1fl/fl mice with Cre and rtTA were fed a doxycycline diet for 4 weeks to obtain mice with HSC-specific Cnr1 deletion (Hsc-Cnr1−/−) (Supplemental Figure 7A).

Quiescent HSCs are characterized by the storage of retinyl esters in their cytoplasm (46). Liver injury and *in vitro* culture drive HSCs to undergo a transdifferentiation to an activated myofibroblast-like cell type, with a distinct feature of losing vitamin A storage (43). To determine if CB-1 was induced during stellate cell activation, HSCs were isolated from wild-type mice and cultured. As previously reported (43), HSCs gradually lost their endogenous retinoid fluorescence.
and morphologically became myofibroblast-like cells during 6-days in culture (Figure 5A). HSCs cultured for 6 days displayed increased expression of genes associated with activation such as \( \alpha SMA \) and \( Coll1A1 \) as well as decreased expression of \( Lrat \), a quiescent HSC-enriched marker (Figure 5B) (45). CB-1 mRNA levels were present at low levels following HSC isolation and did not change as HSCs became activated (Figure 5B).

To investigate a potential role of stellate cell CB-1 signaling in fibrosis, CCl\(_4\) was administered to wild-type and \( Hsc-Cnr1^{+/−} \) mice to induce fibrosis. Chronic exposure of \( Hsc-Cnr1^{+/−} \) and control mice to high dose CCl\(_4\) for 10 weeks via peritoneal administration resulted in extensive liver nodules without significant changes in plasma ALT and AST levels (Figure 5, C and D). CCl\(_4\) administration led to increased expression of HSC activation markers such as \( \alpha SMA \) and decreased expression of \( Lrat \) (Figure 5E). Importantly, there were no significant alterations in CB-1 mRNA levels in HSCs after prolonged CCl\(_4\) exposure (Figure 5E). Similarly, \( Cnr1 \) deletion in HSCs did not affect the development of liver fibrosis induced by CCl\(_4\) as confirmed by PSR staining of liver sections and collagen quantification (Figure 5F and Supplemental Figure 7, B and C). These data suggest that \( Cnr1 \) deletion in HSCs does not prevent liver fibrosis triggered by \textit{in vivo} activation of HSCs by CCl\(_4\).

Next, we analyzed the consequences of HSC-specific \( Cnr1 \) deletion in mice fed the HFD to induce NAFLD. \( Hsc-Cnr1^{+/−} \) and control mice were maintained on HFD for 12 weeks. Liver TG concentrations, steatosis, and fibrosis in control and \( Hsc-Cnr1^{+/−} \) mice were not significantly different (Figure 6, A, B, and C). Glucose tolerance and insulin sensitivities of \( Hsc-Cnr1^{+/−} \) mice
fed the HFD were also comparable to that of controls (Figure 6, D and E). Finally, the expression of genes associated with HSC activation were identical in HSCs obtained from wild-type and *Hsc-Cnr1*−/− mice fed the HFD (Figure 6F). These findings suggest that CB-1 signaling in HSCs does not play a major role in the development of fibrosis or in the development of metabolic alterations associated with high fat diets.

*CNR1 expression is low in human liver.* To determine if CNR1 expression is induced in humans with NASH and/or fibrosis, we assessed CB-1 mRNA expression in livers from healthy controls and NAFLD/NASH patients via *in situ* hybridization (ISH). All control livers were histologically normal, while NAFLD/NASH human donors had varying NAS scores but all had a diagnostic of fibrosis grade 2 (Supplemental Table 1). In addition to the human-specific CB-1 probe, two control probes were included in ISH: a positive control probe for species-specific housekeeping gene PPIB to verify mRNA quality of tissue sections and a negative control probe for DapB to detect nonspecific labeling. In accordance with published findings (47), CB-1 was highly enriched in the neurons of cerebellum, which served as the positive control tissue for the CB-1 probe (Supplemental Figure 8). All liver samples in the study were PPIB positive and DapB negative, as illustrated in the representative liver sections (Figure 7). CB-1 mRNA was expressed at extremely low levels in normal livers and NAFLD/NASH livers with no apparent differences between groups (Figure 7). Based on criteria for ISH scoring (Supplemental Table 2), CNR1 mRNA was detected at a moderate level in endothelial cells, low levels in cholangiocytes and barely expressed in mononuclear cells and cells located within sinusoids (Supplemental Table 3). Thus, consistent with our observations in mice, CB-1 mRNA expression was low in healthy human liver samples and remain unchanged with the development of NASH.
Discussion

The endocannabinoid system and particularly signaling through the CB-1 receptor in liver has been previously implicated as critical for the development of insulin resistance and NAFLD (25-29). To further investigate the possible role of CB-1 receptors in the development and progression of NAFLD and specifically in their ability to regulate lipogenesis, we independently generated mice that lack CB-1 receptors in hepatocytes or stellate cells. In contrast to previously published studies, we did not find that the deletion of CB-1 receptors in hepatocytes altered lipogenesis nor did it protect mice from the development of hepatic steatosis when fed the HSD or HFD. In addition, no changes were measured in insulin or glucose sensitivity in hepatocyte-specific knockout mice fed chow, the HSD or HFD. Similarly, the deletion of CB-1 receptors in stellate cells did not alter the development of NAFLD in HFD-fed mice nor did it prevent the development of cirrhosis in mice administered CCl₄.

As suggested in previous studies, we found the mRNA level of CB-1 to be extremely low in whole liver RNA extracts and they remained low in livers of wild-type mice fed the HSD and HFD. The validation that our construct disrupted CB-1 expression was demonstrated in Cnr1⁰/₀ mice bred to mice that express Cre under the control of SF-1 promoter. SF-1 and CB-1 are highly expressed in the ventral medial hypothalamus (VMH). CB-1 mRNA was absent in the VMH of Cnr1⁰/₀; SF-1-Cre mice (47). As a final attempt to determine if there was an isolated population of cells in liver that might more highly express CB-1, we performed single cell sequencing on livers
of mice fed chow or the HSD. As shown in Figure 4, low levels of CB-1 expression were only found in stellate and cholangiocytes.

Previous reports suggested that the pharmacological inhibition of CB-1 by SR141716A or germline deletion of CB-1 prevented the progression of fibrosis in mouse models of liver injury including CCl₄ (44). Similarly, Rimonabant was reported to reduce fibrosis in rats with CCl₄-induced cirrhosis (48). These results stimulated us to further investigate the expression and function of CB-1 in HSCs. CB-1 mRNA was expressed in HSCs isolated from wild-type mice but the expression was only slightly higher than that found in whole liver RNA extracts by qPCR and it was very low compared to other stellate-specific genes (Figure 5E). A greater role for the CB-1 receptor could be hypothesized if its expression was induced during stellate cell activation; therefore, we measured CB-1 expression by qPCR in HSCs before and after activation in culture. While the expression of genes such as Col1A1 and αSMA were induced in the cultured HSCs during activation, there was no change in expression of CB-1 (Figure 5B). In vivo studies demonstrated that the development of fibrosis induced by CCl₄ was not altered in Hsc-Cnr1⁻/⁻ mice (Figure 5 and Supplemental Figure 7). Based on these results, we conclude that CB-1 signaling in HSCs does not significantly reduce or augment the deposition of collagen or the development of cirrhosis in these models of hepatocyte injury.

While the studies described herein did not suggest that CB-1 is induced by a HSD and HFD or that CB-1 in hepatocytes or stellate cells play a role in the development of NAFLD, it was possible that human livers have higher CB-1 expression and/or that the development of NASH further
enhances CB-1 expression. ISH was used to evaluate the relative expression levels of CB-1 in normal and livers with NASH (Figure 7). The results obtained from the ISH were consistent with those in the mouse studies. CB-1 mRNA levels were very low in hepatocytes and there was no discernable increase in CB-1 expression in livers with histological evidence of NASH (Figure 7).

In summary, our studies suggest that CB-1 signaling in hepatocytes or in stellate cells does not alter the development or progression of NAFLD in response to the HSD or HFD in mice. There is no obvious explanation for the differences in results obtained from the studies described here and those previously published regarding the hepatocyte-specific CB-1 knockout studies. The construct to generate Cnr1fl/fl mice contains the entire open reading frame of Cnr1 gene (32), which is the same as that used previously (49). Moreover, the mouse strain, diet, and duration of diet feeding were identical to that used in previous publications and thus cannot be the cause of the discrepancy. One factor that cannot be controlled for that would be certainly different between centers performing the studies is the microbiome of the mice studied. The microbiome has been postulated to play a role in the development of NAFLD (50). However, if the microbiome is responsible for the differences found in this study compared to others, it would suggest that a specific microbiome is required for CB-1 inhibition to positively impact insulin resistance and NAFLD. Such a requirement would call into question whether this therapeutic approach would have consistent and broad-based positive responses in the general population.

Previous studies suggested that peripheral blockade of CB-1 signaling would be beneficial for the treatment of NAFLD. In fact, Nimacimab, a peripheral antagonist antibody to CB-1 for NASH,
has completed a Phase 1b study. Unfortunately, the results of our animal studies do not suggest that a peripherally acting compound will be successful in treating NAFLD if its mechanism of action is through the hepatocyte or stellate cells. While our results do not rule out the possibility that a peripherally acting CB-1 antagonist could function through other cell types and ultimately alter whole body metabolism in a way that may improve NAFLD, it is also possible that the benefit derived is centrally mediated, which would potentially carry the same unwanted side effects associated with Rimonabant.

Methods

Animals. Cnr1<sup>fl/fl</sup> mice (C57BL/6N background) harboring the conditional floxed Cnr1 alleles were generated using CRISPR-Cas9 at the Transgenic Core Facility of UT Southwestern Medical Center.

The guide RNAs and donor ssODNs were designed to insert one <i>loxP</i> site upstream and another <i>loxP</i> site downstream of exon 2 (Supplemental Figure 1). The gRNA sequences are 5’-TCTGGGTGAGGAGACATGCCTGG-3’ (upstream) and 5’-AGTCTATCGCTGCAGTTGCTCGG-3’ (downstream). Guides were selected using the CRISPR Design Tool (http://tools.genome-engineering.org). Cas9 mRNA, crRNA/tracrRNA, and donor ssODNs (Sigma-Aldrich) were mixed and injected into the cytoplasm of fertilized eggs, which were then transferred into the uterus of pseudo pregnant females to produced F0 founders. Founders containing the dual insertion of the <i>loxP</i> sites were identified via PCR using primer set 5’-agattagcacagagcttat-3’ and 5’-ataaagcttcttgctaatct-3’ for the upstream <i>loxP</i> site, and primer set 5’-tctgggtgagagacatgccttg-3’ and 5’-ggcatgtctcaccaggc-3’ for the downstream <i>loxP</i> site. F0
founders were bred with C57BL/6J mice to obtain F1 mice heterozygous for the floxed Cnr1 allele (Cnr1^{+/fl}), which were then intercrossed to produce homozygous Cnr1^{fl/fl} mice. For hepatocyte-specific deletion of Cnr1, Cnr1^{fl/fl} mice were bred with mice expressing Cre-recombinase under the control of the albumin promotor (Alb-Cre, Jackson Lab, #003574). To confirm that Cnr1 was deleted in hepatocytes, liver- and tail-derived DNA was used for genotyping by PCR with the following primers: 5’-accaccttcctcatgttaacct-3’, 5’-gaccagagacagctccaga-3’, and 5’-tgagggtatatctgtttttgc-3’ (WT 195bp; Flox 233bp; Delta 480bp).

To achieve inducible HSC Cnr1 gene deletion, Cnr1^{fl/fl} mice were bred with TRE-Cre mice (#006234, Jackson Laboratory). Offspring with both targeted alleles and the Cre transgene were bred with mice expressing rtTA driven by Lrat promoter. Cnr1^{fl/fl} mice with Cre and rtTA were fed a doxycycline diet (BioServ, S3888) for 4 weeks to obtain Hsc-Cnr1^{-/-}mice.

**Diet-induced hepatic steatosis.** Mice were housed at room temperature (23˚C) and maintained on a 12 hr light/dark cycle and provided ad libitum access to rodent chow (Harlan, Teklad Global 18% Protein Rodent Diet 2018; 18% kcal from fat, 3.1 kcal/g), HSD (17% kcal from sucrose, 45% kcal from fat, Research Diets D12451) or HFD (60% kcal from fat, Research Diets D12492). Body weights were measured weekly and mice in HFD and HFD studies were euthanized at 22 and 16 weeks of age respectively. For diet-induced obesity studies in HSC-specific Cnr1 knockout mice, Hsc-Cnr1^{-/-} and Cnr1^{fl/fl} mice were fed the HFD supplemented with 600 mg/kg doxycycline (60% kcal from fat, BioServ) for 12 weeks starting at 4 weeks of age.
**CCl₄-induced liver fibrosis.** Hsc-Cnr1⁺/- and Cnr1⁺/⁺ mice were fed the rodent chow described above supplemented with 600 mg/kg doxycycline (BioServ, S3888) for 4 weeks to induce Cnr1 deletion in HSCs. Mice were injected intraperitoneally with either CCl₄ (0.2 ml/kg of body weight, Sigma, 289116) or corn oil (Sigma, C8267) twice a week for 10 weeks. Mice were maintained on doxycycline diet, and body weights were measured weekly after injection and euthanized at 16 weeks of age.

**HSC isolation.** HSCs were isolated and cultured as described (46). RNA was isolated from HSCs for reverse transcription and quantitative real-time PCR analysis as described previously (51).

**Glucose tolerance and insulin sensitivity studies.** To determine glucose tolerance, 16 hr-fasted mice were administered glucose intraperitoneally (1.5 g/kg of body weight for chow-fed and HSD-fed mice). Tail vein blood was collected at 0, 15, 30, 60, and 120 min after glucose injection. To determine insulin sensitivity, 6 hr-fasted mice were administered insulin intraperitoneally (1 U/kg and 2 U/kg of body weight for chow-fed and HSD-fed mice respectively, Humulin R, Lilly). Tail vein blood was collected at 0, 15, 30, 60, and 120 min after insulin injection. Blood glucose levels were quantified using a Bayer Contour glucose meter.

HFD-fed doxycycline-inducible Hsc-Cnr1⁺/- and control mice (Cnr1⁺/⁺) were administered glucose (2 g/kg of body weight; Sigma) via oral gavage or human recombinant insulin (0.75 U/kg
of body weight; Novo Nordisk) via intraperitoneal injection, respectively. Blood glucose levels were determined with glucometer at indicated time points.

*Liver function tests.* Plasma ALT, plasma AST, liver TG and cholesterol levels were measured as previously described (51).

*Histology and collagen quantification.* All histological sections, Hematoxylin & eosin (H&E), trichrome and PSR staining were performed by the Molecular Pathology Core at University of Texas Southwestern Medical Center. Mean area of PSR-stained collagen in each liver section was determined using standard ImageJ software. PSR-stained liver sections demonstrate a red collagen under light microscopy (bright field). The images can be separated into three distinct channels. Collagen in liver sections of CCl₄-injected mice can be quantified by image reduction to green channel followed by automatic calculation of mean red area (Minimum = 0, Maximum = 255). To better reflect the whole tissue, each PSR-stained liver slide was sub-divided into 5-10 small sections for individual quantification, and the average was obtained for plotting.

*RNA isolation and quantitative real-time PCR.* Total RNA extraction from tissue and cells, RT-PCR and quantitative real-time PCR were performed as previously described (51). All data were normalized to the expression of mouse β-actin or apolipoprotein B. The forward and reverse primers for real-time PCR are: Cnr1, 5’-GTACCATCACCAGACCTCCT-3’ and 5’-GGATTCAGAATCATGAAGCACTCCA-3’; ApoB, 5’-CGTGGGCTCCAGCATTCTA-3’ and 5’-TCACCAGTCATTTCGTGCTTGGAGAGCAT-3’; Coll1A1, 5’-CGTGGTGGTTGGAGAGCAT-3’
and 5’-GGTCAGCTGGATAGCGACATC-3’; 

**Lrat**, 5’-

ACACTGACATCATTCTACTCTTTGG-3’ and 5’-

AGTTCAATGATTCTGGTGTGTAAC-3’; 

**aSMa**, 5’-TTCCGCTGCCAGAGACT-3’ and 5’-

GATGCCCGCTGACTCCAT-3’; 

**SrebP-1c**, 5’-GGAGCCATGGATTGCACATT-3’ and 5’-

GGCCGGAGTGACTCTGT-3’; 

**SrebP-2**, 5’-GCCAGGACCAGGTAAAT-3’ and 5’-

ACAAAGTTGCTCTGAAAAACATCA-3’; 

**ChrebP**, 5’-CCTCGCAACTCAGATCTT-3’ and 5’-

CTTTGCAGAGTTGCTCAGCACAA-3’; 

**Acly**, 5’-GCCAGGAGGAGCACACATC-3’ and 5’-

CTTTGCAGAGTTGCTCAGACACAA-3’; 

**Fasn**, 5’-GCCAGGAGGAGCACACATC-3’ and 5’-

AGAGAGCTCCTCTGGACTT-3’; 

**Scd1**, 5’-GCCAGGAGGAGCACACATC-3’ and 5’-

TAGCCTGTAATAAAGATTTCTGCAAACC-3’; 

**Elovl6**, 5’-GCCAGGAGGAGCACACATC-3’ and 5’-

GCTGCGGAACTTCAGGAAAT-3’ and 5’-

AGAGAGCTCCTCTGGACTT-3’; 

**SrebP-1** was detected using a rabbit polyclonal anti-SREBP-1 (IgG-20B12) antibody and SREBP-2 was detected using a rabbit monoclonal anti-SREBP-2 (IgG-22D5) antibody (52, 53). Calnexin was used as an invariant control for membrane protein and was detected using a commercially available polyclonal antibody (Enzo Life Science, Farmingdale, NY, lot: ADI-SPA-860-F). Creb (cAMP response element binding protein) was used as an invariant control protein for nuclear protein immunoblots and was detected using a

**Immunoblots.** Liver membrane and nuclear proteins were isolated from liver as previously described (51). Equal aliquots of liver membrane or nuclear proteins from seven to ten mice per group were pooled and 50 µg of protein was separated by SDS-PAGE and immunoblot analyses were carried out as previously described (51). SREBP-1 was detected using a rabbit polyclonal anti-SREBP-1 (IgG-20B12) antibody and SREBP-2 was detected using a rabbit monoclonal anti-SREBP-2 (IgG-22D5) antibody (52, 53). Calnexin was used as an invariant control for membrane protein and was detected using a commercially available polyclonal antibody (Enzo Life Science, Farmingdale, NY, lot: ADI-SPA-860-F). Creb (cAMP response element binding protein) was used as an invariant control protein for nuclear protein immunoblots and was detected using a
commercially available monoclonal antibody (Invitrogen, Catalog # 35-0900). Bound SREBP-1, SREBP-2, and Calnexin primary antibodies were visualized using peroxidase-conjugated, affinity-purified, light-chain specific, goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories Inc., 111-035-047). Bound Creb antibody was visualized using peroxidase-conjugated, affinity-purified, light-chain specific, goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories Inc., 115-035-072).

*Immunofluorescence microscopy of HSCs.* Freshly isolated HSCs were resuspended in phenol red-free DMEM containing 10% FBS, 5% streptomycin and 1% HEPES, and cultured in glass bottom dishes at 37°C under 5% CO₂. HSCs cultured for 1 day and 6 days were visualized via confocal microscopy (Zeiss LSM 880). Endogenous retinoid fluorescence signal was detected with laser setup for detection of DAPI.

*ISH.* ISH was performed using the RNAscope 2.5 LS Reagent Kit-Red (Cat No. 322150) from Advanced Cell Diagnostics according to the manufacturer’s guidelines on a Leica Bond Rx (Leica Biosystems). The RNAscope LS 2.5 Hs-CNR1 probe (Cat No. 591528) was used to identify human CNR1 mRNA in formalin-fixed paraffin-embedded sections of human cerebellum and liver samples from healthy and diseased donors. Tissues were sectioned at 5 µm thick on Fisherbrand Superfrost Plus microscope slides (Cat No. 12-550-15; Fisher Scientific). The mRNA quality of tissue sections was verified by using a housekeeping gene RNAscope 2.5 LS Positive Control Probe Hs-PPIB (Cat No. 313908), and nonspecific labeling was tested by using RNAscope 2.5 LS Negative Control Probe DapB (Cat No. 312038). Tissue sections were pretreated using the
following conditions: 15 min ER2 and 15 min protease. Following automation, slides were individually dipped into fresh xylene and cover-slipped using VectaMount Permanent Mounting Medium (Cat No. H-5000; VectorLabs).

**Single-cell sequencing.** Two pairs of mice (one pair fed chow, one pair fed the HSD) were used for single cell sequencing. For each pair, one mouse was perfused for hepatocyte preparation, and the other for HSC preparation. HSC isolation was described previously (46). Hepatocytes were isolated using pronase/collagenase digestion. After Nycodenz gradient centrifugation, the entire supernatant was removed and pellet was suspended with 1 ml chilled PBS in 1.5 ml Eppendorf tube. The cell suspension was centrifuged at 800 rpm for 3 min. The supernatant was discarded and the pellet was washed with 1 ml chilled PBS. Hepatocytes were resuspended in chilled PBS for cell counting. The approximate cell viability was between 80% and 90%, as determined by trypan blue staining. The final single cell suspension was used to generate separate libraries for hepatocytes and HSCs via 10X Genomics Chromium Single Cell 3’ Library and Gel Beads Kit (version 3) according to manufacturer’s protocol. Libraries were sequenced on an Illumina NextSeq 500 with high output of 400 million reads.

Raw data was demultiplexed using cellranger (version 3.1.0) and barcode-aware wrapper (version 2.17.1.14) supported by 10X Genomics’ pipeline. Transcriptomes were aligned to Genome Reference Consortium Mouse Build 38 (mm10) using STAR. Approximately 68.5% of sequencing reads were confidently mapped to the mouse genome. Seurat (version 3.2.0) was applied for single cell transcriptome analysis and plots on R (version 3.5.1). Briefly, genes that
were expressed in over three cells are kept as well as cells expressing more than 200 unique genes. Multiplets were excluded by removing cells with greater than 3,000 unique genes. Data was scaled and mitochondrial genes were removed for deep analysis. Then, UMI counts were scaled and variations due to differences in UMI/cell and percent mitochondrial genes were regressed out of the data. Cells in each pair of mice fed a chow or HSD were integrated to better understand cell identity and function (37). The highest variable genes were used for UMAP visualization and clustering. Sub-clustering hepatocytes and HSCs were based on different resolutions. All scRNA-seq data were deposited into GSE182365.

Statistics. Results are expressed as mean ± SEM. Comparisons were performed by two-tailed unpaired Student’s t test or one-way ANOVA analysis followed by post-hoc comparisons using Tukey corrections by GraphPad Prism 7 Software.

Study approval. All animal studies were approved and conducted under the oversight of the UT Southwestern Institutional Animal Care and Use Committee.

Author contributions

SW and QZ conducted experiments. JDH, PES, and JKE designed the research. SW and JDH wrote manuscript. SZ and PES provided founders of Hsc-Cnr1+/− mice. TT, MB, KKB and ML contributed to human CNR1 ISH study. CMC and JKE generated and verified Cnr1+/− mice. GL and JDH revised manuscript.
Acknowledgements

We thank Norma Anderson, Judy Sanchez, Tuyet Dang, and Tessa Edwards for excellent technical assistance, Gervaise Henry and Chelsea Hepler for critical advice on single-cell sequencing experiments, and Chelsea Burroughs and Nancy Heard for assistance with graphics. The work is supported by Supported by the NIH HL-20948.

References


Figures and figure legends

Figure 1. *Cnr1* deletion in hepatocytes does not affect body weight, liver function or diet-induced obesity in 22-week-old mice fed chow or a HSD. Growth curves of chow-fed (A) and HSD-fed (B) *Cnr1<sup>−/−</sup>* and *Hep-Cnr1<sup>−/−</sup>* mice (n = 6-8/group). Body weights were monitored weekly
starting at 5 weeks of age. (C) Plasma ALT and AST levels. (D) Whole liver RNA was extracted for measurement of relative mRNA levels of CB-1, Col1A1, Lrat and αSMA quantified by qPCR. ApoB was used as an invariant control. Values are expressed relative to chow fed Cnr1fl/fl mice, which was arbitrarily set to 1. Corresponding mean CT values are denoted above. (E) Mean area of collagen was obtained by calculating the PSR-stained red area in the image under split green channel, as described in Methods. Results shown as mean ± SEM, assessed by ANOVA. (F) H&E, trichrome and PSR staining of liver sections. Scale bar = 100 µm. All experiments (A-F) were repeated with a separate cohort of mice and with similar results.
Figure 2. Cnr1 deletion in hepatocytes does not affect liver steatosis in mice fed chow or HSD. The mice used are those described in Figure 1. (A and B) Liver TG and cholesterol levels in chow-fed Cnr1^fl/fl and Hep-Cnr1^-/- mice (n = 6-8/group) as well as HSD-fed Cnr1^fl/fl and Hep-Cnr1^-/- mice (n = 6-8/group) were measured before euthanasia at 22 weeks of age. (C and D) Membrane and nuclear fractions of SREBP-1 and SREBP-2 expression in pooled liver protein of chow-fed Cnr1^fl/fl and Hep-Cnr1^-/- mice (n = 6-8/group) as well as HSD-fed Cnr1^fl/fl and Hep-Cnr1^-/- mice (n = 6-8/group) euthanized at 22 weeks of age. Calnexin and Creb as control of membrane and nuclear...
proteins respectively. (E) Total RNA was extracted from each mouse liver, and the relative mRNA expression levels of Srebp-1c, Srebp-2, Chrebp, Acly, Acc1, Fasn, Scd1, and Elovl6 were quantified by real-time PCR. ApoB was used as an invariant control. The values were expressed relative to that of chow fed Cnr1<sup>fl/fl</sup> mice, which was arbitrarily set to 1. Corresponding mean CT values are denoted above. Results shown as mean ± SEM, assessed by ANOVA. mSREBP-1, membrane-bound SREBP-1; nSREBP-1, nuclear form of SREBP-1; mSREBP-2, membrane-bound SREBP-2; nSREBP-2, nuclear form of SREBP-2; Acly, ATP-citrate lyase; Chrebp, carbohydrate response element binding protein; Elovl6, elongation of long chain fatty acids family member 6; Fasn, fatty acid synthase; Scd1, stearoyl CoA desaturase 1.
Figure 3. Hepatocyte-specific Cnr1 deletion does not affect glucose tolerance or insulin sensitivity in mice fed chow or the HSD. Mice used are those described in Figure 1. Glucose and insulin tolerance tests were carried out 2 and 3 weeks prior to euthanasia, respectively. (A and B) Blood glucose levels were measured at indicated times after glucose injection. (C and D) Blood glucose levels were measured at indicated times after insulin injection. (E and F) Plasma blood glucose and insulin levels after euthanasia at 22 weeks of age. Results shown as mean ± SEM, assessed by ANOVA. The experiments were repeated in a separate cohort of mice with similar results.
Figure 4. Single-cell sequencing reveals very low Cnr1 expression in hepatocytes and non-parenchymal liver cells of mice fed chow or HSD. Symbol UMAP plot highlighting six subclusters of main liver cell compartments from scRNA-seq data aggregated from chow-fed wild-type mice (A) and wild-type mice maintained on HSD for 17 weeks (B). Violin plots of
representative hepatocyte zone-specific gene expression from (C) chow-fed wild-type mice and (D) wild-type mice maintained on HSD for 17 weeks: Cyp2f2 (periportal), Cyp2e1 and Cyp1a2 (midzonal/pericentral). Violin plots of HSC landmark genes (Lrat, Colla1, and Acta2) and Cnr1 expression across cell subpopulations from (C) chow-fed wild-type mice and (D) wild-type mice maintained on HSD for 17 weeks.
Figure 5. Cnr1 expression is low in cultured HSCs and Cnr1 deletion in HSCs does not alter CCl4-induced fibrosis. (A) Representative images of freshly isolated HSCs (upper panels) from chow-fed wild-type mice and from isolated HSCs cultured for 6 days (lower panels). Confocal microscopy was performed for detection of retinoid fluorescence (blue). Endogenous retinoid expression was visualized in cytoplasmic lipid droplets of HSCs (left panels). The merged Right panels show the retinoid signal overlaps with lipid droplets in activated HSCs. Scale bar = 50 µm.
(B) Total RNA from freshly isolated HSCs and HSCs cultured for 6 days was extracted for qPCR quantification of Col1A1, Lrat, αSMA and CB-1. β-actin was used as an invariant control. Values were expressed relative to that of freshly isolated HSCs, which was arbitrarily set to 1. Corresponding mean CT values are denoted above. (C) Plasma ALT and AST levels in chow-fed doxycycline-treated Hsc-Cnr1^{-/-} and Cnr1^{fl/fl} mice injected with either CCl_4 or corn oil (5-11/group). (D) Mice described in (C) were euthanized at 16 weeks of age. Total RNA was extracted from HSCs of each mouse, and the relative mRNA levels of Col1A1, Lrat, αSMA and CB-1 were quantified by qPCR. β-actin was used as an invariant control. Values were expressed relative to that of chow-fed doxycycline-treated Cnr1^{fl/fl} mice injected with corn oil, which was arbitrarily set to 1. Corresponding mean CT values are denoted above. Results shown as mean ± SEM. **p < 0.01, ***p < 0.001 assessed by ANOVA. (E) Gross appearance of representative livers of chow-fed doxycycline-treated Cnr1^{fl/fl} (top) and Hsc-Cnr1^{-/-} (bottom) mice injected with CCl_4 for 10 weeks. (F) H&E and trichrome staining of liver from mice described in (E). Scale bar = 200 µm. All experiments (A-F) were repeated with a separate cohort of mice and the results were similar.
Figure 6. Cnr1 deletion in HSCs does not improve diet-induced hepatic steatosis. Cnr1<sup>fl/fl</sup> and Hsc-Cnr1<sup>−/−</sup> mice were switched to a HFD supplemented with doxycycline for 12 weeks, starting at 6 weeks of age. Glucose and insulin tolerance tests were carried out 10 and 11 weeks after HFD feeding, respectively (6-7/group). (A) Representative H&E, trichrome and PSR staining of liver sections. Scale bar = 300 µm. (B) Mean area of collagen was obtained by calculating the PSR-stained red area in the image under split green channel, as described in Methods. (C) Liver TG contents of Cnr1<sup>fl/fl</sup> and Hsc-Cnr1<sup>−/−</sup> (n=5/group) mice after 12 weeks of HSD. (D) Blood glucose levels were measured in mice at indicated times after glucose administration. (E) Blood glucose levels were measured in mice at indicated times after insulin injection. Mice in (D and E) were euthanized at 18 weeks of age. (F) Total RNA was extracted from each mouse liver, and the relative mRNA levels of Col1A1, Lrat, αSMA and CB-1 were quantified by qPCR. β-actin was used as an invariant control. Values were expressed relative to HFD-fed doxycycline-treated
Cnr1<sup>fl/fl</sup> mice, which was arbitrarily set to 1. Corresponding mean CT values are denoted above. Results shown as mean ± SEM, assessed by ANOVA.
Figure 7. CB-1 mRNA expression in control and NASH human livers. Left panel: Representative H&E staining of livers from normal individuals (n=3) or patients with NASH (n=7). Middle panel: Representative ISH staining of PPIB housekeeping gene showing the detection of red punctate dots in hepatocytes of normal (n=3) and NASH livers (n=7). Right panel: Representative ISH revealing a negative CB-1 mRNA expression in hepatocytes of normal (n=3) and NASH livers (n=7). Scale bar = 50 µm.
### Supplementary Table 1. Human Liver Tissue Information

<table>
<thead>
<tr>
<th>Donors</th>
<th>Gender</th>
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### Supplementary Table 2. Histological Assessment and Scoring of *In-situ* Hybridization

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<tr>
<td>76-100%</td>
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<td>Many coalesced dots and not countable</td>
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*Staining distribution is determined by the approximate qualitative percentage of positive cells within this cell type population in the whole tissue section examined.*

*Staining intensity is determined by the number of dots per cell.*
### Supplementary Table 3. *In-situ* Hybridization of CNR1 mRNA Expression in Human Liver Tissues

<table>
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<th>Probes</th>
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<td>C2</td>
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<td>Mononuclear cells</td>
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* low positivity in large ducts.

# low abundance of CNR1 mRNA; all present at close proximity in liver parenchyma/sinusoids.