Original Article

MOLECULAR METABOLISM

Sympathetic overdrive and unrestrained adipose lipolysis drive alcohol-induced hepatic steatosis in rodents

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ABSTRACT

Objective: Hepatic steatosis is a key initiating event in the pathogenesis of alcohol-associated liver disease (ALD), the most detrimental organ damage resulting from alcohol use disorder. However, the mechanisms by which alcohol induces steatosis remain incompletely understood. We have previously found that alcohol binging impairs brain insulin action, resulting in increased adipose tissue lipolysis by unrestraining sympathetic nervous system (SNS) outflow. Here, we examined whether an impaired brain—SNS—adipose tissue axis drives hepatic steatosis through unrestrained adipose tissue lipolysis and increased lipid flux to the liver.

Methods: We examined the role of lipolysis, and the brain—SNS—adipose tissue axis and stress in alcohol induced hepatic triglyceride accumulation in a series of rodent models: pharmacological inhibition of the negative regulator of insulin signaling protein-tyrosine phosphatase 1β (PTP1b) in the rat brain, tyrosine hydroxylase (TH) knockout mice as a pharmacogenetic model of sympathectomy, adipocyte specific adipose triglyceride lipase (ATGL) knockout mice, wildtype (WT) mice treated with β3 adrenergic agonist or undergoing restraint stress.

Results: Intracerebral administration of a PTP1b inhibitor, inhibition of adipose tissue lipolysis and reduction of sympathetic outflow ameliorated alcohol induced steatosis. Conversely, induction of adipose tissue lipolysis through β3 adrenergic agonism or by restraint stress worsened alcohol induced steatosis.

Conclusions: Brain insulin resistance through upregulation of PTP1b, increased sympathetic activity, and unrestrained adipose tissue lipolysis are key drivers of alcoholic steatosis. Targeting these drivers of steatosis may provide effective therapeutic strategies to ameliorate ALD.

Keywords Sympathetic outflow; Adipose triglyceride lipase; Tyrosine hydroxylase; β3 adrenergic agonism; Insulin signaling

1. INTRODUCTION

Excessive alcohol consumption is the third leading cause of preventable deaths in the US accounting for health care costs of almost $250 billion a year [1]. Alcohol associated-liver disease (ALD) is one of the most detrimental consequences of alcohol abuse because it can progress to cirrhosis, liver failure and death [2]. The prevailing paradigm of early ALD pathogenesis proposes that alcohol induced steatosis is the "first hit" that increases vulnerability of the liver to a "second hit" leading to steatohepatitis [3]. While steatosis in the setting of chronic alcohol consumption has been thoroughly studied, the pathophysiological mechanisms accounting for the early hepatic steatosis induced by alcohol remain poorly understood. Alcohol binge drinking, defined in humans as sufficient alcohol consumption to increase blood alcohol concentration level to $\geq$0.08 g/dl [2], causes early steatosis in humans and rodents. Hence, experimental binge drinking models are commonly used and particularly valuable to study the early biological mechanisms leading to alcohol induced steatosis.

Here, we examined whether an impaired brain—SNS—adipose tissue axis is a key driver of hepatic steatosis through unrestrained adipose tissue lipolysis and increased lipid flux to the liver. Lipolysis occurs primarily in adipose tissue and is principally driven by increased sympathetic outflow, norepinephrine release and adrenergic signaling in adipocytes. Since alcohol binging results in increased sympathetic outflow [4] and unrestrained adipose tissue lipolysis [5], here we set out to examine whether sympathetically driven increased efflux of adipose tissue lipolysis-derived lipids to the liver is an important driver of alcohol-induced hepatic steatosis. An increase in adipose tissue lipolysis after alcohol consumption was first described in the 1960s [6] and was subsequently confirmed employing multiple approaches including tracer dilution techniques and metabolic flux analyses

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Abbreviations

ALD	alcohol-associated liver disease
DNL	de novo lipogenesis
ACC	acetyl-CoA carboxylase
ATPCL	ATP citrate lyase
VLDLs	very low-density lipoproteins
PTP1b	protein-tyrosine phosphatase 1β
WAT	white adipose tissue
CNS	central nervous system
aCSF	artificial cerebrospinal fluid
ICV	intracerebroventricularly
IP	intraperitoneally
ORO	oil red O
NEFA	nonesterified fatty acids
ATGL	adipose triglyceride lipase
WT	wildtype
iAAKO	inducible adipose tissue specific ATGL knockout
SNS	sympathetic nervous system
TH	tyrosine hydroxylase
NE	norepinephrine
HSL	hormone-sensitive lipase

[4,7,8]. However, the mechanisms through which lipolysis is increased by alcohol and their importance in steatosis remain poorly understood. In previous work, we have shown that binge alcohol induces adipose tissue insulin resistance and unrestrains lipolysis. Insulin inhibits lipolysis primarily by two mechanisms. First, insulin antagonizes β-adrenergic signaling in adipocytes [9] and, second, it dampens sympathetic outflow to adipose tissue through signaling in the brain [10,11]. Similar to obesity and type 2 diabetes, alcohol rapidly induces brain insulin resistance by increasing the expression of the negative insulin signaling regulator PTP1b, resulting in hepatic and adipose tissue insulin resistance, increased sympathetic outflow and unrestrained lipolysis in adipose tissue [5]. Inhibition of PTP1b enhances brain insulin signaling in several conditions associated with brain insulin resistance. As alcohol leads to brain insulin resistance and brain insulin suppresses adipose tissue lipolysis, we hypothesized that enhancing brain insulin signaling via inhibition of PTP1b would restrain adipose tissue lipolysis, reduce lipolytic flux to the liver and prevent alcoholic steatosis.

Adipose tissue dysfunction is a hallmark of metabolic disease characterized by dysregulated lipolysis, reduced de novo lipogenesis (DNL) [12], and low-grade inflammation [13]. Of note, alcohol induced inflammation also occurs in the hypothalamus [5,14], although the mechanisms remain unclear. As the increased efflux of nonesterified fatty acids (NEFA) released through adipose lipolysis can be pro-inflammatory [15], we hypothesized that unrestrained lipolysis may account for the alcohol induced inflammation in adipose tissue and the hypothalamus.

The goal of this study was to test the hypothesis that alcohol binging promotes hepatic steatosis by unrestraining sympathetic outflow and lipolysis in part due to impaired insulin action and in turn increasing lipid efflux from adipose tissue to the liver. If this model is correct, at least three interventions should prevent or ameliorate alcohol binge-induced fatty liver and inflammation in adipose tissue and the hypothalamus, namely enhancing brain insulin signaling via PTP1b inhibition, dampening sympathetic outflow, and suppressing adipose tissue lipolysis.

2. RESULTS

2.1. Intracerebral inhibition of PTP1b ameliorates alcohol binge-induced hepatic steatosis in rats

We have previously demonstrated that alcohol binging impairs brain insulin action and its ability to regulate liver and adipose tissue metabolism by inducing the negative regulator of insulin signaling PTP1b in rats [5]. Intracerebroventricular (ICV) inhibition of PTP1b prevented alcohol binge-induced glucose intolerance [5]. To examine the role of impaired brain insulin signaling in alcohol-induced hepatic steatosis, we tested whether ICV inhibition of PTP1b ameliorates alcohol binge-induced hepatic steatosis in rats. To this end, we placed osmotic mini-pumps to continuously infuse the small molecule PTP1b inhibitor CPT-157633 (CPT, 0.2 mg/day) or artificial cerebrospinal fluid (aCSF) ICV two days prior to a three-day alcohol binge challenge (Figure 1A). Alcohol was administered intraperitoneally (IP, 3 g/kg) daily for 3 days and rats were sacrificed 24 h after the last binge when blood alcohol was undetectable in circulation [5]. While alcohol administration by oral gavage is a more physiological route of administration, in this rat study we resorted to IP administration of alcohol to avoid dislodging the ICV cannulas while performing gavage. IP and oral alcohol administration have been found to produce comparable results for a number of biological readouts in rodents [5]. Binged rats exhibited a two-fold increase in hepatic triglyceride content compared to control rats that received an isocaloric glucose solution (Figure 1B). ICV infusion of CPT protected rats from alcohol binge induced hepatic steatosis as determined by measuring hepatic triglyceride content after Folch extraction (Figure 1B) and oil red O [16] staining (Figure 1C). Of note, we previously found that intracerebral administration of CPT lowered insulin levels 8–10 h after the last alcohol binge, likely as a result of improved insulin sensitivity, but the change was transient and did not persist at later time points [5]. These findings suggest that impaired central insulin signaling contributes to alcohol binge induced hepatic steatosis.

Brain insulin action is an important determinant of adipose tissue insulin action [11]. Alcohol induces brain insulin resistance and unrestrains adipose tissue lipolysis [10,11]. Since fatty acids are believed to stimulate proinflammatory signaling in adipose tissue [15,17], we next tested whether central inhibition of PTP1b attenuates alcohol induced WAT inflammation by inhibiting lipolysis and reducing plasma NEFA. ICV CPT reduced the alcohol induced increase in mRNA levels of the proinflammatory cytokines MCP-1, TNF-α, and IL1β in epigonadal WAT (Figure 1D). Plasma NEFA levels were suppressed in the CPT treated alcohol binged rats (Figure 1E), suggesting that central inhibition of PTP1b restores brain insulin action, restrains lipolysis and ameliorates alcohol induced WAT inflammation, likely through restoration of brain insulin resistance. A limitation of the studies shown in Figure 1C–E is however that the N of the glucose groups was small, so we were underpowered to detect small to moderate differences in NEFAs between the glucose control versus alcohol groups.

Increased hepatic DNL is believed to be a major contributing pathway to hepatic steatosis in models of chronic alcohol feeding [18]. To assess whether hepatic DNL also contributes to the early alcoholic steatosis, we analyzed liver mRNA expression of the transcription factor ChREBP involved in lipogenic gene transcription and of the key lipogenic enzymes fatty acid synthase (FAS). We also assessed protein expression or activation of FAS, ATP-citrate lyase (ATPCL) and acetyl-CoA carboxylase (ACC) in rats subjected to binge drinking. The mRNA levels of FAS or ChREBP and protein expression or activation ATPCL were not increased but surprisingly reduced after alcohol treatment,

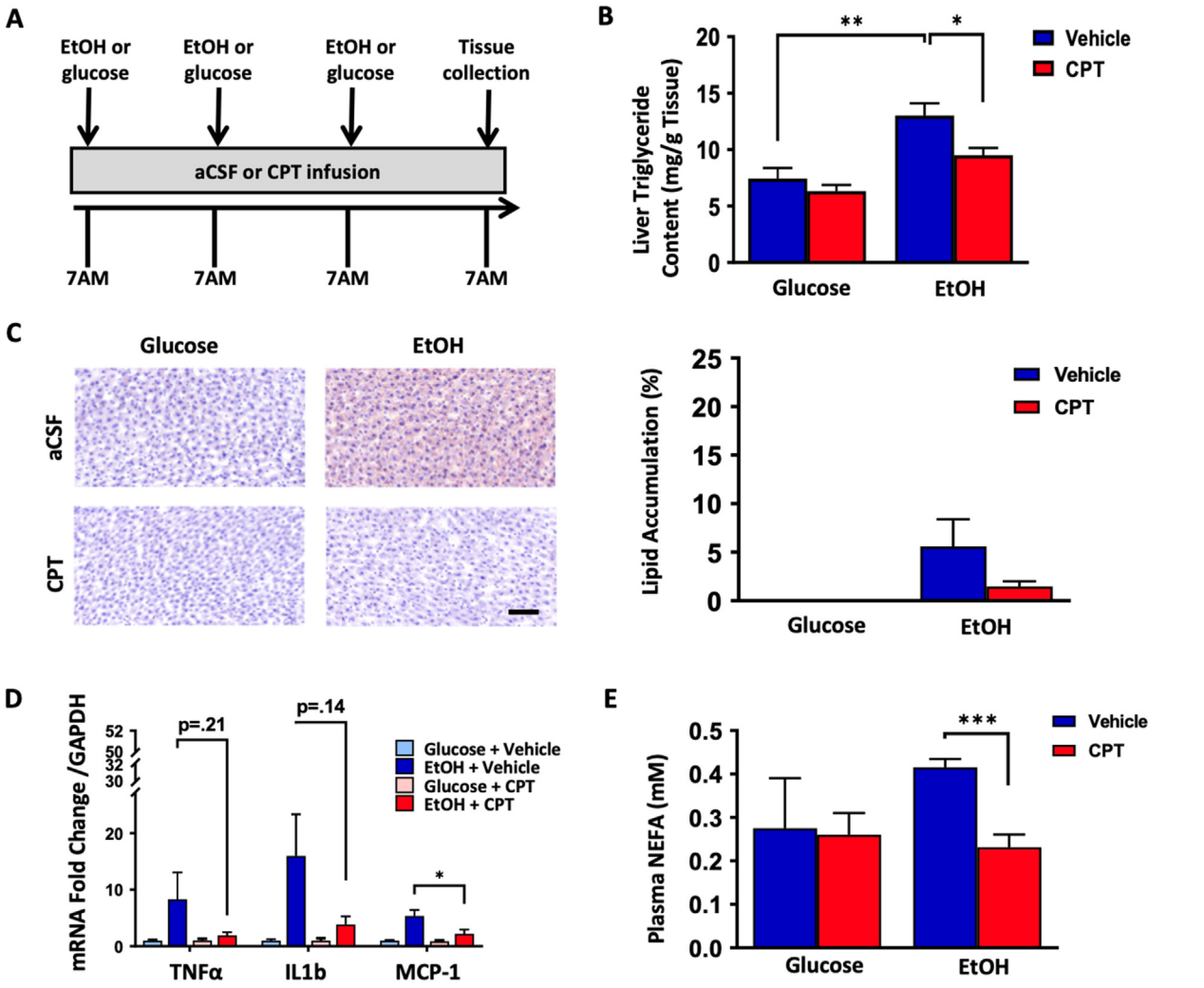

Figure 1: Binge drinking induced hepatic steatosis is prevented by central inhibition of PTP1b in rats. A. Experimental protocol; alcohol (EtOH; 3 g/kg) or isocaloric glucose administered IP. **B.** Triglyceride content of livers as determined after Folch extraction (n = Glucose Veh 5, Glucose CPT 6, EtOH Veh 10, EtOH CPT 9). **C.** ORO staining of hepatic lipids. Left: representative images of lipid droplets in hepatocytes that are stained red with ORO; Right: quantification of percentage of hepatocytes that stained positive for ORO in liver samples (n = Glucose Veh 2, Glucose CPT 2, EtOH Veh 7, EtOH CPT 6); Scale bar = 100 μm. **D.** Pro-inflammatory cytokine expression in WAT was ameliorated by inhibiting central PTP1b as assessed by RT-qPCR (n = Glucose Veh 2, Glucose CPT 2, EtOH Veh 5, EtOH CPT 6). **E.** Plasma NEFA levels after an alcohol binge were reduced by inhibiting central PTP1b (n = Glucose Veh 2, Glucose CPT 2, EtOH Veh 6, EtOH CPT 7). *P < 0.05, **P < 0.01, and ***P < 0.001 according to Tukey post-hoc tests following two-way ANOVA.

except for ACC where protein levels were unchanged and activity was moderately increased as indicated by reduced inhibitory phosphorylation (Figure S1A and B). Central inhibition of PTP1b did not result in a decrease of expression or activation of these enzymes (Figure S1A and B), suggesting that prevention of hepatic steatosis upon PTP1b inhibition in the brain is unlikely due to reduced hepatic DNL, but primarily explained by an inhibition of lipolysis.

2.2. Inhibition of adipose tissue lipolysis prevents alcohol binge induced hepatic steatosis in mice

To directly test the role of adipose tissue lipolysis as a driver of alcohol induced hepatic steatosis and adipose tissue inflammation, we studied mice devoid of the lipolysis initiating enzyme adipose triglyceride lipase (ATGL) in adipocytes (AAKO). The deletion of ATGL in WAT was confirmed by Western Blot (Figure S2A) and functionally validated by assessing cold tolerance, which was markedly impaired in the AAKO (Figure S2B), as previously reported [19]. AAKO and WT littermates received three doses of ethanol (5 g/kg) or an isocaloric glucose solution via gavage every 12 h to model binge drinking (Figure 2A). In this study and in all subsequent studies in mice, animals received alcohol by oral gavage. Ethanol markedly reduced body weight in WT mice, while the weight loss was partly prevented in AAKO mice (Figure 2B, S2E–F), suggesting that increased adipose tissue lipolysis contributes to ethanol-induced weight loss. Circulating triglyceride levels nearly doubled after alcohol binging in WT mice, while triglycerides were not significantly different after alcohol in AAKO mice (Figure 2C), suggesting that unrestrained adipose tissue lipolysis contributes to the alcohol binge induced hypertriglyceridemia. We did not detect a significant difference in insulin levels in binged AAKO mice compared to WT controls (Figure S2D).

AAKO mice were completely protected from hepatic triglyceride accumulation after binging as assessed biochemically and through ORO staining (Figure 2D and E). AAKO mice exhibited lower plasma NEFA after alcohol binging compared to WT counterparts (Figure 2F),

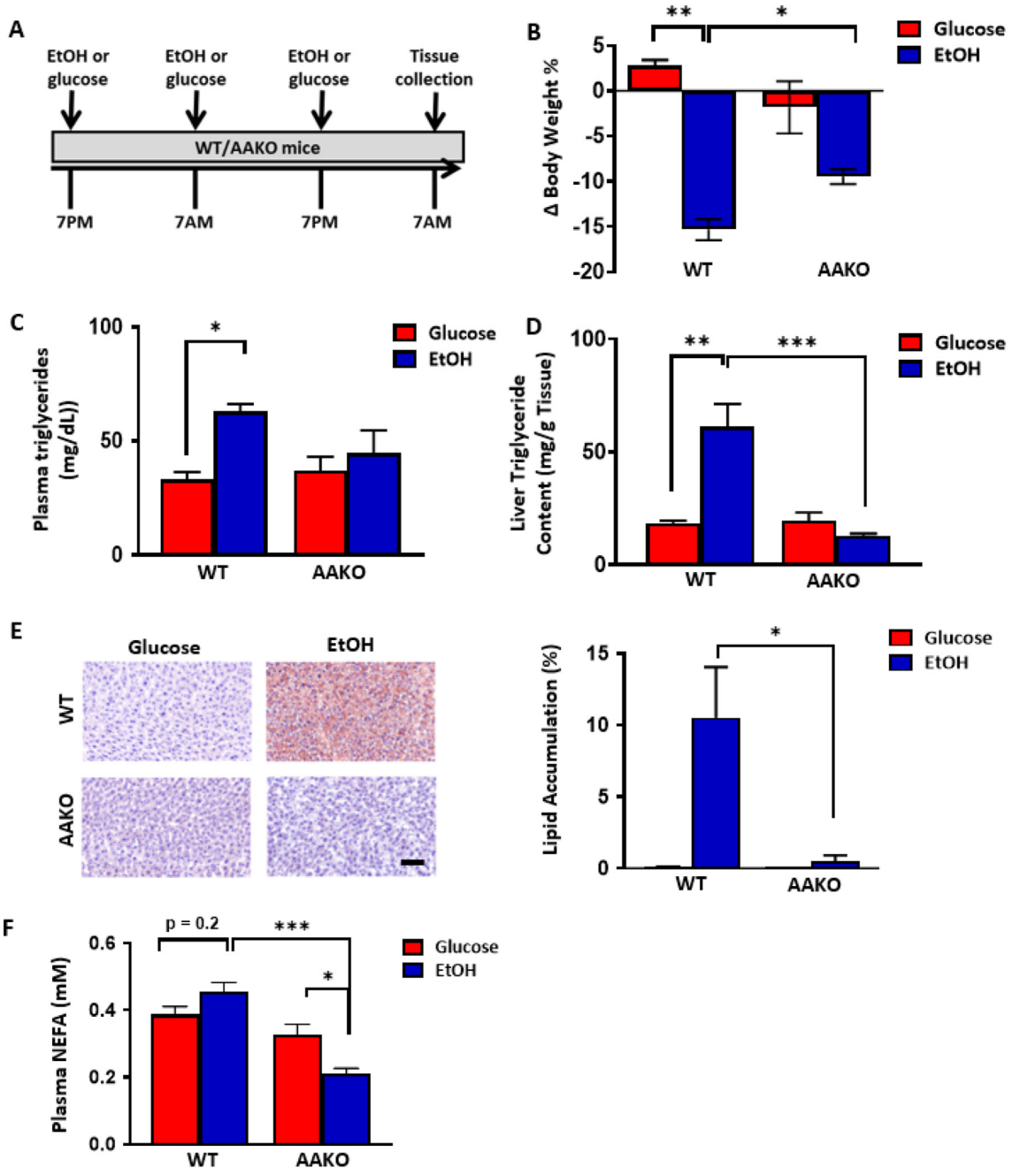

Figure 2: Reducing adipose tissue lipolysis prevents alcohol binging-induced hepatic steatosis in mice. A. Experimental scheme alcohol (EtOH 5 g/kg) or isocaloric glucose administered by oral gavage. **B.** Changes in body mass after EtOH treatment (n = Glucose WT 3, EtOH WT 6, Glucose AAKO 3, EtOH AAKO 5). **C.** Alcohol binging induced hypertriglyceridemia is prevented in the AAKO mice. **D.** Alcohol binging induced hepatic steatosis was prevented in AAKO mice (n = Glucose WT 9, EtOH WT 9, Glucose AAKO 10, EtOH AAKO 8). **E.** ORO staining of lipid in the livers. Left: representative images of lipid droplets in hepatocytes that are stained red with ORO; Right: Quantification of percentage of hepatocytes that stained positive with ORO (n = Glucose WT 6, EtOH WT 10, Glucose AAKO 5, EtOH AAKO 8); Scale bar = 100 μm. **F.** Plasma fatty acid levels were markedly lower in AAKO mice after alcohol binging compared to those in WT mice (n = Glucose WT 6, EtOH WT 9, Glucose AAKO 5, EtOH AAKO 9). *P < 0.05, **P < 0.01, and ***P < 0.001 according to Tukey post hoc tests following two-way ANOVA (B, C, D, E, F and H).

consistent with the suppression of alcohol induced lipolysis by genetic deletion of ATGL. Unexpectedly, we did not observe an increase in NEFA in the WT ethanol group compared to the glucose control. Of note, unchanged plasma NEFA levels do not rule out an increase in lipolysis as plasma levels are not just a reflection of NEFA release but also of uptake or utilization in various tissues, which may also be altered by alcohol binging. We confirmed the prevention of alcohol induced hepatic steatosis in inducible adipose tissue specific ATGL knockout (iAAKO) mice (Figure S3D), thereby establishing that developmental effects do not account for protection against steatosis observed in the AAKO mice. ATGL deletion in WAT of the iAAKO was validated as described above for the conditional AAKO (Figure S3B and C). In line with the genetic loss of function studies, pharmacological inhibition of lipolysis by administration of acipimox (IP 10 mg/kg), a

niacin derivative and nicotinic acid analog, reduces cAMP production and inhibits lipolytic enzymes [20,21], trended to reduce hepatic triglyceride content (Figure S3E). In aggregate, these studies demonstrate that adipose tissue lipolysis is a key driver of the early alcoholic hepatic steatosis. Moreover, the expression of the proinflammatory TNFα and MCP-1 trended to be lower in WAT of AAKO mice (Figure S2C), suggesting that adipose tissue lipolysis contributes to the alcohol induced adipose tissue inflammation and dysfunction.

2.3. Increased SNS outflow drives alcohol binge induced hepatic steatosis in mice

Adipose tissue lipolysis is principally initiated and maintained by the sympathetic nervous system (SNS) through activation of adrenergic signaling by norepinephrine [22]. Alcohol induced fat mass loss and hepatic triglyceride accumulation are associated with increased circulating norepinephrine [4], suggesting that increased sympathetic nervous system activity after alcohol ingestion takes part in the development of alcoholic hepatic steatosis. To probe the contribution of the sympathetic nervous system to alcohol-induced hepatic steatosis we studied a mouse model in which tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine synthesis, is inducibly ablated in the periphery but not in the CNS (THKO), resulting in markedly lower norepinephrine (NE) levels in peripheral tissues as shown previously [23]. THKO mice and littermate controls were subjected to alcohol binge (4 g/kg). A lower alcohol dose was used in this study due to increased mortality in this cohort of mice after alcohol binges. The THKO mice were protected from alcohol-induced elevation in hepatic triglycerides (Figure 3A), demonstrating that reducing production and release of catecholamines from the SNS can prevent alcohol induced hepatic steatosis.

The protection of THKO mice from alcohol induced steatosis suggests that a stress induced increase of SNS outflow and adrenergic signaling in WAT is an important driver of hepatic steatosis. To probe a direct role of adrenergic signaling in adipose tissue in fatty liver, we next examined whether β3 adrenergic stimulation, which activates adrenergic signaling predominantly in adipocytes [24], is sufficient to cause hepatic steatosis in the absence of an alcohol binge. Indeed, IP injection of the β3-selective adrenergic agonist CL-316,243 (CL, 1 mg/kg) increased hepatic triglyceride accumulation independent of alcohol binging (Figure S4A), demonstrating that increased lipolysis *per se* can induce hepatic steatosis. Figure S4B, Figure S4C

We next tested whether increased adrenergic signaling in adipocytes via β3 adrenergic stimulation and alcohol binge drinking act synergistically to induce hepatic steatosis. To this end, we administered a low dose of CL (0.01 mg/kg) and/or a subclinical dose of alcohol (3 g/kg), i.e. which alone does not induce hepatic steatosis (Figure 3B Glucose vs. EtOH of PBS groups). While these treatments individually did not induce steatosis, when combined they resulted in a two-fold

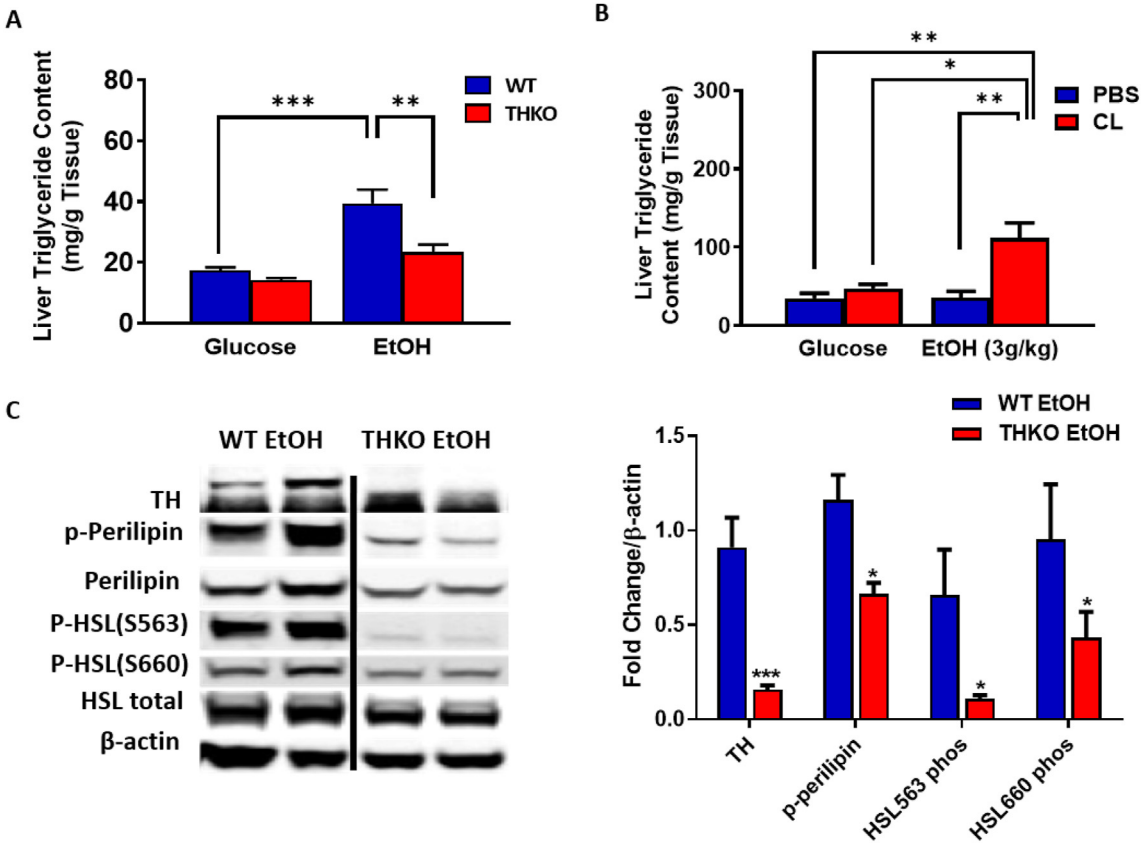

Figure 3: Reduction in catecholamine synthesis and SNS induced norepinephrine release prevents binge drinking induced hepatic steatosis in mice. A. Alcohol (4 g/kg) binging induced liver triglyceride content increase was prevented in THKO mice (n = Glucose WT 10, Glucose THKO 9, EtOH WT 9, EtOH THKO 8). **B.** β3 adrenergic agonism (CL, 0.01 mg/kg) promotes hepatic steatosis in mice treated with a subclinical alcohol dose (3 g/kg) that does not increase liver triglycerides by itself (n = Glucose PBS 6, Glucose CL 6, EtOH PBS 8, EtOH CL 10). **C.** Deletion of TH protein in THKO mice. HSL and perilipin phosphorylation were markedly reduced as determined by Western blotting. Left: representative immunoblots. Right: Quantification normalized to beta actin (n = EtOH WT 6, EtOH THKO 7). Dash line indicates cutting from the same blot. *P < 0.05, **P < 0.01, and ***P < 0.001 according to two-way ANOVA followed by Tukey post-hoc tests (A, B) or Student's t-test (C).

increase in liver triglyceride content (Figure 3B), demonstrating that stress induced lipolysis driven by sympathetic activation can synergize with alcohol to promote alcoholic hepatic steatosis. No change in body mass (Figure S4B) or plasma triglycerides (Figure S4C) was observed in CL-treated binged animals compared to binged animals that did not receive CL. These findings suggest that the association between stress and alcoholic hepatic steatosis, which is often ascribed to increased alcohol consumption under stress, may also have a pathophysiological basis independent of the amount of alcohol consumed.

Consistent with a role of sympathetic activation in the alcohol induced weight loss, the decrease in body mass after alcohol binging trended to be reduced in the THKO mice (Figure S5A). Fat mass loss was prevented in the THKO (Figure S5B), while lean mass was not altered (Figure S5C), suggesting that SNS outflow is an important contributor to the fat mass loss after an alcohol binge. Consistent with this notion, we found that levels of phosphorylated perilipin and phosphorylated HSL were lower in adipose tissue of THKO mice (Figure 3C, and Figure S6) supporting the notion that a reduction in sympathetic outflow prevents fat mass loss through a reduction in lipolysis. We detected no significant difference in insulin levels in binged THKO mice compared to WT controls (Figure S5H).

2.4. Acute stress and alcohol synergize to induce hepatic steatosis in mice

Susceptibility to alcohol induced fatty liver is higher in the setting of stress [25,26]. However, whether stress plays a direct role in liver injury is unclear because stressed individuals tend to drink more alcohol [27]. As stress activates the SNS, we examined whether stress enhances alcohol-induced hepatic steatosis. Mice were placed in a restraining tube for one hour before each administration of a subclinical dose of alcohol (3 g/kg), which by itself does not cause hepatic steatosis, while control groups were left in their home cages (Figure 4A). Hepatic triglyceride content in alcohol binged mice that were stressed increased two-fold compared to alcohol binged mice that were not subjected to restraint stress (Figure 4B). Unexpectedly, the synergism of stress and alcohol resulted in lower HSL activation (Figure 4C), which we speculate may be due to catecholamine resistance secondary to sympathetic overstimulation as it is seen in obesity [28]. In addition, stress and alcohol synergized to reduce the lipogenic capacity of WAT (Figure 4D), consistent with increased sympathetic activity as shown above. The loss of fat mass (Figure S7A) and the increase of HSL activation in the stress only group (Figure 4C) indicates that stress itself is a potent adrenergic stimulus of lipolysis in WAT.

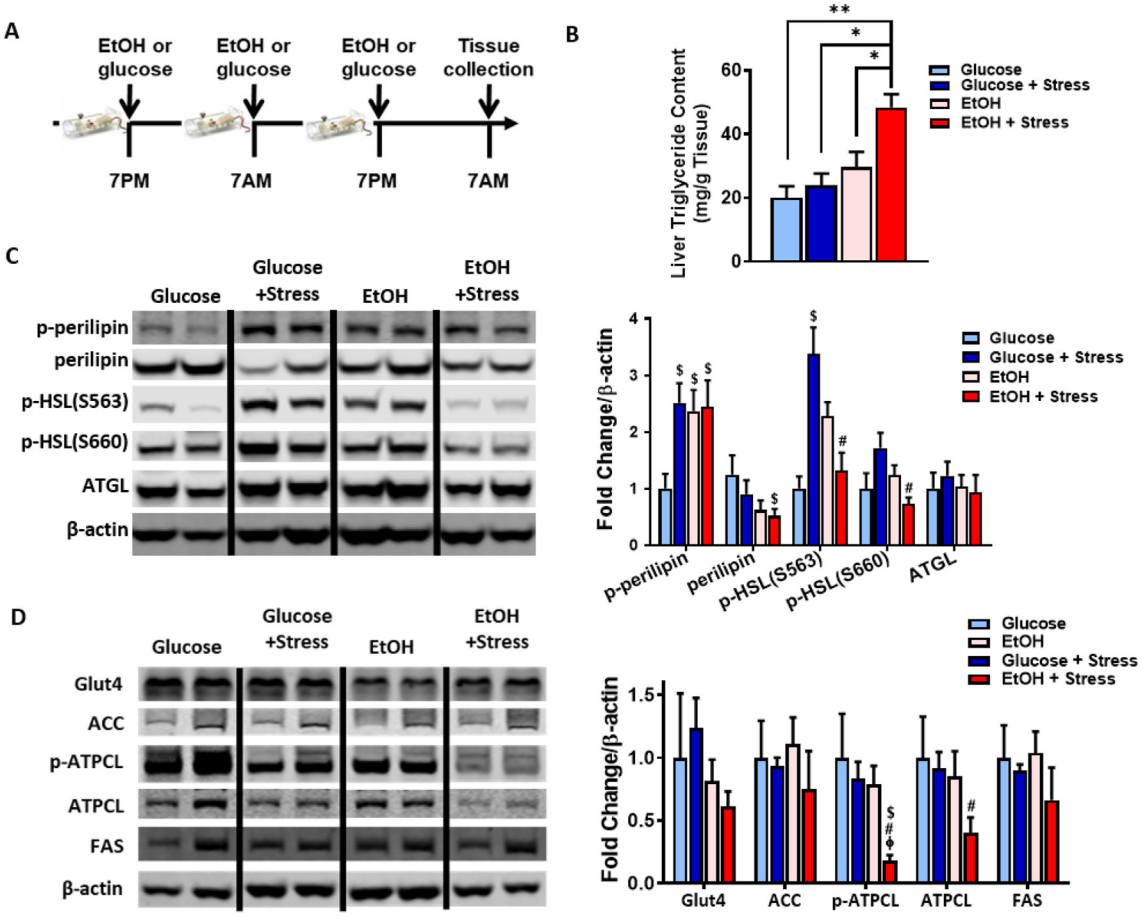

Figure 4: Stress exacerbates alcohol binging induced hepatic steatosis in mice. A. Experimental protocol. **B.** Restraint stress synergizes with a subclinical alcohol dose (3 g/kg) on liver triglyceride content. **C.** Co-exposure of mice to stress and alcohol paradoxically resulted in lower HSL activation, possibly due to catecholamine resistance due to chronic sympathetic over stimulation. Left: representative immunoblots. Right: quantification normalized to beta actin (n = Glucose 5, Glucose + Stress 6, EtOH 7, EtOH + Stress 7). **D.** Stress and alcohol synergize to reduce lipogenic capacity in WAT. Left: representative immunoblots. Left: representative immunoblots. Right: quantification normalized to beta actin Right: quantification normalized to beta actin (n = Glucose 5, Glucose + Stress 6, EtOH 7, EtOH + Stress 7). *P < 0.05, **P < 0.01, and ***P < 0.001 according to two-way ANOVA followed by Tukey post-hoc tests. $, # and Φ mean P < 0.05 vs Glucose, EtOH, and Glucose + Stress groups correspondingly.

These findings suggest that stress does synergize with alcohol in worsening hepatic steatosis.

2.5. Ablation of peripheral catecholamine synthesis prevents alcohol binge-induced reduction of WAT DNL and stress signaling in mice

A key characteristic of healthy adipose tissue is a strong lipogenic capacity and high protein expression of de novo lipogenic enzymes [29], while in metabolic disease adipose tissue DNL is often markedly suppressed [30,31]. Hence, we speculated that alcohol binging impairs adipose tissue function by reducing lipogenic capacity in adipose tissue due to unrestrained sympathetic outflow. If this notion is correct, peripheral TH deletion should prevent alcohol-induced decrease in lipogenic capacity. Indeed, alcohol binging decreased levels of the de novo lipogenic proteins ACC, ATPCL, and FAS, and lowered levels of activated ATPCL in WT mice while THKO mice were protected from this decrease in DNL enzymes (Figure 5A). These findings suggest that the alcohol induced suppression of WAT lipogenic capacity is a consequence of increased SNS outflow.

Adipose tissue dysfunction in metabolic disease is further characterized by increased activity of inflammatory and stress signaling mediators such as STAT3, NF-κB, and p38 MAPK (mitogen activated protein kinase) [32–34] in adipose tissue. Hence, we examined whether alcohol binging induces stress signaling pathways in WAT and whether induction of these pathways is attenuated by the inhibition of the SNS. In line with the increased MCP-1 expression in WAT after alcohol treatment (Figure S6A), we found that alcohol binging (3 g/kg) significantly increased levels of phosphorylated NF-κB p65, p38 and STAT3 in WAT (Figure 5B), indicating that cellular stress and inflammation are features of alcohol induced adipose tissue dysfunction. Further, THKO mice were protected from alcohol binging induced cellular stress signaling as demonstrated by unaltered phosphorylation levels of NF-κB p65, p38 and STAT3 in WAT, indicating that increased SNS activity accounts for the alcohol induced stress signaling in WAT.

2.6. WAT lipolysis drives alcohol binging induced hypothalamic inflammation in mice

Alcohol, similar to obesity, induces neuroinflammation [35], which is commonly attributed to direct toxicity of alcohol in the brain. Alcohol binging markedly increased proinflammatory signaling molecules such as MCP-1, IL-1b and TNFα in the hypothalamus (Figure 6A). Since excess free fatty acids can be proinflammatory, we hypothesized that an alternative mechanism through which alcohol causes neuroinflammation is an overactive brain—SNS—adipose tissue axis, where unrestrained sympathetic outflow induces increased lipolytic flux that drives hypothalamic inflammation. To test whether increased SNS and lipolysis contribute to alcohol induced hypothalamic inflammation, we examined whether the reduction of adrenergic signaling in THKO mice or a reduction in lipolysis in AAKO mice prevented the alcohol induced hypothalamic inflammation. Indeed, THKO mice were protected from alcohol induced elevation in mRNA levels of hypothalamic MCP1, TNFα and IL1β (Figure 6A), and AAKO mice showed a similar trend, although this did not reach statistical significance (Figure 6B). Conversely, inducing WAT lipolysis through β3 adrenergic agonism or restraint stress resulted in hypothalamic inflammation when mice were subjected to a subclinical dose (3 g/kg) of alcohol (Figure 6C and D). These findings suggest that alcohol induced neuroinflammation is at least in part driven by unrestrained SNS activation and adipose tissue lipolysis.

3. DISCUSSION

Here we examined the role of a brain—SNS—adipose tissue axis in early alcohol induced hepatic steatosis, adipose tissue dysfunction and hypothalamic inflammation (Figure 7). Key findings from our studies are: 1) central inhibition of PTP1b ameliorates alcohol induced steatosis and WAT inflammation, likely via restoration of brain insulin signaling; 2) inhibition of adipose tissue lipolysis or catecholamine production prevents alcohol induced hepatic steatosis as well as

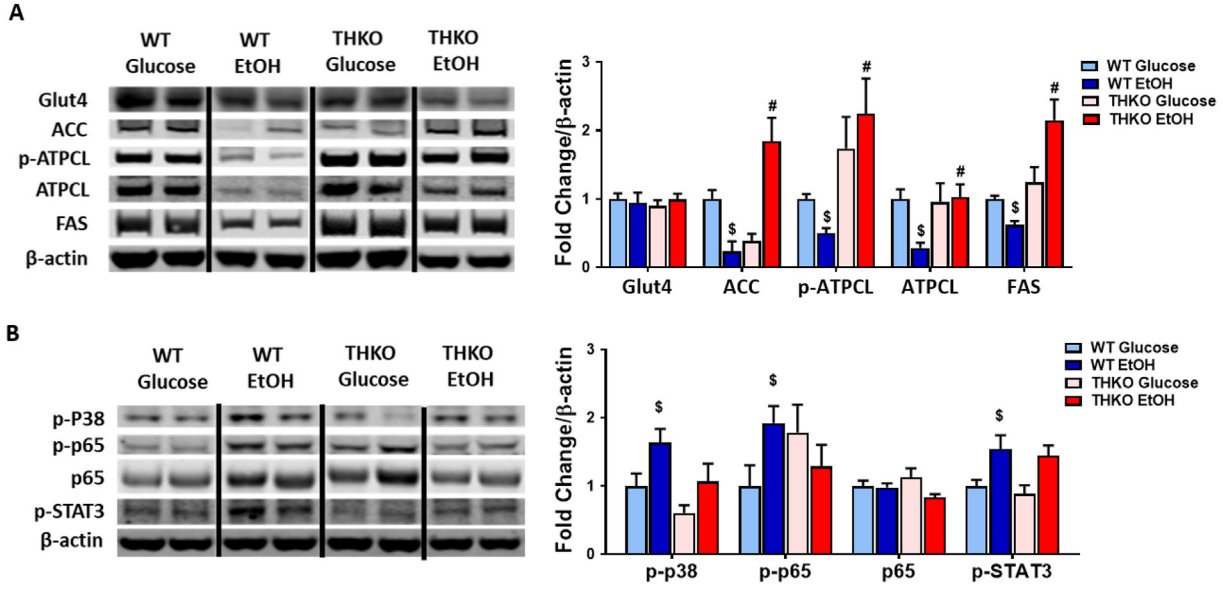

Figure 5: Alcohol binge-induced suppression of lipogenic capacity and stress signaling in adipose tissue are mediated through SNS in mice. A. Alcohol (4 g/kg) binging reduced lipogenic capacity of adipose tissue, which was prevented in THKO mice. Left: representative immunoblots. Right: quantification normalized to beta actin (n = WT Glucose 5, WT EtOH 6, THKO Glucose 5, THKO EtOH 7). **B.** Alcohol binging-induced stress and inflammatory signaling in WAT is ameliorated in the THKO. Left: representative immunoblots. Right: quantification normalized to beta actin mice (n = WT Glucose 5, WT EtOH 6, THKO Glucose 5, THKO EtOH 7). $ and # mean $P < 0.05$ vs WT Glucose and WT EtOH groups correspondingly according to two-way ANOVA followed by Tukey post-hoc tests.

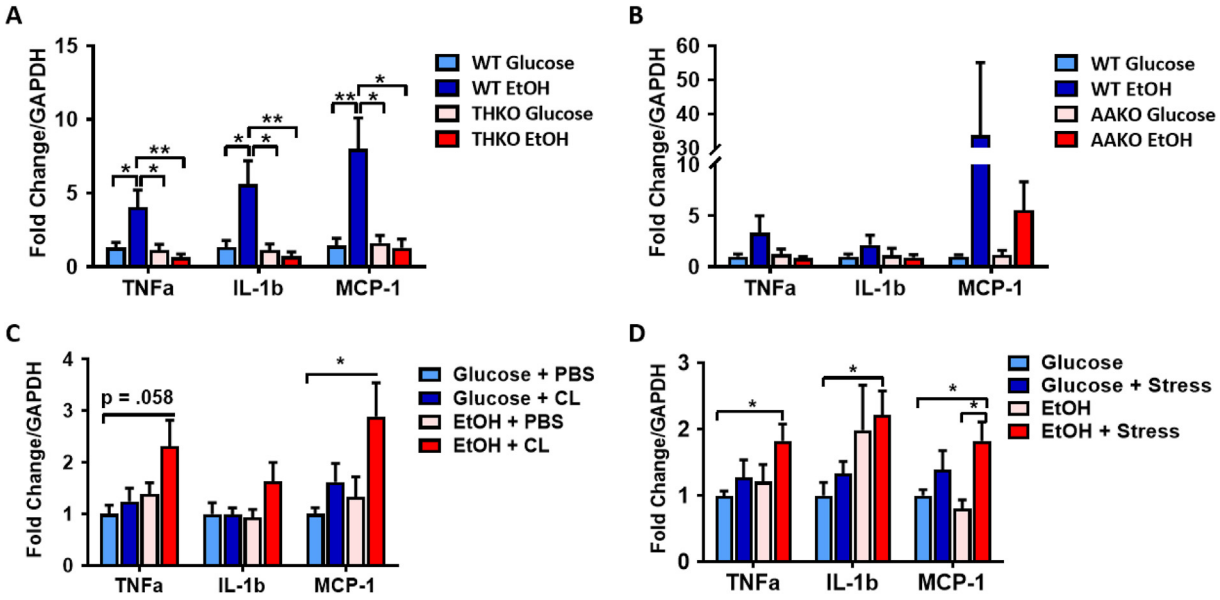

Figure 6: Alcohol binge-induced lipolysis drives hypothalamic inflammation in mice. Alcohol binging induced mRNA levels of pro-inflammatory cytokine expression in the hypothalamus in: **A.** THKO mice (n = WT glucose 10, WT EtOH 9, THKO glucose 10, THKO EtOH 10); **B.** AAKO mice (n = WT glucose 6, WT EtOH 10, AAKO glucose 5, AAKO EtOH 9); **C.** mice that were IP injected with β3 agonist (0.01 mg/kg CL-316,243) or vehicle (PBS) (n = Glucose PBS, 6 Glucose CL 6, EtOH PBS 8, EtOH CL 9) **D.** mice that were subjected to restrained stress in combination with a subclinical dose of ethanol (3 g/kg) (n = Glucose 6, Glucose + Stress 7, EtOH 7, EtOH + Stress 9). *P < 0.05, and **P < 0.01 according to two-way ANOVA followed by Tukey post-hoc tests.

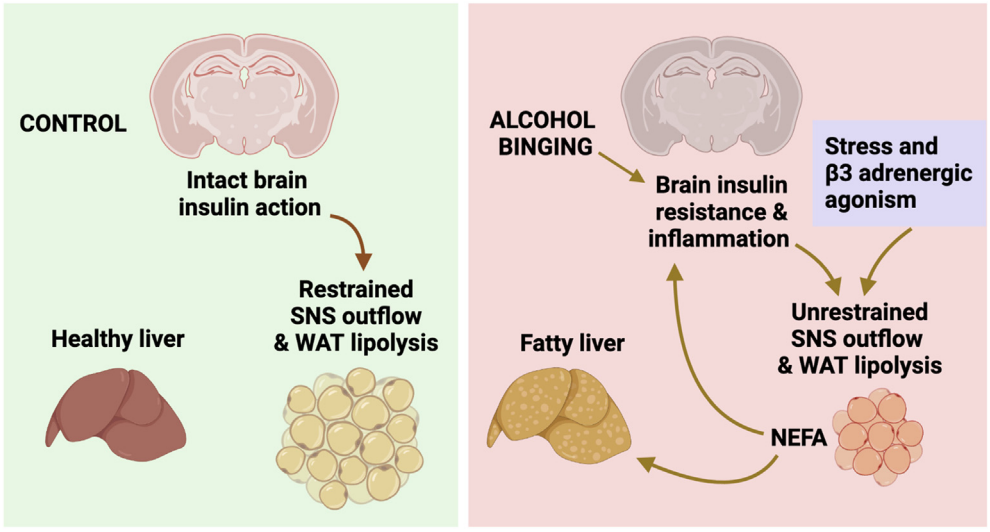

Figure 7: Model of how a dysregulated brain-adipose axis may contribute to alcohol induced hepatic steatosis. Alcohol binging impairs central insulin signaling which results in unrestrained sympathetic outflow to adipose tissue and induction or unrestraining of lipolysis. Nonesterified fatty acids (NEFA) derived from WAT lipolysis drive hepatic steatosis, adipose tissue dysfunction and hypothalamic inflammation. Stress or β3 adrenergic agonism synergize with alcohol to drive hepatic steatosis through increased adipose tissue lipolysis. Alcohol induced steatosis, adipose tissue dysfunction and hypothalamic inflammation can be ameliorated by enhancing brain insulin signaling, dampening peripheral catecholamine synthesis, or by reducing WAT lipolysis.

inflammation in WAT and hypothalamus; and 3) activation of the SNS via β3 adrenergic agonism or restraint stress synergized with alcohol in inducing hepatic steatosis. While lipolysis has been shown to be increased in models of ALD [4,7,8], this study establishes adipose tissue lipolysis as a key driver of early alcoholic hepatic steatosis. Further, our studies identify brain insulin resistance and unrestrained SNS outflow as important culprits of alcohol induced adipose tissue dysfunction and hepatic steatosis.

Alcohol impairs central insulin signaling through the induction of PTP1b which results in whole-body insulin resistance at least in part by unrestraining SNS outflow and WAT lipolysis [5,10]. Our study demonstrated that central inhibition of PTP1b markedly reduced the alcohol induced hepatic steatosis, likely due to enhanced brain insulin signaling resulting in restored inhibition of SNS outflow and adipose tissue lipolysis. While we cannot exclude a contribution of other signaling pathways to participate in the hypothalamic control of hepatic

lipid handling that alcohol may disrupt, the fact that PTP1b inhibition restored insulin sensitivity in alcohol binged rats [5] suggests that insulin signaling in the brain is playing a pivotal role. Besides enhancing insulin signaling, PTP1b inhibition can have anti-inflammatory effects in the brain. However, we have shown previously that ICV administration of the anti-inflammatory IKKβ inhibitor PS1145 was insufficient to restore brain insulin action in the setting of alcohol binging [5]. This suggests that the effect of PTP1b inhibition on lipolysis and steatosis in this study is primarily driven by restoration of brain insulin signaling and consequent modulation of SNS outflow and adipose tissue lipolysis rather than due to an anti-inflammatory effect. The observation that AAKO mice are completely protected from alcohol binge-induced hepatic steatosis provides direct evidence that adipose tissue lipolysis is an important driver of alcohol induced fatty liver and suggests that pharmacological targeting of adipose tissue lipolysis holds promise for the treatment of alcoholic steatosis and possibly further progression to more advanced manifestations of ALD. Of note, lipolysis inhibitors that target ATGL should be targeted to WAT and bypass the liver, as inhibition of hepatic ATGL slows fatty acid oxidation in the liver and may negate the benefits of lipolysis inhibition. Hence, lipolytic inhibitors that selectively target WAT lipolysis such as niacin derivatives may be more promising pharmacological approaches to ameliorate alcohol induced hepatic steatosis. The finding that AAKO mice are also protected from alcohol induced adipose tissue inflammation and fat mass loss supports the notion that elevation of adipose tissue lipolysis accounts in part for the weight loss observed in alcohol abuse [36]. Obesity is similarly characterized by adipose tissue dysfunction and alcohol-induced liver injury is worse in obese rats or human subjects with higher BMI [37,38] suggesting that preexisting adipose tissue dysfunction increases the risk to alcoholic steatosis. This is consistent with our finding that mice with higher BMI lost more fat mass after alcohol binges (Figure S8). Our studies corroborates the work by another group that examined the role of lipolysis in different models of alcohol feeding in mice, including chronic alcohol feeding [39], suggesting that lipolysis may continue to be an important driver of hepatic steatosis in more advanced stages of ALD.

Multiple mechanisms have been proposed to contribute to hepatic steatosis after alcohol binging including increased hepatic DNL [40], reduced hepatic β-oxidation [41], insufficient hepatic lipid secretion [42], and increased lipid delivery to the liver [7]. Support for the DNL hypothesis stems from observations that mRNA levels of lipogenic enzymes such as FAS [5], ACC and ATPCL and SREBP1c are upregulated in the liver of alcohol fed rodents [40,43]. However, an increase in DNL is not consistently observed and mRNA levels of DNL enzymes may not reflect the protein expression and/or lipogenic rates. Further, in studies where protein levels of FAS and SREBP1c were found to be moderately increased after alcohol exposure, an isocaloric control group was not included [43,44]. It is therefore unclear whether the increase in hepatic lipogenic proteins observed in those studies was due to a specific effect of alcohol intoxication or simply to the caloric load of an alcohol binge. Indeed, in this study, we find that after alcohol binging protein levels of DNL enzymes are rather decreased in the liver (Figure S1A), suggesting that an increase in hepatic DNL is unlikely to be an important mechanism underlying the early alcohol induced steatosis.

β-Oxidation is under the transcriptional control of PPARα, whose expression in the liver is decreased after short term alcohol feeding in rodents. However, PPAR-α null mice do not exhibit worsened alcohol induced steatosis [45], challenging the notion that inhibition of hepatic β-oxidation is a primary driver of steatosis. Reduced lipid mobilization from the liver, principally through the secretion of very low-density lipoproteins (VLDLs) may lead to lipid retention in the liver. However, VLDL secretion was not altered by alcohol ingestion in human subjects [46], suggesting that reduced hepatic lipid secretion is not a major driver of steatosis. The notion that increased lipid influx to the liver can lead to hepatic lipid overload, overwhelming the liver's ability to export and/or utilize lipids is supported by evidence that increased dietary fat intake worsens alcohol induced liver steatosis [47]. However, while increased lipid ingestion might be a contributing factor in steatosis development [48], there is no evidence that alcohol consumption increases dietary lipid intake nor absorption and hence is unlikely to be a primary culprit of alcohol induced steatosis. An alternative pathway that increases lipid delivery to the liver is adipose tissue lipolysis and consequent release of free fatty acids. Indeed, this study highlights the key role of unrestrained lipolysis due to brain insulin resistance and unrestrained sympathetic outflow to adipose tissue.

The SNS activation of adrenergic signaling in adipocytes is the principal inducer of adipose tissue lipolysis [22]. Brain insulin resistance, such as seen after alcohol binging [5], appears to increase sympathetic outflow to adipose tissue [10]. Our study revealed that a model of peripherally restricted sympathectomy prevents alcohol induced hepatic steatosis. Vice versa, β3 adrenergic agonism, which mimics the SNS mediated activation of adipose tissue lipolysis, worsened binge drinking induced hepatic steatosis (Figure 3B), indicating that the increased adrenergic signaling drives WAT lipolysis and subsequent hepatic steatosis. A key role of sympathetically driven adipose tissue lipolysis in alcohol induced steatosis is consistent with the finding that alcohol induced fat mass loss and hepatic triglyceride accumulation are associated with elevated circulating norepinephrine in mice subjected to chronic alcohol binge [4]. Our finding that adipose tissue DNL is suppressed after alcohol binge and that this effect is completely reversed in the THKO mice (Figure 5A), which are protected from hepatic steatosis (Figure 3A), raises the question of whether a suppression of adipose tissue DNL after alcohol contributes to hepatic steatosis. It is possible that impaired adipose tissue DNL after alcohol binging may contribute to hepatic steatosis due to reduced synthesis of metabolically beneficial lipids such as the insulin sensitizing palmitoleate [49]. Future studies focusing on adipose tissue DNL may elucidate additional ways in which impaired adipose tissue function in alcohol binging contributes to ALD.

It is unclear whether the association between stress and ALD is explained by increased alcohol consumption during stress or whether stress synergizes with alcohol in causing an overdrive of the sympathetic nervous system thereby increasing susceptibility to alcoholic liver disease independent of the alcohol dose. Similar to alcohol binging, psychological stress increases SNS activity, which we find synergizes with alcohol to induce hepatic steatosis. Although stress in humans is mostly social stress rather than restraint stress, our studies provide a conceptual framework to understand the biological effects of psychological stress on alcohol induced hepatic injury.

An interesting finding of this study is that unrestrained SNS and impaired adipose tissue function contribute to alcohol binging induced hypothalamic inflammation. We speculate that the increased release of free fatty acids from adipose tissue may drive lipotoxicity and inflammation in the hypothalamus, a brain region that is more permeable to circulating lipids. Alternatively, increased cytokines release from adipose tissue in circulation could reach the brain and contribute to neuroinflammation. While additional studies are needed to clarify the mechanism through which unsuppressed lipolysis induces neuroinflammation, our studies indicate that rebalancing the brain—SNS—adipose tissue axis and reducing lipolysis may provide promising approaches to reduce alcohol induced neuroinflammation. Identifying

drivers of alcohol induced neuroinflammation is important as neuroinflammation is believed to contribute to the cognitive impairment, addiction, and various neuropsychiatric disorders of alcoholism [50]. Our findings also suggest that hypothalamic and brain inflammation may not be a direct effect of alcohol on the CNS but rather a consequence of alcohol induced adipose tissue dysfunction and lipolysis. A similarly disrupted brain—SNS—adipose tissue axis may also be at play in obesity induced non-alcoholic steatosis, given the many similarities in the pathogenesis of these conditions including brain insulin resistance and adipose tissue dysfunction as described above.

In summary, we have examined the role of a brain—SNS—adipose axis in driving early hepatic triglyceride accumulation in a rodent model of repeated binge drinking. Whether this axis continues to drive hepatic steatosis and other manifestations of ALD in chronic alcohol feeding will be examined in future studies. Evidence of increased lipolysis in rodent models of chronic alcohol feeding [4,7,8] and of autonomic dysfunction in chronic alcohol abuse in humans [51] warrants further investigation of the contribution of this brain—SNS—adipose axis to later stages of ALD.

4. METHODS

4.1. Animals

Animal protocols were approved by the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee. All animal experiments were conducted according to ARRIVE guidelines. Rodents were housed under controlled temperature, humidity, and light cycles (12:12 h). Standard chow diet (Rodent Diet 5001; LabDiet, St. Louis, MO) and water were available ad libitum. Female Sprague Dawley rats and mice on a C57BL/6J background were used throughout the study as females are more susceptible to alcohol-associated liver injury than males [52,53]. 12- to 14-week-old female Sprague Dawley rats (Charles River Laboratories) were implanted with osmotic minipumps (Durect Corporation, model 1004) to infuse into the lateral ventricle either artificial cerebrospinal fluid (aCSF) or the PTP1b inhibitor CPT-157633 as previously described [5]. Alcohol or an isocaloric glucose solution was administered by IP in rats daily at 7 am for a total of 3 doses as indicated. IP administration was used in the rat study to avoid destabilizing catheters placed which often occurs with the procedure of oral gavage. In all subsequent studies in mice, alcohol or an isocaloric glucose solution was given at 7 am or 7 pm for a total of 3 doses with 12-hour interval by oral gavage, a more physiological administration route. In all studies, animals that received alcohol were pair-fed with animals that received isocaloric glucose to ensure equal food intake in binged and control animals. Twelve hours after the last alcohol injection when blood alcohol levels were undetectable, the animals were anesthetized with isoflurane, decapitated, and harvested tissues were flash frozen in liquid nitrogen and stored at −80 °C until further analyzed. Isocaloric glucose was chosen to control for the caloric load of alcohol.

Adipose tissue specific deletion of ATGL (AAKO) was achieved by crossing B6N.129-Pnpla2^{tm1Eek} (ATGL$^{flox/flox}$, kindly provided by Dr. Erin E. Kershaw and backcrossed onto the C57BL/6J strain for 10 generations) with B6N.FVB.Tg (Adipoq-Cre)1evr/J mice (stock No. 028020, Jackson Laboratory, Bar Harbor ME). The inducible AAKO (iAAKO) mice were generated by crossing B6N.129-Pnpla2^{tm1Eek} mice with C57BL/6-Tg(Adipoq-cre/ERT2)1Soff mice (stock No. 025124, Jackson Laboratory, Bar Harbor ME). The mice were induced by IP tamoxifen (75 mg/kg every other day for five doses). The deletion of ATGL was functionally validated prior to the alcohol binge study by assessing cold tolerance, which is known to be impaired in models of adipose tissue specific deletion of ATGL [19]. Mice were placed in a 4-degree room in the random fed state for up to 2 h. Food was removed at the beginning of the test. The body temperature of the mice was measured with a rectal thermometer.

The inducible peripheral tyrosine hydroxylase (TH) KO mice were derived as described [23] and induced by IP administration of tamoxifen as described above.

4.2. Pharmacological studies

Lipolysis was inhibited through IP administration of 10 mg/kg acipimox or stimulated through the β3 adrenergic agonist CL (0.01 mg/kg or 1 mg/kg) as indicated. Acipimox and CL were administered immediately before gavage.

4.3. Restraint stress model

Mice were restrained in restraint tubes for one hour and the control littermates were left in the home cage without restraint stress. The restrained mice were then returned to their home cage at which time point all mice were subjected to the above binge protocol or an isocaloric glucose load which was repeated every 12 h for a total of three times.

4.4. Liver histology and lipid measurement

Frozen liver samples were embedded in optimal cutting temperature compound (OCT, cat# 25608-930, VWR, Radnor, PA) and sectioned at a thickness of 7 μm. Slides were fixed and stained with an ORO [16] and counterstained with Carazzi's hematoxylin (Histoserv Inc, MD). ORO-stained slides were scanned with magnification of 10× and 40×, and at least four 40× images were captured per animal. Percent of tissue stained positive for lipids was calculated using Bioquant (Bioquant Image Analysis Corporation, Nashville, TN). Liver lipids were extracted using a protocol based on the Folch extraction method as previously described [54].

4.5. Western Blot

Western Blot analysis was performed as previously described [5]. Primary antibodies are listed in the Supplementary materials. All quantifications were performed from samples run on the same blot. Levels of phosphorylated proteins were normalized to the housekeeping genes GAPDH or β-actin.

4.6. RNA extraction and gene expression analysis

Homogenized tissue was processed using RNeasy Kit (Qiagen, Valencia, CA) to isolate RNA according to manufacturer's protocol. Quantity and quality of the extracted RNA was assessed using SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA). cDNA was then synthesized using Superscript III Reverse Transcription Supermix; cat# 11752050 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. The resulting cDNA was used to determine gene expression levels via qPCR using SYBR green (Thermo Fisher Scientific, Waltham, MA). Relative gene expression was measured using the READER-VENU Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA), normalized to the expression of GAPDH, and plotted as fold change compared to the control group.

4.7. Plasma assays

Blood samples were collected either from the tail vein or via cardiac puncture in EDTA coated tubes and triglyceride and non-esterified fatty acids (NEFA) levels were determined with a NEFA kit from Wako Chemicals USA Inc. following the manufacturer's protocol.

4.8. Quantification and statistic analysis

Statistical analysis was performed using the two-tailed Student's t-tests or ANOVA as indicated and followed up with a Bonferroni post-hoc test unless otherwise stated.

AUTHOR CONTRIBUTIONS

C.Z. designed the research, performed the experiments (Figures 1C–E, 2E–H, 3–6, Figures S1, S3E, S3–S9), analyzed the data, and wrote the paper. H.H.R. performed the studies and analyzed the data in Figures 1 and 2, participated in research design and manuscript writing. L.L. contributed to studies presented in Figures 3–6 and S3–S9. G.M., K.S. and C.L. assisted with experiments in Figure S3. L.W., A.S., H.E.E., S.M.S. and C.L. helped with experiments in Figures 1–2, and S2A. M.A.B. wrote the manuscript. C.B. conceived the studies, obtained funding, designed the research, wrote the paper, and supervised the project.

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DECLARATION OF COMPETING INTEREST

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: CB received research support from Pfizer and consulted for Boehringer and Novo Nordisk. All other authors declare no conflict of interests.

DATA AVAILABILITY

Data will be made available on request.

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Figure 7 was created with https://www.biorender.com.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2023.101813.

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