Title: Loss of the brain-liver axis prevents hepatic steatosis in mice.

Authors

Jiyeon Hwang[1,2], Junichi Okada[1,2], Li Liu[1,2], Jeffrey E. Pessin[1,2,3], Gary J. Schwartz[1,2], and Young-Hwan Jo[1,2,3]*

Affiliations

[1]The Fleischer Institute for Diabetes and Metabolism

[2]Division of Endocrinology, Department of Medicine

[3]Department of Molecular Pharmacology, Albert Einstein College of Medicine, NY, USA

*** Lead contact: young-hwan.jo@einsteinmed.edu**

Departments of Medicine and Molecular Pharmacology

Albert Einstein College of Medicine,

1300 Morris Park Ave, Bronx, NY 10461

Tel: 718-430-3495

Abstract

Hepatic lipid metabolism is regulated by the autonomic nervous system of the liver, with the sympathetic innervation being extensively studied, while the parasympathetic efferent innervation is less understood despite its potential importance. In this study, we investigate the consequences of disrupted brain-liver communication on hepatic lipid metabolism in mice exposed to obesogenic conditions. We found that a subset of hepatocytes and the bile duct are innervated by parasympathetic nerves originating from the dorsal motor nucleus of the vagus. The elimination of the brain-liver axis by deleting parasympathetic cholinergic neurons innervating the liver prevents hepatic steatosis and promots browning of inguinal white adipose tissue (ingWAT). The loss of the brain-liver axis also raises hepatic *Cyp7b1* expression and fasting serum bile acid levels. Furthermore, knockdown of the G protein-coupled bile acid receptor 1 gene in ingWAT reverses the beneficial effects of the loss of the brain-liver axis, leading to the reappearance of hepatic steatosis in the experimental groups. However, deleting the brain-liver axis has a small but significant effect on body weight, which is accompanied by an increase in energy expenditure. Therefore, altering parasympathetic cholinergic innervation of the liver could offer a potential therapeutic approach for enhancing hepatic lipid metabolism in obesity and diabetes.

Introduction

Non-alcoholic fatty liver disease, particularly its progressive form known as non-alcoholic steatohepatitis, is now the leading cause of liver transplantation in Western countries (Burra et al., 2020). NAFLD is characterized by the accumulation of excessive amounts of fat in the liver, which is determined by multiple factors including the uptake of fatty acids by hepatocytes, *de novo* lipogenesis (DNL), mitochondrial and peroxisomal fatty acid oxidation, and lipid export from the liver [1,2]. These pathways are downstream targets of the autonomic nervous system. A recent study reveals that significant alterations in sympathetic innervation to the liver, such as swollen axonal varicosities, nerve fiber retraction, and abnormal axonal sprouting, are associated with NAFLD in mice and humans [3], supporting the critical role of sympathetic nerve plasticity in hepatic lipid metabolism. However, it has been demonstrated that the vagus nerve exerts a significant influence on the regulation of hepatic lipid metabolism. For example, activation of central leptin receptors reduces hepatic fat accumulation, which requires hepatic vagal innervation in lean and high-fat fed rodents [4]. Similarly, the anti-steatotic effect of leptin is dependent on vagal innervation in humans [5]. In addition, central infusion of oleic acid suppresses triglyceride-rich very low-density lipoproteins (VLDL-TG) secretion through hepatic vagal innervation in rats [6]. The hepatic vagus nerve regulates the development of NAFLD via altering hepatic inflammation [7]. However, the challenge in interpreting the results of hepatic vagal denervation is that it affects both vagal sensory and parasympathetic cholinergic fibers, which pass through the hepatic vagus nerve. As a result, it is unclear what role parasympathetic cholinergic innervation of the liver plays in regulating hepatic lipid metabolism.

Dynamic crosstalk between the liver and white adipose tissue (WAT) appears essential for hepatic fat accumulation and the development of NAFLD [8]. VLDL-TG synthesized in the liver is secreted and transported to adipose tissue for storage in the postprandial state [9]. Fasting increases lipolysis in adipocytes, and the free fatty acids released from WAT are transported to the liver for energy metabolism [9]. In a human study, most fatty acids constituting VLDL-TG are derived from adipose tissue [10]. However, in obesity, adipose tissue can no longer effectively store lipids, thus redirecting them toward other organs, primarily the liver [8]. Increased lipolysis, along with elevated cytokine release from WAT, causes hepatic steatosis [8]. Interestingly, the liver has the capability to control lipid uptake and lipolysis in WAT by activating bile acid (BA) signaling pathways [11]. Vagotomized rats exhibit reduced expression of the cholesterol 7 alpha-hydroxylase (*Cyp7a1*) gene, the rate-limiting enzyme in the classic bile acid synthesis pathway in the liver, while displaying increased bile acid levels in the serum and bile, in part via enhanced intestinal passive absorption of BAs [12]. Biliary epithelial cells in rats express the

muscarinic acetylcholine receptor type 1 and its activation by carbachol reduces bile flow [13]. The parasympathetic cholinergic innervation to the liver may potentially exert a regulatory effect on bile acid synthesis and outflow from the liver, which could subsequently result in a decrease in lipolysis within the white adipose tissue. In fact, a recent 3D/Airyscan imaging study has demonstrated vesicular acetylcholine transporter (vAChT)-positive parasympathetic nerve terminals on the bile duct in human liver tissues [14].

In this study, we aimed to investigate the impact of the loss of the brain-liver axis on hepatic ipid metabolism in mice fed a high-fat diet (HFD). Our anterograde and retrograde neuronal mapping and 3D imaging of cleared liver tissues revealed that a subset of hepatocytes and the bile duct received vAChT-positive nerve terminals from the DMV. The removal of the brain-liver axis prevented the development of hepatic steatosis. This decrease in hepatic fat accumulation was linked to the beiging of ingWAT caused by activation of the G protein-coupled bile acid receptor 1 (GPBAR1 or TGR5) in ingWAT.

Results

Parasympathetic cholinergic innervation of the liver

In our previous study [15], we showed that a subset of DMV cholinergic neurons projected to the liver. To extend and further confirm the presence of DMV cholinergic neurons innervating the liver, we injected a Cre-dependent viral tracer (adeno-associated virus (AAV).PHP.eB-FLEX-tdTomato [16]) into the liver of choline acetyltransferase (ChAT)-IRES-Cre ($ChAT^{Cre}$) mice [17]. The $ChAT^{Cre}$ transgenic strain has been used to selectively label parasympathetic cholinergic neurons [18]. At 3 weeks post-viral infection, we performed double immunocytochemistry with anti-ChAT and anti-tdTomato antibodies on the brain sections and found that the DMV displayed a small number of tdTomato-positive cells (Fig. 1A). Importantly, all tdTomato-positive cells in the DMV were ChAT-expressing cells (n = 72 out of 72 neurons from 3 mice), and approximately 10 % of DMV cholinergic neurons expressed tdTomato (range, 7–14 %; n= 72 out of 660 cholinergic neurons from 3 mice). We investigated whether the liver receives cholinergic innervation by immunostaining with an antibody against the presynaptic cholinergic marker vesicular acetylcholine transporter (vAChT). Our analysis revealed the presence of vAChT-positive nerve terminals scattered throughout the liver parenchyma, including in the periportal, midlobular, and pericentral zones (Fig. 1B). Hence, our results are consistent with prior findings that a subset of DMV cholinergic neurons innervate the liver [15,19,20].

Given that DMV cholinergic neurons projected to the liver, we injected an anterograde AAV1 encoding a Cre-inducible tdTomato and a synaptophysin-GFP (sypGFP) fusion protein

separated by the sequence of a T2A self-cleaving peptide (AAV1-FLEX-tdTomato-T2A-sypGFP [21]) into the DMV of ChAT^{Cre} mice (Fig. 1C). Under these experimental conditions, we anticipated that tdTomato would be expressed in DMV cholinergic neurons, and that sypGFP would move forward to the axon terminals of DMV cholinergic neurons. As shown in Fig. 1C, immunostaining revealed that the DMV cholinergic neurons were positive for tdTomato, indicating that the DMV cholinergic neurons were successfully infected. Immunolabeling of liver sections further revealed numerous sypGFP-positive nerve terminals in a subset of hepatocytes and bile duct epithelial cells cholangiocytes (Fig. 1C and D). SRY-Related HMG Box Gene 9 (SOX9)-positive cholangiocytes were surrounded by vAChT-positive nerve terminals (Fig. 1D). 3D imaging analysis of cleared liver tissue collected from ChAT^{Cre} mice injected with AAV1-FLEX-tdTomato-T2A-sypGFP further conformed the presence of cholinergic nerve terminals around the portal vein and in a subset of hepatocytes (Fig. 1E, F, G, and Movie S1), consistent with a recent findings of parasympathetic cholinergic innervation of the bile duct in human liver tissues [14].

In addition to cholinergic input, Western blot analysis of liver tissue homogenates revealed the presence of muscarinic ACh receptor types 1, 2, 4, and 5 (CHRM1, 2, 4, and 5), but not CHRM3 (Fig. S1A). As CHRM4 was highly expressed compared to other CHRM subtypes, we immunolabeled the liver tissues with an anti-CHRM4 antibody. CHRM4 was detected in areas adjacent to vAChT-positive terminals (Fig. S1B). Similarly, CHRM2 was also found in hepatocytes (Fig. S1C). Our analysis of cleared liver tissues using 3D imaging techniques, as well as antero- and retrograde neural tracing support the interpretation that the liver is a downstream target of the parasympathetic cholinergic neurons.

Loss of the brain-liver axis prevents hepatic lipid accumulation

The role of DMV cholinergic neurons innervating the liver in controlling hepatic lipid metabolism remains unexplored. We examined the physiological impact of hepatic cholinergic loss-of-function on hepatic fat accumulation. For these experiments, we used a retrograde AAV (AAVrg) encoding a Cre-inducible diphtheria toxin subunit A fragment (dtA) that induces apoptosis of target cells [22]. AAVrg-FLEX-dtA and AAVrg-FLEX-GFP as control were injected into the livers of the ChAT^{Cre} mice (Fig. 2A). To validate cholinergic cell death in the DMV, we sacrificed mice at 10 weeks post-viral inoculation and stained the DMV cholinergic neurons with an anti-ChAT antibody. The number of DMV cholinergic neurons was significantly lower in ChAT^{Cre} mice that received retroAAV-FLEX-dtA than in the controls (Fig. 2B, C and Fig. S2A and B). This reduced number of DMV cholinergic neurons was strongly associated with a lack of vAChT-positive nerve terminals in the livers of ChAT^{Cre} mice that received AAVrg-FLEX-dtA (Fig. 2D). Importantly, measurement of ACh levels revealed a significant reduction in hepatic ACh

content in the experimental mice, whereas there was no difference in plasma ACh levels between the groups (Fig. 2E and F). These results support the interpretation that our experimental approach effectively ablates parasympathetic cholinergic neurons innervating the liver.

We then examined whether deleting the brain-liver axis alters hepatic lipid metabolism during HFD feeding. To address this question, we first investigated if there are differences in the liver histology between the groups. Liver tissues were labeled with liver zonation markers such as the pericentral marker glutamine synthetase (GS) and the periportal marker E-cadherin (E-cad) [23]. The expression pattern of these zonation markers in the livers of the control group fed a standard chow diet demonstrated that GS- and E-cad-positive areas were clearly separated in the liver (Fig. S3A). Interestingly, high-fat feeding robustly reduced E-cad expression in periportal hepatocytes in the livers of control mice compared to control mice fed a standard chow diet (Fig. 3A), consistent with disrupted E-cad expression in chronic liver disease in mice [24]. Surprisingly, deleting the parasympathetic cholinergic input to the liver in $ChAT^{Cre}$ mice fed HFD restored the expression of E-cad in periportal hepatocytes (Fig. 3B). In contrast, GS was similarly expressed in pericentral hepatocytes in both control and experimental mice fed HFD (Fig. 3A and B).

Hematoxylin and eosin (H & E) staining revealed a clear difference in the macroscopic appearance of the livers of $ChAT^{Cre}$ mice receiving AAVrg-FLEX-GFP and AAVrg-FLEX-dtA. Control mice exhibited an increased number of large and small lipid droplets and ballooned hepatocytes compared to experimental mice (Fig. 3C and D). Oil Red O staining further showed that $ChAT^{Cre}$ mice without DMV cholinergic neurons innervating the liver contained fewer lipid droplets in the liver than controls (Fig. 3E and F). Our results suggest that parasympathetic cholinergic input to the liver regulates hepatic steatosis during high-fat diet feeding.

As there was a lack of lipid droplets in the livers of the experimental mice, we measured the hepatic and plasma triglyceride (TG) levels. $ChAT^{Cre}$ mice without parasympathetic cholinergic neurons innervating the liver on high-fat feeding exhibited reduced TG levels in the liver compared to controls (Fig. 3G). This was associated with decreased plasma TG levels (Fig. 3G). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of liver tissues revealed a significant decrease in the expression of the lipogenic transcription factor sterol regulatory element-binding protein 1-c (*Srebp-1*) in the experimental mice relative to the controls. The experimental mice also exhibited downregulation of its target genes, including acetyl-coenzyme A carboxylase 1 (*Acaca*), fatty acid synthase (*Fasn*), stearoyl-CoA desaturase-1 (*Scd1*) (Fig. 3H). In addition, the $ChAT^{Cre}$ mice without parasympathetic cholinergic input to

the liver had increased expression of peroxisome proliferator-activated receptor alpha (PPAR-a), the central transcriptional regulator of fatty acid oxidation relative the control group. As a result, there was increased carnitine palmitoyltransferase 1a (*Cpt1a*) and acyl-CoA dehydrogenase medium chain (*Acadm*) mRNA expression in the experimental mice relative to controls (Fig. 3H). In contrast to male mice, high-fat feeding did not cause any changes in liver histology or fat accumulation in both control and experimental females (Fig. S4A-C). In fact, hepatic steatosis was less common in C57BL/6 females than in males during high-fat feeding [25-27].

Hepatic vagal denervation results in an increase in hepatic interleukin 6 (*Il6*) gene expression in mice, which subsequently modulates the expression of hepatic gluconeogenic enzymes. In our experiments, where we selectively deleted liver-projecting cholinergic neurons, we observed a significant decrease in Il6 gene expression (Fig. S5A). Notably, there was no significant difference in gluconeogenic gene expression between the experimental and control groups (Fig. S5B). Furthermore, we investigated whether the loss of parasympathetic cholinergic innervation affects sympathetic input to the liver. We used immunostaining with an anti-tyrosine hydroxylase (Th) antibody and found no detectable difference in Th-positive nerve fibers (Fig. S6A and B). Additionally, hepatic norepinephrine levels were similar between the groups (Fig. S6C). Hence, these findings suggest that the loss of parasympathetic cholinergic neurons innervating the liver prevents the development of hepatic steatosis during HFD feeding in male mice.

Loss of the brain-liver axis causes beiging of ingWAT

It shas been shown that the WAT does not receive parasympathetic cholinergic innervation in rodents [28,29], and that separate DMV cholinergic neuron subtypes have distinct targets even within the same organ, such as the glandular stomach [30]. This may suggest the presence of liver-specific DMV cholinergic neurons. Based on these findings, we hypothesized that parasympathetic cholinergic input to the liver may play a role in the crosstalk between the liver and WAT, particularly in the context of increased lipolysis in WAT, which can lead to hepatic steatosis in fasted and insulin-resistant mice [8,31]. To investigate this further, we examined whether there are metabolic alterations in WAT, particularly in ingWAT, which has a beiging capacity [32]. Our results showed that the size of ingWAT in experimental mice was smaller than that in control mice (Fig. 4A), and histological analysis of ingWAT revealed an increase in large-sized adipocytes in control mice compared to the experimental groups (Fig. 4B). Additionally, there was a significant reduction in adipocyte area in $ChAT^{Cre}$ mice without DMV cholinergic neurons innervating the liver (Fig. 4C and D), suggesting that a loss of DMV cholinergic input to the liver prevents ingWAT hypertrophy.

We then examined if there is any connection between these unexpected outcomes and the process of beiging in ingWAT. RT-qPCR analysis revealed that deleting DMV cholinergic neurons innervating the liver significantly upregulated the expression of Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha (PGC1a) in ingWAT, which is required for the expression of mitochondrial biogenesis and thermogenic genes in brown fat cells [33,34] (Fig. 4E). Likewise, the expression of PR (PRD1-BF1-RIZ1 homologous)-domain containing 16 (PRDM16), a transcription factor that plays a key role in browning and thermogenesis of subcutaneous WAT [35], was significantly increased in experimental mice relative to controls (Fig. 4E). The upregulation of these genes was associated with a robust increase in the mRNA expression of uncoupling protein 1 (*Ucp1*), iodothyronine deiodinase 2 (*dio2*), and cell death-inducing DFFA-like effector A (*Cidea*) (Fig. 4E). Western blotting and immunostaining further showed an increase in UCP1 protein levels in ingWAT of ChAT^{Cre} mice receiving AAVrg-FLEX-dtA to the liver (Fig. 4F and G). In addition, daily measurement of ingWAT temperature revealed a significant elevation in the ingWAT temperature of the experimental mice in the dark phase (Fig. 4H). These data support that a loss of parasympathetic cholinergic neurons innervating the liver induces beiging of ingWAT, resulting in an increase in thermogenesis.

WAT lipolysis in obesity can result in an excess of fatty acids and glycerol in the liver [36]. This lipid influx can cause hepatic TG synthesis, which results in NAFLD [37]. We investigated whether eliminating cholinergic input to the liver from the DMV would decrease HSL-mediated lipolysis in ingWA. The Western blot analysis of ingWAT demonstrated a decrease in the levels of phospho-HSL in the experimental mice (Fig. 4I). Moreover, we observed a decrease in plasma glycerol levels in the experimental mice compared to the control mice (Fig. 4J). Overall, the loss of hepatic cholinergic innervation led to an increase in the expression of thermogenic genes while simultaneously reducing lipolysis in ingWAT.

Increased bile acid secretion induces ingWAT beiging via TGR5.

What causes the beiging of the ingWAT in mice without DMV cholinergic neurons innervating the liver? Previous studies has indicated that bile acid and its corresponding receptor TGR5 are crucial in the browning of WAT [11,38]. We thus investigated whether the loss of function of liver-innervating cholinergic neurons affects the expression of genes related to bile acid synthesis, such as *Cyp7a1* and *Cyp7b1*. We found that hepatic cholinergic loss of function upregulated expression of the *Cyp7b1* gene involved in an alternative bile acid synthesis pathway [39] (Fig. 5A). Furthermore, our spatial transcriptomic analysis of the liver revealed that ChAT^{Cre} mice without DMV cholinergic neurons innervating the liver displayed a trend toward an increase in *Cyp7b1* gene expression in all three zones (Fig. 5B), whereas *Cyp7a1* expression

was not altered, as observed in qPCR experiments (Fig. 5A and 5B). Elevated *Cpy7b1* expression led to a substantial increase in fasting serum bile acid levels in ChAT^{Cre} mice that received AAVrg-FLEX-dtA. (Fig. 5C). In our further analysis of the fasting serum bile acid profiles in both control and experimental mice, we observed a significant increase in primary bile acids such as cholate and chenodeoxycholate, which was attributed to the loss of hepatic cholinergic input (Fig. 5D).

We then examined whether the expression of its corresponding receptor, TGR5, is altered in the ingWAT of the experimental mice. Our observations revealed elevated levels of both TGR5 gene and protein expression in the ingWAT of the experimental mice compared to the control group (Fig. 6A). This suggests that the increase in serum bile acids and *Tgr5* expression in the ingWAT leads to beiging of the ingWAT in the experimental mice. To investigate this possibility, we administered *Tgr5* siRNA or control siRNA into the ingWAT of ChAT^{Cre} mice without liver-innervating cholinergic neurons to exclusively knock down *Tgr5* in the ingWAT (Fig. 6B). Ten weeks after a viral infection, we conducted RT-qPCR and Western blot analyses on samples and found that the *Tgr5* gene and protein levels in ingWAT were significantly reduced following the injection of *Tgr5* siRNA (Fig. 6C), demonstrating the effectiveness of our approach in knocking down *Tgr5* expression. Our experiments also revealed that the number of large-sized adipocytes and crown-like structures in the ingWAT of mice that received *Tgr5* siRNA was increased (Fig. 6D), accompanied by a notable increase in adipocyte area (Fig. 6E and F).

We examined whether *Tgr5* knockdown impacts HSL-mediated lipolysis in ingWAT. Our findings showed an increase in phospho-HSL levels in the ingWAT (Fig 7A), which subsequently led to higher levels of plasma glycerol (Fig. 7B). Considering the well-established connection between elevated plasma glycerol levels and lipid accumulation in the liver, we sought to determine if *Tgr5* knockdown results in hepatic steatosis. Our results revealed the existence of large lipid droplets and ballooned hepatocytes in the liver sections of mice that received *Tgr5* siRNA (Figure 7C). Furthermore, Oil Red O staining confirmed the accumulation of lipids in the livers of mice treated with *Tgr5* siRNA in the ingWAT (Figure 7D). Additionally, we found that the knockdown of the Tgr5 gene in the ingWAT of ChAT^{Cre} mice without DMV cholinergic neurons to the liver resulted in a significant increase in hepatic TG content, which is indicative of enhanced lipid accumulation in the liver (Fig. 7E). Collectively, our findings support the idea that cross-talk between the liver and ingWAT through elevated bile acid signaling plays a vital role in maintaining the lipid metabolism balance in both the liver and ingWAT.

Deleting the brain-liver axis has a slight but significant effect on body weight

We also investigated whether the disruption of the brain-liver axis could impact body weight in mice fed HFD. After 10 weeks of high-fat diet feeding, we observed a small but significant difference in body weight gain between the control and experimental groups (Fig. S7A). The experimental mice gained less weight than the control mice during the HFD period. This decrease in weight gain was closely related to a significant reduction in fat mass in the experimental group, while lean mass was similar between the two groups (Fig. S7B). When we compared adipose tissue mass collected from controls with that of adipose tissue from the experimental group, we found a significant decrease in the mass of ingWAT (Fig. S7C). It is important to note that both groups consumed an equal amount of food during the light and dark phases (Fig. S7D).

The reduction in body weight, despite no change in energy intake, may be due to an increase in energy expenditure. To assess metabolic rate, we placed animals in metabolic cages and observed that the experimental mice consumed more oxygen and produced more carbon dioxide during the dark phase than the controls (Fig. S7E and F). These findings indicate an increase in total energy expenditure in the experimental mice (Fig. S7G). However, the respiratory exchange ratio was similar between the two groups (Fig. S7H). Notably, the experimental mice exhibited more locomotor activity during the dark phase (Fig. S7I and J). Collectively, these results suggest that deleting liver-projecting cholinergic neurons plays a role in increasing energy expenditure.

Loss of the brain-liver axis improves insulin tolerance in male mice fed HFD

We evaluated whether deleting the brain-liver axis affects glucose homeostasis in mice fed a high-fat diet, as the liver plays a crucial role in regulating systemic glucose levels [40]. To assess glucose intolerance and insulin sensitivity, we measured blood glucose and insulin levels. Our results showed that basal glucose levels were lower in ChATCre mice receiving AAVrg-FLEX-dtA injection than in the controls (Fig. S8A). Additionally, the loss of DMV cholinergic neurons innervating the liver led to a significant reduction in fasting glucose levels (Fig. S8B).

To evaluate whether $ChAT^{Cre}$ mice without parasympathetic cholinergic neurons innervating the liver exhibit any changes in their ability to dispose a glucose load, we conducted intraperitoneal glucose tolerance tests (i.p.GTT) and found no significant difference in blood glucose levels in response to a bolus of glucose (i.p., 2 g/kg) between the groups (Fig. S8C and D). However, the experimental mice showed increased insulin sensitivity (Fig. S8E). During the insulin tolerance tests (ITT) (i.p., 1 U/kg), blood glucose levels were significantly lower in $ChAT^{Cre}$ mice injected with AAVrg-FLEX-dtA than in controls (Fig. S8E). Furthermore, the

improved insulin tolerance in the experimental group was associated with reduced plasma insulin levels relative to those in the controls (Fig. S8F). These results indicate that the loss of DMV cholinergic input to the liver enhanced insulin sensitivity in ChATCre male mice fed HFD.

Intriguingly, a growing number of studies have revealed sex-specific differences in metabolic homeostasis, diabetes, and obesity in both rodents and humans [41-47]. For instance, premenopausal women were relatively protected from diseases associated with metabolic syndrome [48]. Female rodents were less prone to developing diet-induced obesity (DIO), insulin resistance, hyperglycemia, and hypertriglyceridemia [41,42,45,46]. In particular, C57BL/6 female mice were less likely to develop DIO and hyperglycemia than C57BL/6 male mice during high-fat feeding [45-47,49,50]. Our study also found that control female mice did not develop DIO or hyperglycemia (Fig. S9A-F), and hepatic cholinergic loss of function had no effect on body weight or glucose homeostasis (Fig. S9A-F). In other words, both groups did not show any notable differences in body weight, food intake, body composition, blood glucose levels, GTT, and ITT compared with the controls (Fig. S9A-H).

Discussion

The current study provides concrete evidence for the existence of the cholinergic system in the mouse liver. Our research showed that DMV cholinergic neurons directly projected to hepatocytes and cholangiocytes and that hepatocytes expressed muscarinic ACh receptors. Our 3D imaging analysis of cleared liver tissue also revealed the presence of cholinergic nerve terminals around the portal vein. Additionally, our study found that deleting cholinergic neurons innervating the liver prevented the development of hepatic steatosis during HFD feeding, which was closely linked to the browning of ingWAT. $ChAT^{Cre}$ mice without cholinergic input to the liver displayed elevated bile acid synthesis, primarily through the CYP7B1-mediated alternative pathway, leading to elevated serum bile acid levels. Importantly, the experimental mice exhibited elevated *Tgr5* gene expression in ingWAT, which was found to be critical in mediating the beneficial effects of the loss of hepatic cholinergic function. Specifically, knockdown of the *Tgr5* gene in ingWAT completely abolished these beneficial effects and led to the reappearance of hepatic steatosis in the experimental groups. These findings suggest that TGR5 is a crucial mediator of the metabolic benefits conferred by loss of hepatic cholinergic function. Taken together, our results suggest that the brain-liver axis plays an essential role in bile acid synthesis and secretion. The interorgan crosstalk via bile acids is also critical for the development of hepatic steatosis.

The liver plays a major role in controlling energy metabolism, including macronutrient metabolism, lipid homeostasis, and energy storage [9]. Like other visceral organs [30,51,52], the liver is innervated by the vagus nerve [53], and vagal innervation to the liver regulates these metabolic functions [4,7,54,55]. Parasympathetic (or vagal) efferent neurons innervating the liver are located in the DMV [56]. Prior anatomical studies with retrograde neuronal tracers such as cholera toxin B, pseudorabies virus, and AAV encoding a Cre-inducible reporter protein demonstrated that the mouse liver receives parasympathetic nerve innervation from the DMV [15,19,20]. DMV motor neurons are acetylcholine (ACh)-expressing neurons [30]. We recently showed that hepatocytes received direct DMV cholinergic input and expressed muscarinic ACh receptor genes [15]. Importantly, a short-term change in the activity of DMV cholinergic neurons projecting to the liver regulated hepatic glucose output in lean mice [15]. In contrast to these prior findings, recent 3D imaging studies revealed a paucity of parasympathetic cholinergic innervation in the mouse liver [3,57], indicating that hepatic metabolism is primarily regulated by the sympathetic nervous system. However, a recent elegant study by the Tang research group clearly demonstrated the presence of vAChT-positive nerve terminals in the bile duct of human liver tissues, suggesting that the liver receive parasympathetic cholinergic innervation [14].

In fact, accumulated studies have shown that parasympathetic innervation to the liver controls hepatic glucose output, lipid metabolism, and bile formation [4,6,7,54,58]. However, there is limited information available on the detailed neuroanatomy of parasympathetic innevation of the liver, leading some to question its importance in controlling hepatic metabolism. [3,57]. In addition, although hepatic branch vagotomy altered multiple physiological processes, including glucose homeostasis [54,58], hepatic lipid metabolism [4,6], and hepatic inflammatory response [7], studies involving hepatic branch vagotomy have not provided conclusive evidence supporting the role of the parasympathetic cholinergic system in liver function. The hepatic branches of the vagus nerve also contained sensory nerves that transmit interoceptive information from the liver to the brain. Despite this, several studies have used virus-mediated tracing methods to clearly demonstrate the presence of DMV cholinergic neurons that innervate the liver [15,19,20]. Our study, which utilized anterograde tracing techniques alongside 2D immunostaining and 3D imaging of cleared liver tissue, uncovered direct synaptic connections between cholinergic neurons in the DMV and the liver. The elimination of DMV cholinergic neurons that innervate the liver led to a substantial reduction in the number of vAChT-positive nerve terminals and hepatic ACh levels. It is possible that differences in the morphology of preganglionic and postganglionic neurons may contribute to the discrepancy between our findings and recent 3D imaging studies [3,57]. While sympathetic postganglionic neurons and sensory neurons, particularly c-fibers, have

unmyelinated nerve fibers, the nerve fibers of parasympathetic cholinergic preganglionic neurons are myelinated [59,60]. This myelin sheath may make it difficult to stain cholinergic nerve fibers in the liver parenchyma. In our study, we detected cholinergic nerve terminals but not nerve fibers, whereas TH-positive nerve fibers were clearly visible. Our previous functional analysis [15] and current neuroanatomical results support the fact that the liver is a crucial target of parasympathetic cholinergic efferent neurons.

Compared to the experimental groups, $ChAT^{Cre}$ mice fed HFD showed elevated basal and fasting glucose levels and higher basal insulin levels, which led to hyperglycemic and hyperinsulinemic conditions during HFD feeding. These conditions caused hepatic steatosis in control mice. Interestingly, the deletion of parasympathetic cholinergic input to the liver reversed the detrimental effects of high-fat feeding on insulin sensitivity and hepatic fat accumulation. The effects observed were partly due to the decrease in hepatic *de novo* lipogenesis and increase in fatty acid oxidation in the livers of $ChAT^{Cre}$ mice, which lack DMV cholinergic innervation. In other words, when DMV cholinergic innervation to the liver was deleted, genes associated with lipogenesis were expressed at lower levels, while genes involved in fatty acid oxidation were expressed at higher levels, leading to reduced levels of triglycerides. Hence, our results suggest a significant role for parasympathetic cholinergic innervation in the regulation of hepatic lipid metabolism under obesogenic conditions.

Simple hepatic steatosis resulted from an imbalance between the delivery and production of fat in the liver and its subsequent secretion and metabolism [61]. Excessive dietary fat led to increased plasma concentrations of TGs and free fatty acids (FFAs), resulting in increased uptake into hepatocytes [62]. In our preparations, both groups consumed the same amount of food, implying that there may not be a substantial difference in the daily intake of fat. Additional sources of fatty acids can also be obtained from the breakdown of TGs stored in adipocytes, which is mainly controlled by insulin [63]. Insulin regulates systemic lipid metabolism by suppressing lipolysis in adipocytes. This mechanism is the primary way that insulin controls the breakdown of fats in these cells. As a result, insulin typically increases the uptake of FFAs from lipoproteins and decreases their release from adipocytes [62]. In contrast, insulin resistance depressed both of these functions [62]. $ChAT^{Cre}$ mice, which lack DMV cholinergic neurons innervating the liver, displayed enhanced insulin sensitivity compared with the control group. This metabolic profile, including decreased adiposity, lower levels of plasma glycerol, and reduced fat mass, may contribute to the improved insulin sensitivity observed in experimental mice. By improving insulin resistance in adipocytes, the flow of FFAs and glycerol to the liver

was limited, preventing the development of hepatic steatosis in ChAT^{Cre} mice that lack cholinergic innervation to the liver during HFD feeding.

We should highlight that disrupting the brain-liver axis caused the browning of ingWAT, which was a process that enhances glucose uptake and significantly contributes to systemic glucose disposal. This improvement in glucose disposal can ultimately lead to better insulin sensitivity [36]. Hepatocytes secrete various signaling molecules, such as bile acids, which can trigger WAT browning through TGR5 [11,38,64]. Daily administration of the TGR5 agonist for 1 week resulted in a significant decrease in subcutaneous white adipose tissue (scWAT) mass and the upregulation of browning marker genes, including *Ucp1* and *Cidea* [11]. ChAT^{Cre} mice lacking liver-projecting cholinergic neurons showed a significant reduction in WAT mass and adipocyte size. Interestingly, these mice also exhibited an increase in thermogenic gene and protein expression in ingWAT, leading to an elevation in nocturnal ingWAT temperature. The upregulation of *Cyp7b1* and bile acid levels due to the loss of hepatic cholinergic innervation may lead to browning of ingWAT as a result of crosstalk between the liver and ingWAT.

Communication between ingWAT and other organs, including the liver and muscle, is essential for the proper functioning of systemic metabolism [63]. Our current study revealed that the crosstalk between the liver and ingWAT through bile acids played a significant role in the progression of hepatic steatosis. Remarkably, silencing *Tgr5* in the ingWAT completely reversed the beneficial effects of the loss of hepatic cholinergic innervation. *Tgr5* knockdown resulted in enlarged adipocytes, leading to hypertrophy. Surprisingly, knockdown of *Tgr5* that was restricted to ingWAT induced lipid accumulation in the livers of ChAT^{Cre} mice lacking cholinergic innervation. In other words, the absence of hepatic cholinergic innervation failed to prevent the onset of hepatic steatosis following the silencing of *Tgr5* in ingWAT, suggesting that the ingWAT makes a crucial contribution to the development of hepatic steatosis. In fact, knockdown of *Tgr5* in ingWAT elevated plasma glycerol levels, indicating a role of TGR5 in regulating lipolysis in ingWAT. While our study provides evidence for crosstalk between the liver and ingWAT via TGR5, it is possible that TGR expressed in central neurons may also play a role in the observed effects as activation of hypothalamic TGR5 suppressed food intake and prevented diet-induced obesity [65,66].

In conclusion, our study provides substantial evidence for the presence of parasympathetic cholinergic input to the liver, which constitutes a key component of the brain-liver axis. This axis is involved in the synthesis and secretion of bile acids, and the interorgan communication between the liver and ingWAT via bile acids plays a crucial role in the development of systemic insulin resistance and hepatic steatosis under obesogenic conditions.

Therefore, targeting parasympathetic cholinergic input to the liver may offer a promising avenue for treating systemic insulin resistance and its associated hepatic steatosis in obesity.

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Figure legends

Figure 1. Parasympathetic cholinergic system in the mouse liver

A. Images of confocal fluorescence microscopy showing co-expression of tdTomato and ChAT in a subset of DMV cholinergic neurons in ChAT^{Cre} mice receiving an injection of AAV.Php.eB-tdTomato into the liver, representing cholinergic neurons innervating the liver (white arrowheads). Scale bar, 30 μm

B. Images of confocal fluorescence microscopy showing the expression of vAChT-positive nerve terminals (green) in the parenchyma of the liver (inset: higher-magnification view of vAChT-positive terminals). Scale bar, 70 μm

C. Images of confocal fluorescence microscopy showing tdTomato-positive cholinergic neurons in the DMV (upper panel; white arrowheads) and sypGFP-positive nerve terminals (bottom panel; white arrowheads) in the liver of ChAT^{Cre} mice injected with AAV1-FLEX-tdTomato-T2A-sypGFP into the DMV. A small number of hepatocytes and cholangiocytes received parasympathetic cholinergic innervation (inset). Blue: nucleus staining with DAPI. Upper panel: scale bar, 30 mm; lower panel: scale bar, 20 μm

D. Images of confocal fluorescence microscopy showing that SOX9-positive cholangiocyte cells receive vAChT-positive nerve terminals (white arrowheads). Scale bar: 10 μm

E. and **F**. Schematic illustration of our experimental configurations (E). Image of the liver after tissue clearing (F).

G. Z-stack image showing parasympathetic cholinergic nerve terminals (green) around the periportal area in cleared liver tissue collected from ChAT^{Cre} mice injected with AAV1-FLEX-tdTomato-T2A-sypGFP (upper panel). Bottom panel: 2D image of cholinergic nerve terminals shown in the upper panel. White arrowheads indicate the cholinergic nerve terminals. Scale bar: 50 μm

Figure 2. Loss of the brain-liver axis by deleting parasympathetic cholinergic neurons innervating the liver

A. Schematic illustration of the experimental configurations, where AAVrg-FLEX-tdTomato (control, o) and AAVrg-FLEX-dTA (•) viral vectors were injected directly into the livers of ChAT^{Cre} mice.

B. Images of confocal fluorescence microscopy showing reduced numbers of cholinergic neurons in the DMV of ChAT^{Cre} mice receiving an injection of AAVrg-FLEX-dtA compared with the controls. Scale bar, 80 μm, cc: central canal, 12N: hypoglossal nucleus

C. Summary graph showing the number of cholinergic neurons on the left and right sides of the DMV in the control (n= 4 mice) and experimental mice (n= 4 mice). Unpaired *t*-test, **$p<0.01$

D. Images showing a lack of vAChT-positive nerve terminals in the liver parenchyma of ChAT$^{Cre + AAVrg\text{-}FLEX\text{-}dtA}$ mice (right panel). Left panel: control. Scale bar, 50 μm

E and **F**. Summary plot showing a significant decrease in hepatic (control, n= 7 mice; experimental mice, n= 6 mice), but not plasma (control, n= 11 mice; experimental mice, n= 8 mice), ACh contents in ChAT$^{Cre + AAVrg\text{-}FLEX\text{-}dtA}$ mice compared to the controls. Unpaired *t*-test, *p<0.05

Figure 3. Deleting the brain-liver axis prevents hepatic steatosis during high-fat diet feeding.

A and **B.** Macroscopic appearance of livers of ChAT^{Cre} mice receiving AAVrg-FLEX-GFP and AAVrg-FLEX-dtA. E-Cad expression was lower in the control group than in the experimental group. Scale bar, 100 μm

C and D. H & E staining of liver tissues revealed increased numbers of lipid droplets and ballooned hepatocytes in the controls compared to those in the experimental mice (top panel). Scale bar, 100 mm. Bottom panel: Higher magnification view of liver tissue in the black dotted square. Scale bar, 30 μm

E and F. Oil red O staining (red) of liver tissues from ChAT^{Cre} mice with and without hepatic cholinergic input. The experimental group did not exhibit fat accumulation in the liver parenchyma during high fat feeding. Nuclear staining (blue): hematoxylin, Scale bar, 100 μm

G. Graphs showing hepatic (n= 9 vs. 8 mice) and plasma (n= 11 vs. 12 mice) TG levels in the controls and experimental mice. There was a significant reduction in both hepatic and plasma TG levels in the experimental group relative to those in the control group (unpaired *t*-test, **p<0.01, ***p<0.001).

H. Summary graphs showing changes in hepatic mRNA expression of enzymes involved in lipid metabolism. *Srebp1*c mRNA expression was significantly downregulated, while *Ppara* mRNA expression was increased in the livers of ChAT^{Cre} mice receiving AAVrg-FLEX-dtA relative to controls (control, n= 8 mice; experimental, n= 8 mice, unpaired t-test, *p<0.05, **p<0.01).

Figure 4. Loss of the brain-liver axis induces beiging of ingWAT.

A. Representative images of the ingWAT of control and experimental mice.

B. Representative images of adipocytes in the ingWAT of control and experimental groups. Scale bar, 100 μm.

C and **D.** Plots showing the distribution of the average adipocyte size (left panel). Right panel: The mean cell area was significantly smaller in the experimental groups than in controls

(control, n=4 mice; experimental, n=5 mice, unpaired *t*-test, *p<0.05)

E. Summary graph showing changes in thermogenic gene expression in ingWAT of ChAT^{Cre} mice with and without DMV cholinergic neurons innervating the liver. Ablation of liver-projecting cholinergic neurons significantly upregulated the thermogenic gene expression (unpaired *t*-test, *p<0.05, **p<0.01).

F and G. Western blot images of ingWAT showing an increase in UCP1 expression in experimental mice. Images of confocal fluorescence microscopy showing increased UCP1 expression in the ingWAT of experimental mice (**H**). Scale bar, 50 µm.

H. Summary plots showing ingWAT temperature in the light and dark phases (control, n=5 mice; experimental, n= 4 mice, unpaired *t*-test, **p<0.01)

I. Western blot images showing a decrease in pS660-HSL expression in ingWAT of experimental mice relative to controls.

J. Summary plot showing plasma glycerol levels in the control (n= 8 mice) and experimental mice (n= 8 mice, unpaired *t*-test, *p<0.05)

Figure 5. Deleting the brain-liver axis elevates serum bile acid levels.

A. Graph showing mRNA expression of the enzymes involved in bile acid synthesis in liver. While there was no difference in *Cyp7a1* gene expression, the loss of hepatic cholinergic input significantly upregulated *Cyp7b1* mRNA expression (control (open circle), n=7-8 mice; experimental (closed black circle), n=7 mice).

B. Graphs showing *Cyp7a1* and *Cyp7b1* gene expression in the periportal (PP), midlobular (Mid), and pericentral (PC) zones in the livers of the controls and the experimental groups.

C. Graph showing fasting serum bile acid levels in the control (n=6 mice) and experimental groups (n=5 mice, unpaired t-test, *p<0.05).

D. Graph showing fasting serum bile acid profile in the control (n=4 mice) and experimental (n= 4 mice) mice. The Mann-Whitney test revealed a statistically significant difference between the two groups (*p<0.05).

Figure 6. Liver-ingWAT communication contributes to beiging of ingWAT via bile acids.

A. Graph showing mRNA and protein expression levels of *Tgr5* in ingWAT. There was a significant increase in TGR5 expression in the ingWAT of ChAT^{Cre} mice that received AAVrg-FLEX-dtA (control (open circle), n=7 mice; experimental (closed circle), n=7 mice, unpaired *t*-test, **p<0.01).

B. Schematic illustration showing our experimental configurations.

C. Reduction in *Tgr5* gene and protein levels in ChAT^{Cre} mice following administration of AAVrg-FLEX-dtA to the liver (black circle) and another injection of *Tgr5* siRNA into both sides of the ingWAT (blue circle)(unpaired *t*-test, *** P <0.001).

D. Representative images of ingWAT sections stained with H&E. ChAT^{Cre} mice without DMV cholinergic neurons innervating the liver receiving an injection of *Tgr5* siRNA into ingWAT exhibited larger adipocytes compared to the control mice (upper panel). Scale bar, 50 μm Bottom panel: higher magnification view of the black dotted square. Arrow represents crown-like structure. Scale bar, 100 μm

E and **F**. Plot showing the distribution of average adipocyte size. The mean cell area was significantly larger in the experimental group receiving *Tgr5* siRNA than in the experimental group receiving control siRNA (**I**) (control, n=5 mice; experimental, n=5 mice, unpaired *t*-test, *p<0.05)

Figure 7. *Tgr5* knockdown in ingWAT causes hepatic steatosis in ChAT^{Cre} mice receiving AAVrg-FLEX-dtA during high-fat diet feeding.

A. Gel images showing the Western blot analysis of ingWAT from experimental mice revealed an increase in the expression of pS660-HSL when compared to the control group.

B. Graph showing plasma glycerol levels in the controls (black circle, n= 6 mice) and the experimental groups (blue circle, n= 5 mice). Unpaired *t*-test, **p<0.01

C and D. Representative images of liver sections stained with H&E (**C**) and Oil Red O (**D**). Upper panel: scale bar, 100 μm; bottom panel: higher magnification view of the black dotted square scale bar, 50 μm

E. Graph showing hepatic TG levels in the controls (n= 5 mice) and the experimental groups (n= 6 mice) . Unpaired *t*-test, *p<0.05

Experimental procedures

Ethics statement

All mouse care and experimental procedures were approved by the Institutional Animal Care Research Advisory Committee of the Albert Einstein College of Medicine and performed in accordance with the guidelines described in the NIH Guide for the Care and Use of Laboratory Animals. Viral injections were performed under isoflurane anesthesia.

Animals

Eight-nine-week-old ChAT^{Cre} mice were purchased from Jackson Laboratory (JAX stock # 006410)[67]. We backcrossed them to C57BL/6J mice for several generations, and the mutant mice were bred together to make this line homozygous. Mice were housed in cages under conditions of controlled temperature (22 °C) with a 12:12 h light-dark cycle and fed a high-fat diet (HFD; Research Diets, D12492; 20% calories by carbohydrate, 20% by protein, and 60% by fat) for 10-12 weeks, with water provided *ad libitum*. Mice were euthanized with an overdose of isoflurane at the end of the experiment.

Western Blotting

Proteins were isolated from the liver using CelLytic™ MT (Sigma, C3228) in the presence of a protease inhibitor/phosphatase inhibitor cocktail (Thermo Fisher Scientific, 78443). Proteins from the ingWAT were extracted using a protein extraction kit for adipocyte tissue (Invent, AT-022).

Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, 23225). Proteins (30-50 ug) were prepared by adding laemmli sample buffer (Bio-Rad, 1610747) and heated at 95°C for 5min. The samples were then separated using 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was incubated with 5% w/v non-fat dry milk or 5% BSA for 1 h at room temperature and immunoblotted with anti-HSL (phospho-serine 660) (1:1000, Cell Signaling Technology, 45804S), anti-HSL (1:1000, Cell Signaling Technology, 4107), rabbit anti-UCP1(1:1000, Abcam, ab234430), rabbit anti-CHRM1 (1:200, Alomone Labs, AMR-001), rabbit anti-CHRM2 (Alomone Labs, AMR-002), rabbit anti-CHRM3 (Alomone labs, AMR-006), rabbit anti-CHRM4 (Alomone labs, AMR-004), rabbit anti-CHRM5 (Alomone Labs, AMR-005), and rabbit anti-β-actin (Cell Signaling, 4970S) antibodies. The specificity of anti-CHRM1-5 antibodies was tested by preincubation with the blocking peptide (CHRM1 with BLP-MR001, CHRM2 with BLP-MR002, CHRM3 with BLP-MR006, CHRM4 with BLP-MR004, and CHRM5 with BLP-MR005). Following incubation with primary antibodies, the membrane was washed three times in TBS-T and then incubated with an anti-rabbit IgG, HRP-linked antibody (1:10,000, Cell Signaling Technology,

7074) for 2 h at room temperature. ECL reagents were applied to the membrane and protein bands were detected using an Odyssey Fc imaging system (Li-COR).

Immunostaining to examine the hepatic cholinergic system

To examine DMV cholinergic neurons innervating the liver, $ChAT^{Cre}$ mice received an AAV.Php.eB-flex-tdTomato (titer; $1.9x10^{13}$ pfu/ml, Addgene 28306-PHP.eB) injection into the liver. We also injected AAV-phSyn1(s)-FLEX-tdTomato-T2A-SypGFP-WPRE (Addgene 51509-AAV1) into the DMV (500 nl of 4 x 10^{12} pfu/ml per site; coordinate; AP: -7.5 mm, ML: ±0.1 mm, DV: -3.5 mm) to label cholinergic nerve terminals in the liver. Mice were killed 5 and 10 weeks post-viral inoculation.

Mice were anesthetized with isoflurane (3%) and transcardially perfused with pre-perfusion solution (9 g NaCl, 5 g sodium nitrate, 10,000 U heparin in 1L distilled water) followed by 4% paraformaldehyde solution. Liver sections were stained with a rabbit anti-GFP antibody (Rockland, 600-401-215, 1:500) to visualize cholinergic nerve terminals. Brainstem sections were labeled with rabbit anti-RFP (Rockland, 600-401-379, 1:500) and goat anti-ChAT antibodies (Millipore, AB144P, 1:200). The expression of muscarinic ACh receptors CHRM2 and 4 was examined using rabbit anti-CHRM2 (Alomone, AMR-002, 1:200) and rabbit anti-CHRM4 (Alomone, AMR-004, 1:200) antibodies. Cholinergic nerve terminals were stained with goat anti-vAChT (Millipore, ABN100) and goat anti-ChAT (Millipore, AB144P) antibodies. The tissue samples were incubated with AlexaFluor 568 donkey anti-goat IgG (Invitrogen, A11057, 1:200), AlexaFluor 568 donkey anti-rabbit IgG (Invitrogen, A11011, 1:200), and AlexaFluor 488 donkey anti-rabbit IgG (Invitrogen, A21206, 1:200). The tissues were washed, dried, and mounted with VECTASHIELD medium containing DAPI. Images were acquired using a Leica SP8 confocal microscope.

Clear, unobstructed brain imaging cocktails and computational analysis (CUBIC) tissue clearing of the brainstem and liver tissues

The CUBIC method [68] was used to clear liver and brainstem tissues. Mice were perfused with 4% (w/v) paraformaldehyde (PFA) in PBS. Liver and brain tissues were collected and post-fixed in 4% PFA at 4C° for 24 h. Tissue samples were sectioned using a vibratome at a thickness of 300 μm. The sections were immersed in CUBIC reagent 1(25wt% Urea, 25wt% Quadrol, 15wt% Triton X-100) with gentle shaking at 37C° for 3 days. After decolorization (transparent tissues), the sections were washed with PBS three times at RT for 2 h and then incubated in a blocking solution composed of 1% donkey serum, 0.5% BSA, 0.3% Triton X-100, and 0.05% sodium azide for 3 h at RT with rotation. The tissue samples were incubated with

goat anti-ChAT (Millipore, AB144P, 1:200), rabbit anti-RFP (Rockland, 200-301-379 1:200), and rabbit anti-GFP (Rockland, 600-401-215, 1:200) antibodies for 5 days at RT with mild shaking, and washed three times with PBS (each for at least 2 h). Following wash-out, the tissue samples were incubated with AlexaFluor 568 donkey anti-goat IgG (1:200, Invitrogen, A11057), AlexaFluor 568 donkey anti-rabbit IgG (1:200, Invitrogen, A11011), and AlexaFluor 488 donkey anti-rabbit IgG (1:200, Invitrogen, A21206) for 3 days at room temperature. Tissues were washed and immersed in CUBIC-reagent 2 (50wt% Sucrose, 25wt% Urea, 10wt% Triethanolamine, and 0.1%(v/v) Triton X-100) for 1 d and mounted with a mixture of mineral oil and silicone oil. Z-stack images were taken with a Leica SP8 confocal microscope, and 3D construction was performed with the AIVIA community (version 12.1).

Viral injection to delete DMV cholinergic neurons innervating the liver

pAAV-mCherry-FLEX-dtA (Addgene, 58536) was subcloned and packaged in an AAV serotype retrograde (Applied Biological Materials, Inc.). A total volume of 10 -20µl of AAVrg-mCherry-FLEX-dtA (titer, >1 × 10^{12} pfu/ml) was injected into the medial and left lobes of the livers of the $ChAT^{Cre}$ mice. AAVrg-FLEX-GFP viruses (titer, >1x10^{13} pfu/ml) were used as the controls.

To quantify cell death over time, the mice were killed 10-12 weeks after viral inoculation. Mice were anesthetized with isoflurane (3%) and transcardially perfused with PBS and heparin, followed by 10% neutral buffered formalin solution (Sigma, HT501128). Brainstem and liver tissues were embedded in paraffin to create paraffin blocks. Paraffin blocks were cut into 10 µm sections for the brainstem and 5 µm sections for liver tissues at our histology and comparative pathology core facility.

Coronal sections of the brainstem from approximately −7.2 to −7.6 mm posterior to bregma were stained with an anti-ChAT antibody (Millipore, AB144P). Cell counting of DMV cholinergic neurons was performed using the ImageJ software (version FIJI). The adipocyte cell area was measured using the Adiposoft plugin (version 1.16) in the Image J software.

Measurement of body weight, body composition, and blood glucose

Body weight was measured weekly at 9 am. Body composition for fat mass and fat-free mass was assessed by ECHO MRI at our animal physiology core. Blood samples were collected from the mouse tail, and a small drop of blood was placed on the test strip of a glucose meter. Non-fasting basal glucose levels were measured at 9:00 am. Fasted blood glucose levels were measured after overnight fasting at 10 weeks post-viral injection.

Assessment of energy expenditure and locomotor activity

To examine if the loss of function of hepatic cholinergic input regulates energy expenditure and locomotor activity, we performed indirect calorimetry on mice fed HFD for 10 weeks. Mice were individually housed in the calorimeter cages and acclimated to the respiratory chambers for at least 2 days prior to gas exchange measurements. Indirect calorimetry was performed for 3 days at the end of 10 weeks using an open-circuit calorimetry system. O_2 consumption and CO_2 production were measured for each mouse at 10-min intervals over a 24-h period. The respiratory exchange ratio was calculated as the ratio of CO_2 production over O_2 consumption. Since there was no difference in food intake, VO_2 was normalized for body lean mass. Locomotor activity in X-Y and Z planes was measured by infrared beam breaks in the calorimetry cages.

Assessment of glucose tolerance and insulin tolerance

For GTT, the experimental and control mice were fasted for 15 h (6:00 pm–9:00 am) at 10 weeks post-viral inoculation. Sterile glucose solution was intraperitoneally administered at a concentration of 2 g/kg (glucose/body weight) at time 0. Blood glucose levels were measured at 15, 30, 60, 90, and 120 min after glucose injection. Blood glucose levels versus time after glucose injection were plotted, and the area under the curve was calculated and compared between the experimental and control groups.

For ITT, the mice were fasted for 4 h (9:00 am to 1:00 pm). Blood glucose levels were measured at 0, 15, 30, 60, 90, and 120 min following i.p. injection of insulin (1 U/kg). We immediately injected glucose (2 g/kg) if the mice appeared ill owing to insulin-induced hypoglycemia.

RT-qPCR analysis

Liver tissues were collected from the experimental and control groups 10-12 weeks post viral inoculation. Liver tissues were homogenized in Trizol reagent (ThermoFisher Scientific, 15596-018), and total RNAs were isolated according to the manufacturer's instructions. First-strand cDNAs were synthesized using the SuperScript III First-Strand synthesis kit (ThermoFisher Scientific, 18080-051). qPCR was performed in sealed 96-well plates with SYBR Green I master Mix (Applied Biosystems, A25742) using the Quant Studio 3 system (Applied Biosystems). qPCR reactions were prepared in a final volume of 20 µl containing 2 µl cDNAs, and 10 µl of SYBR Green master mix in the presence of primers at 0.5 µM. Glyceraldehyde 3-phosphate dehydrogenase *(Gapdh)* for liver and 18S ribosomal RNA (*18s rRNA*) for white adipocyte tissue were used as an internal control for quantification of each sample. Amplification was performed under the following conditions: denaturation at 95 °C for 30 seconds, followed

by 40 cycles of denaturation at 95 °C for 30 seconds, and annealing/extension at 60 °C for 1 minute.

We examined the following genes; *Srebp1c* (NM_001358315.1), *Chrebp* (NM_021455.5), *Acly* (NM_134037.3), *Acaca* (NM_133360.3), *Fasn* (NM_007988.3), *Scd1* (NM_009127.4), *Ppara* (NM_011144.6), *Prdm16* (NM_027504), *Cpt1a* (NM_013495.2), *Cpt2* (NM_009949.2), *Acadm* (NM_007382.5), *Acadl* (NM_007381.4), *G6pase* (NM_008061.4), *Pck1* (NM_011044.3), *Slc2a2* (NM_031197.2), *Pgc1a* (NM_008904.3), *Ucp1* (NM_009463.3), *Dio2* (NM_010050.4), *Cidea* (NM_007702.2), *Gpbar1* (NM_174985.1), *Cyp7a1* (NM_007824), *Cyp7b1* (NM_007825) (Table S1). All the primer sequences will be validated with "Primer BLAST" primer design program to ensure specificity for the target gene. Melt curves and gel electrophoresis were analyzed to confirm the specificity of the PCR products. Relative gene expression was determined using a comparative method ($2^{-\Delta\Delta CT}$).

Spatial Transcriptomics

Livers were snap-frozen in liquid nitrogen and thawed briefly on ice, followed by embedding in a cryomold (Sakura, 4565) filled with Neg-50 (Epredia, 6502). The cryomold was then frozen by placing into a container with pre-chilled 2-methylbutane on dry-ice. The samples were cryosectioned (Cryostar NX70 Cryostat, -18C for blade, -18 ^{o}C for specimen head, set at 10mm thickness) and placed on a pre-chilled Visium Spatial Gene Expression Slide (10x Genomics, PN-2000233). The tissue sections were processed following 10x Genomics protocols (PN-1000187, PN-1000215). The adaptors and sequencing primers were obtained from Illumina. The libraries were sequenced by Novogene using the NovaSeq 6000 (PE150) instrument targeting 80,000 reads per spot. Fastq files were aligned against mouse mm10 reference and histology images were processed with Spaceranger (2.0). Pericentral, mid-zone and periportal clusters were identified based on Graph-Based clusters and known zonation markers (*Cyp2e1*, *Glul*, *Gulo* for pericentral, *Cyp2f2*, *Hsd17b13*, *Sds* for periportal). Violin plots were generated using Loupe Browser (6.4.1).

Measurement of hepatic and plasma TG and ACh levels

Liver samples were harvested from mice with or without liver-innervating cholinergic neurons. Tissue samples were weighed and homogenized in 1 mL of diluted NP-40 solution to solubilize triglycerides. The samples were centrifuged at 16,000 × g to remove insoluble material. The TG levels were measured using a TG assay kit (Cayman Chemical, 10010303).

For the acetylcholine assay, liver tissues were homogenized in PBS. The homogenates were then centrifuged at 5,000 g for 5 min. The supernatants were collected, and ACh levels were determined using an acetylcholine ELISA kit (Novus, NBP2-66389).

Measurement of plasma insulin levels and fasting serum bile acid levels

Blood samples from the experimental and control groups were collected from the retroorbital plexus using heparinized capillary tubes. Whole blood was centrifuged at 12,000 × g for 10 min, and plasma was separated and stored at -20°C until use. Plasma insulin concentrations were determined using two-site sandwich ELISA kits (Mercodia, cat# 10-1247-0). To analyze fasting total bile acid levels in the serum, fasting blood samples were collected without anticoagulant and determined using mouse total bile acid (Crystal Biochem, Cat#80471). Bile acid profiling was performed at our metabolomic core facility.

Statistics

All statistical results are presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism, version 10.0. Two-tailed *t*-tests were used to determine the p-values for each pairwise comparison. The Mann-Whitney U test was performed to evaluate the differences in bile acid composition between the groups. Time-course comparisons between groups were analyzed using a two-way repeated-measures analysis of variance (RM ANOVA) with Sidak's correction for multiple comparisons. Data were considered significantly different when the probability value was less than 0.05.

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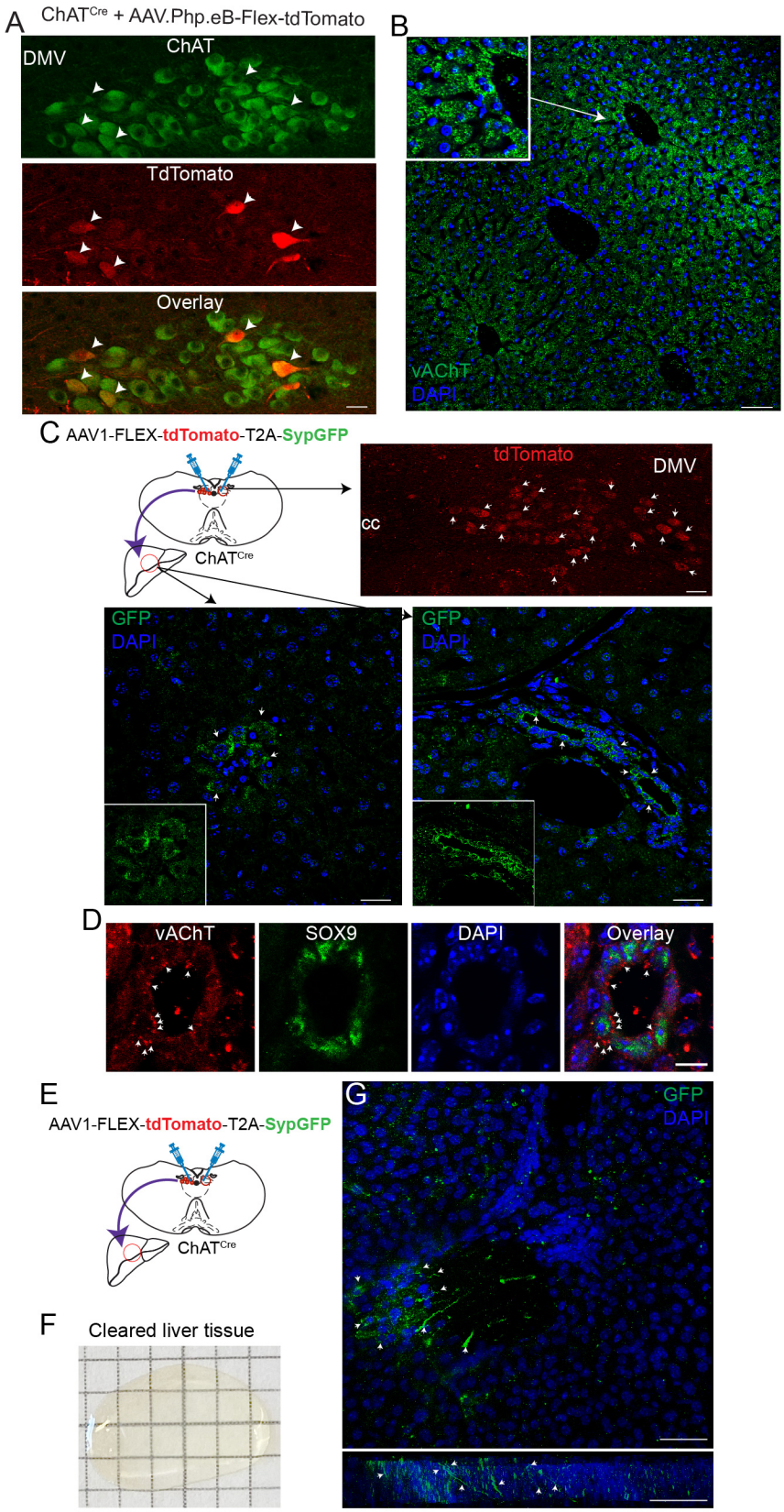
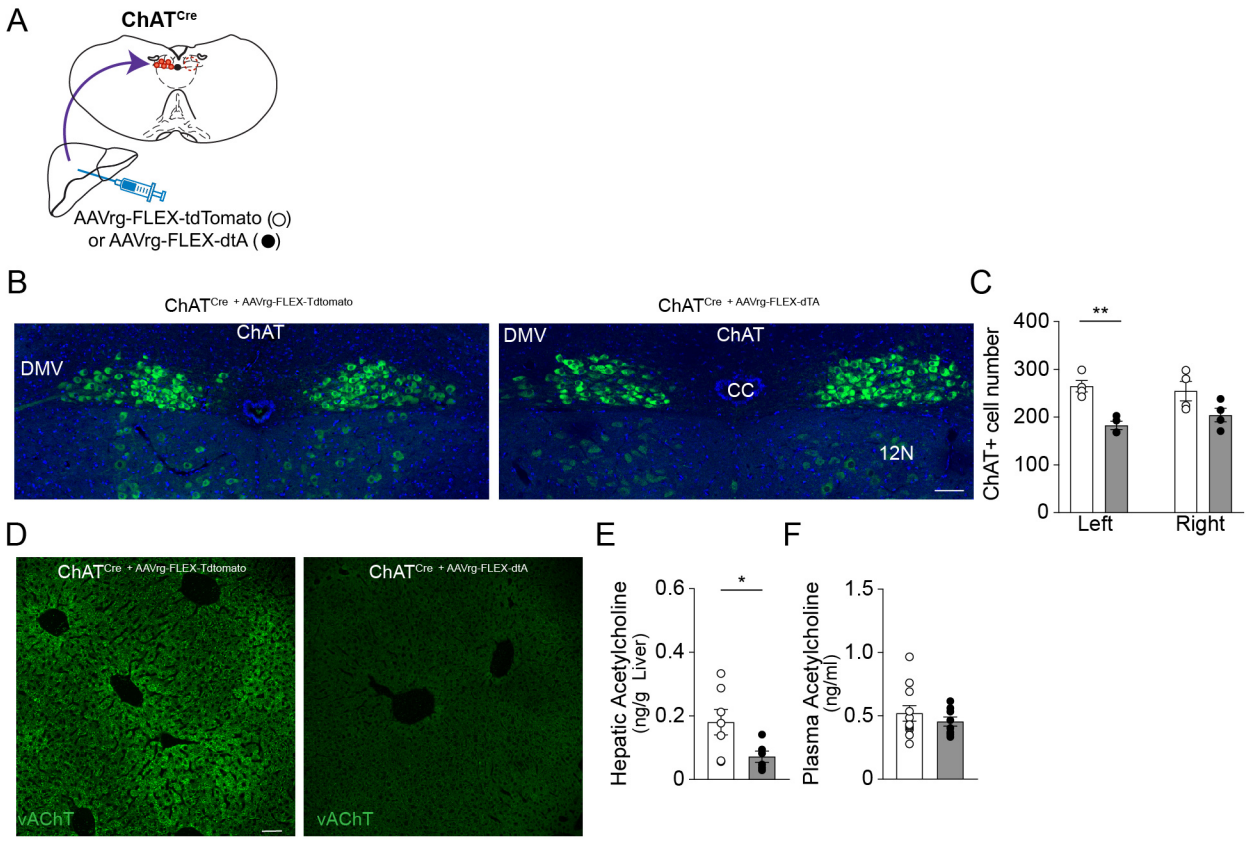


Figure 1. Hwang et al.



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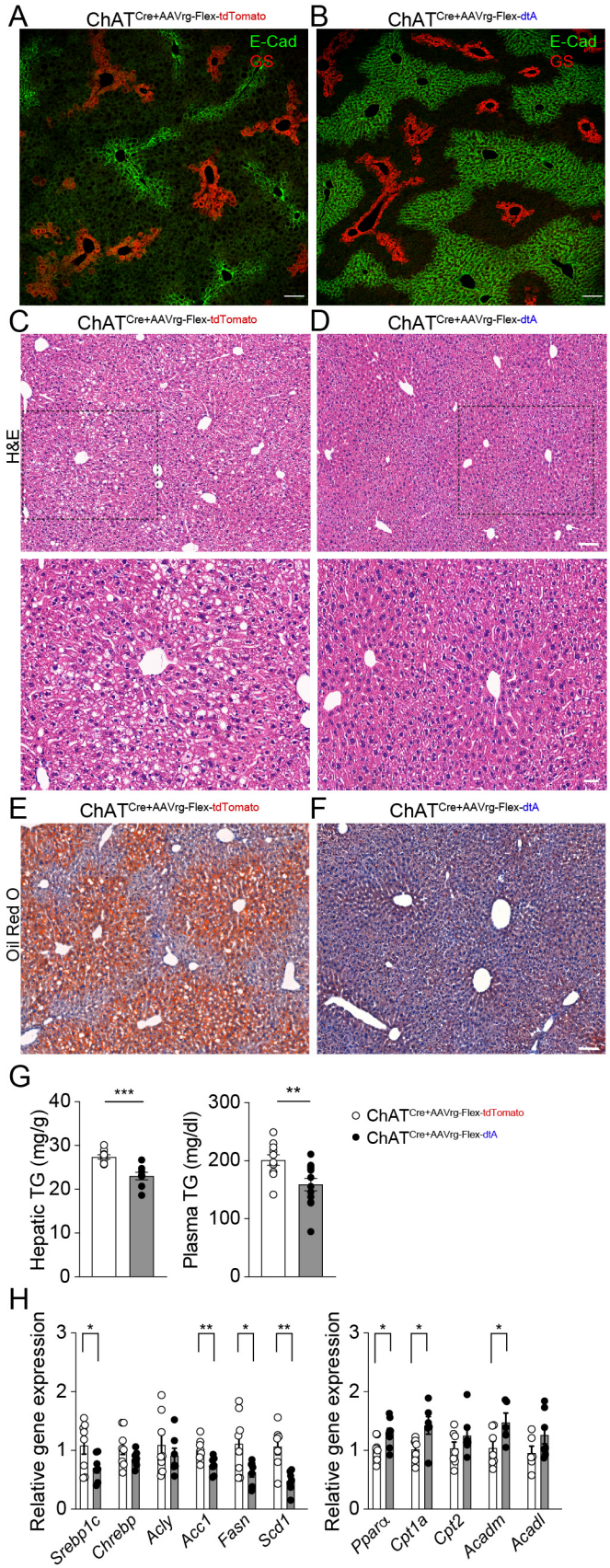

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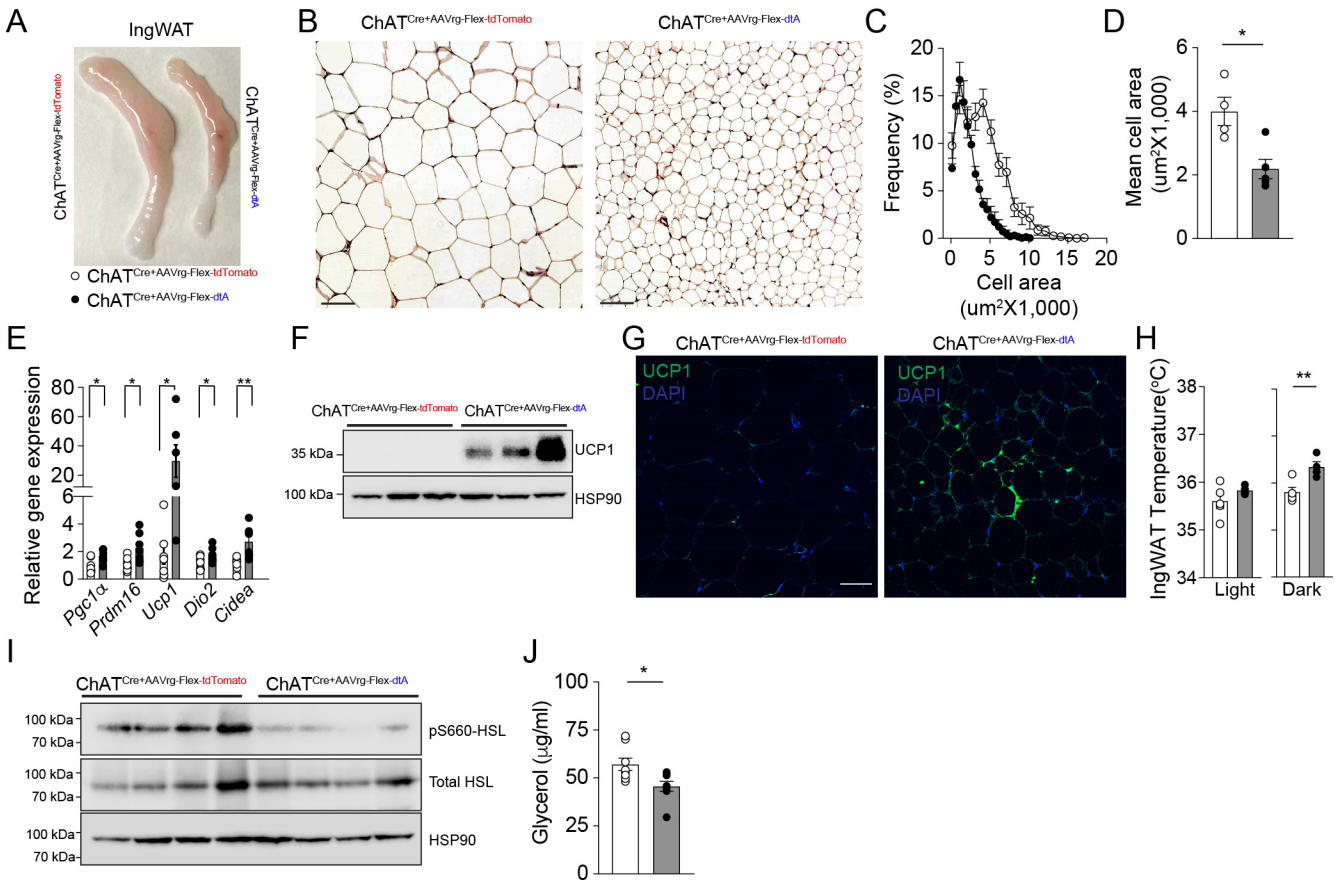

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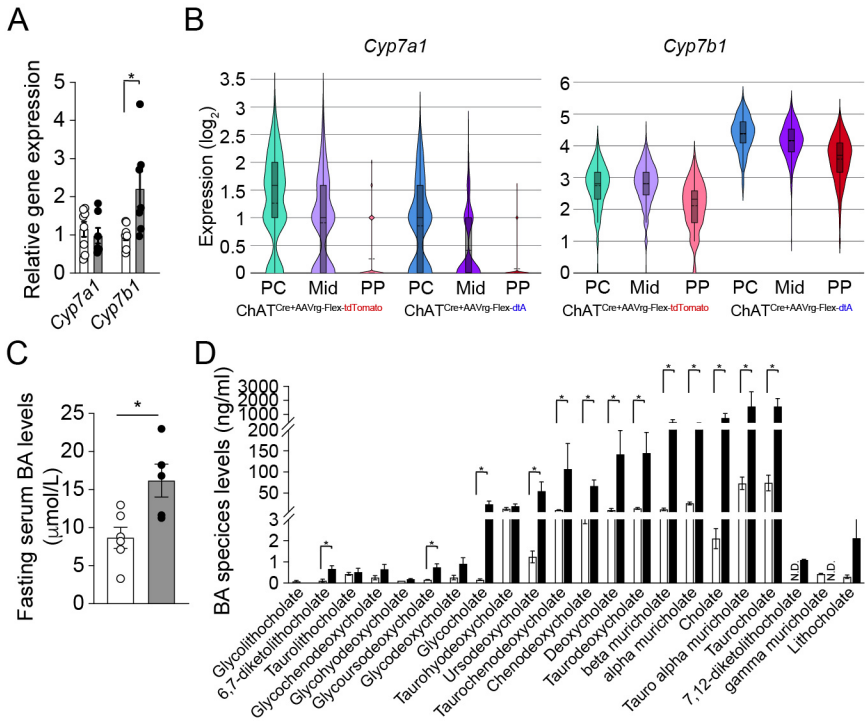

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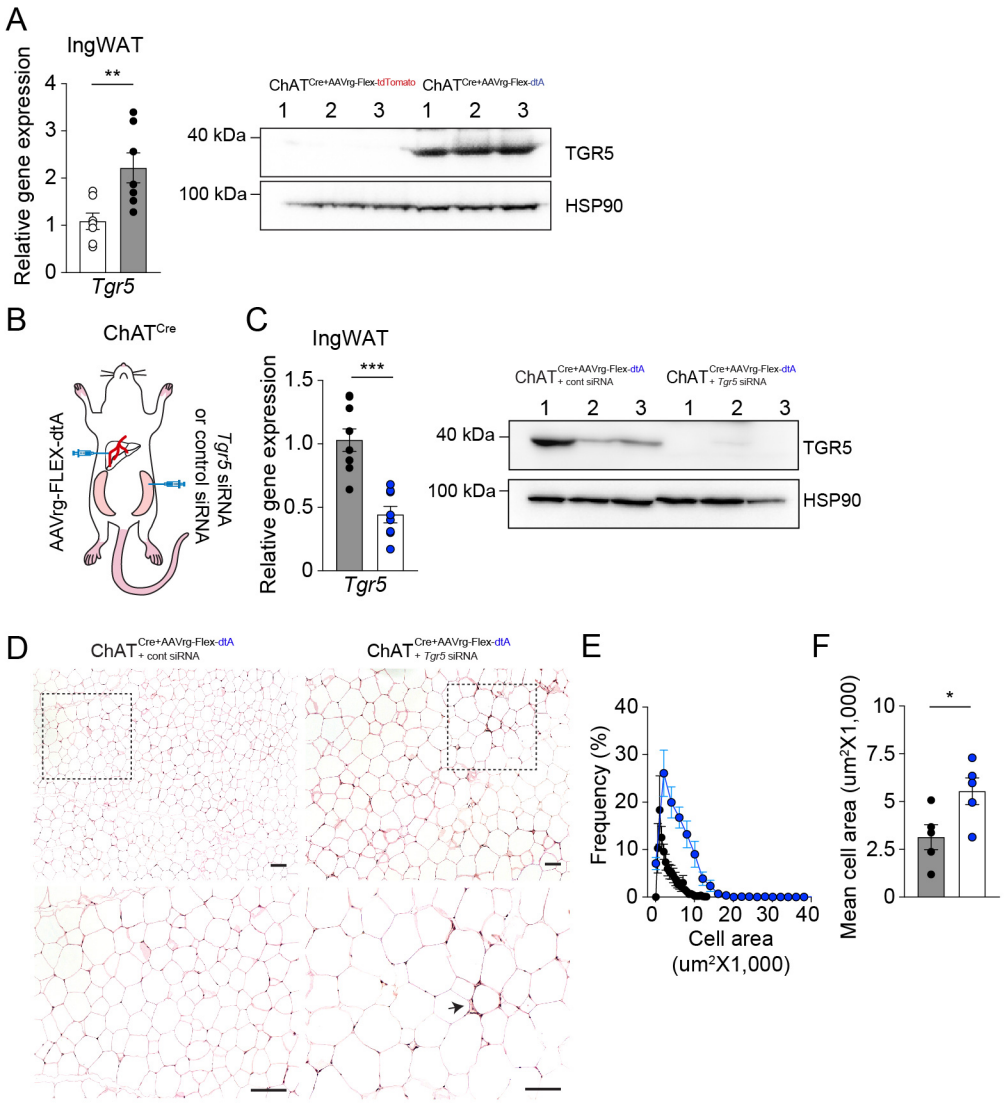

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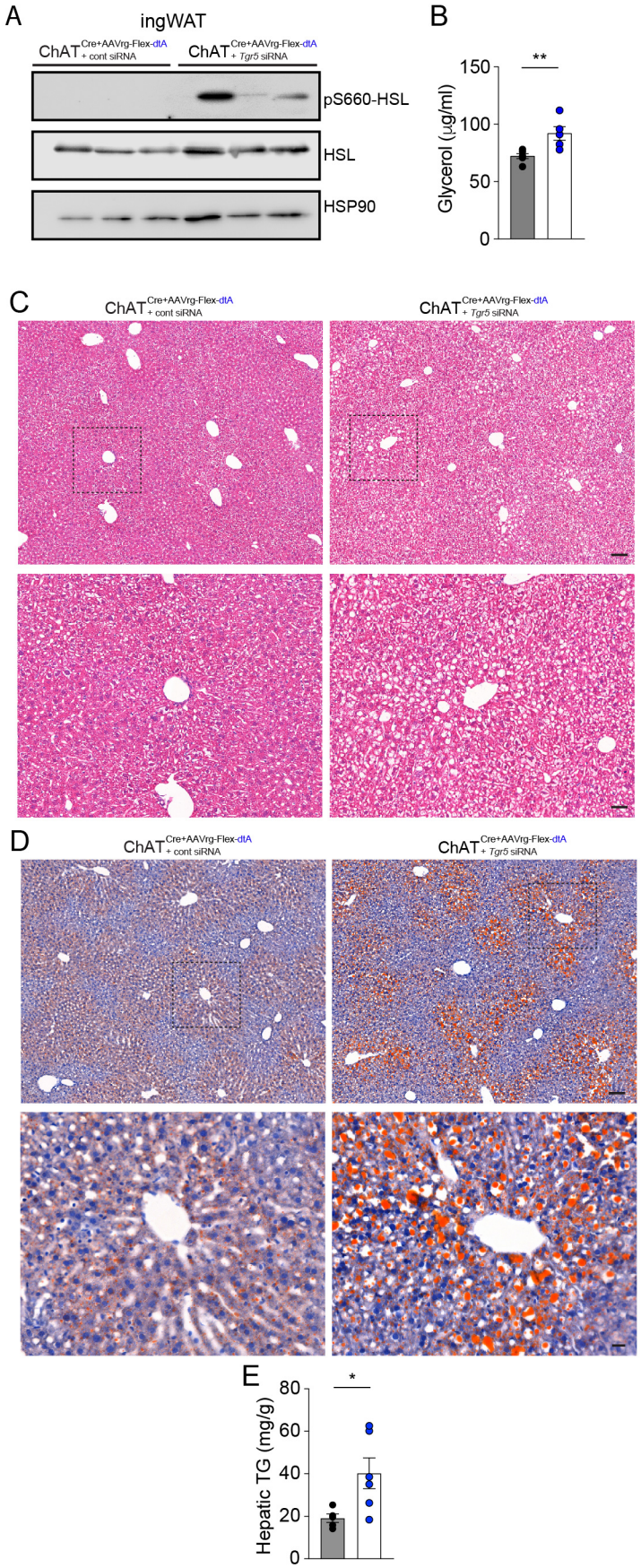

Figure 7. Hwang et al.