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ETHANOL DYSREGULATES SIRT1-MEDIATED LIPOGENIC SIGNALING  
PATHWAYS THROUGH  $\alpha 4^*$ -NICOTINIC ACETYLCHOLINE RECEPTORS IN  
HEPATOCYTES

By  
Caitlin Christina Wilkerson  
B.S., Bellarmine University, 2020

A Master's Thesis  
Submitted to the Faculty of the  
School of Medicine of the University of Louisville  
in Partial Fulfillment of the Requirements  
for the Degree of

Master of Science  
in Pharmacology and Toxicology

Department of Pharmacology and Toxicology  
University of Louisville  
Louisville, KY

December 2023

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A Master's Thesis Approved on

June 22nd, 2023

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## DEDICATION

I would like to dedicate this thesis, first, to my mother, Kelly, for her unending support and encouragement throughout every step of this process; and to the rest of my family, chosen and biological: Scott, Cameron, Kayla, Claudia, and Meagan for their confidence in me and always reminding me to smile.

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## ABSTRACT

### ETHANOL DYSREGULATES SIRT1-MEDIATED LIPOGENIC SIGNALING PATHWAYS THROUGH $\alpha 4^*$ -nAChRs IN HEPATOCYTES

Caitlin C. Wilkerson

June 22<sup>nd</sup>, 2023

Alcohol-associated liver disease (ALD) is a broad spectrum of diseases ranging from steatosis to severe hepatic cirrhosis, none of which have any FDA approved therapies. Downregulation of hepatic Sirtuin 1 (Sirt1) activity by ethanol has been shown to lead to an upregulation of the pro-lipogenic gene targets under its jurisdiction, resulting in increased lipogenesis and triglyceride accumulation. It was hypothesized that ethanol acts on  $\alpha 4^*$ -nicotinic acetylcholine receptors ( $\alpha 4^*$ -nAChRs) in hepatocytes to decrease Sirt1 expression, which ultimately dysregulates lipid metabolic homeostasis. Ethanol's effects were investigated in cultured AML12 hepatocytes and in WT primary mouse hepatocytes acutely exposed to ethanol. Consistent with the hypothesized mechanism of action, ethanol treated hepatocytes showed a significant decrease in Sirt1 mRNA expression and an increase in subsequent lipogenic genes compared to untreated cells. Pre-treatment with  $\alpha 4^*$ -nAChR inhibitor, dihydro- $\beta$ -erythroidine, blocked ethanol's downregulation of Sirt1 and the subsequent upregulation of its downstream targets. Taken together, these data support a role for  $\alpha 4^*$ -nAChRs expressed by hepatocytes in mediating the early effects of ethanol on the liver.



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## INTRODUCTION

According to the World Health Organization (WHO), chronic ethanol use accounts for 5.1% of the global disease and injury burden [1]. Much of the chronic toxicity induced by excessive ethanol consumption manifests as pathologies in the liver since this is the primary site of ethanol metabolism in mammals [2]. Chronic ethanol consumption and misuse leads to impaired hepatic lipid metabolism, but the initial targets exploited by ethanol to induce early liver injury remain elusive, making it increasingly difficult to design therapies against these pathologies. These facts taken together highlight the need for characterizing the early alcohol signaling mechanism in hepatocytes, and for the development of therapies targeting steatosis to mitigate the progression and severity of ALD.

### **Alcohol Associated Liver Disease**

Approximately 1 billion people worldwide are classified as heavy alcohol drinkers, 90 percent of whom will develop fatty liver or steatosis, the first of many pathologies comprising alcohol associated liver disease (ALD) [2, 3]. ALD is a broad spectrum of diseases ranging from steatosis to severe hepatic cirrhosis and/or hepatocellular carcinoma, none of which have any FDA approved therapies. In addition, ALD is one of the most commonly diagnosed liver diseases around the world and, as of 2017, accounted for 25% of the deaths by cirrhosis [4]. Generally, the severity of ALD is determined in a dose-response relationship by the amount

and duration of ethanol consumption, though different biologic, genetic, and environmental factors are also known to influence susceptibility [4, 5].

Chronic ethanol consumption and misuse leads to impaired lipid metabolism, first manifesting as steatosis. Next in the spectrum of ALD comes steatohepatitis, followed by increasing fibrosis that leads to further inflammation and scarring in the liver [4, 6]. This excessive inflammation ultimately results in alcohol associated hepatitis and cirrhosis, which may result in hepatocellular carcinoma [4]. Though abstinence from ethanol consumption is known to reverse early-stage ALD, progression to other stages generally requires steatosis as a “first-hit,” altering the hepatic landscape and priming the liver for “second-hits” and progressive stages of the disease [4, 7].

### **Ethanol’s Effects on Lipid Metabolism**

Excessive ethanol consumption leads to steatosis, broadly, by impairing hepatic lipid metabolism. Specifically, ethanol has been shown to decrease rates of hepatic fatty acid  $\beta$ -oxidation and very low density lipoprotein (VLDL) export while increasing rates of lipogenesis and lipid import [8]. For example, chronic ethanol consumption has been shown to increase the expression of hepatic fatty acid transporter CD36 which does not usually play a role in hepatic lipid import [8, 9]. To increase the hepatic production of triglycerides (TG), ethanol promotes the activity of enzymes and regulatory factors orchestrating *de novo* lipogenesis and inhibits those involved in fatty acid  $\beta$ -oxidation and VLDL production and transport [8].

Although the exact mechanisms resulting in ALD are not fully understood, ethanol consumption is known to impact the biological processes involved in and regulating lipid anabolism [2]. When ethanol is fully oxidized to acetate, the ratio of  $\text{NAD}^+/\text{NADH}$  is decreased which favors hepatic inhibition of fatty acid  $\beta$ -oxidation and further promotes stressful conditions within the cell [7]. As a result of its oxidation, ethanol inhibits the expression and activity of Sirtuin 1 (SIRT1), one of the key metabolic energy sensors regulating lipid metabolism [10, 11]. SIRT1, a member of the silent information regulator 2 family, belongs to a group of highly conserved  $\text{NAD}^+$ -dependent protein and/or histone deacetylases [11]. Due to the dependency of Sirt1 on  $\text{NAD}^+$ , ethanol administration leads to SIRT1 inhibition because of the decreased  $\text{NAD}^+/\text{NADH}$  ratio observed following consumption [2, 8, 11].

There are 7 mammalian Sirtuins, but SIRT1 is most extensively studied in the context of fatty liver diseases because it is responsible for deacetylating important pro-lipogenic transcription factors such as sterol regulatory element binding protein 1 (SREBP-1c, Srebp-1c). In humans, SREBP-1c acetylation following ethanol-induced SIRT1 inhibition results in the upregulation of hepatic TG synthesis through transcriptional induction of SREBP-1c's downstream pro-lipogenic gene targets [11, 12]. When active, SREBP-1c promotes the transcription of genes such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN), both essential in de novo lipogenesis and known to be impacted by alcohol consumption [13, 14].

In concert with SIRT1, AMP-activated protein kinase (AMPK) is a key metabolic protein kinase and regulator of lipid metabolism through phosphorylation of lipogenic target enzymes, including ACC and SREBP-1c [8, 15, 16]. In fed states, AMPK's phosphorylation hinders the activity of ACC, whose activity results in malonyl CoA formation. Malonyl CoA serves as both an inhibitor of fatty acid  $\beta$ -oxidation through inhibition of carnitine palmitoyltransferase 1 (CPT 1) and a precursor for fatty acid synthesis [17]. This said, inhibition of malonyl CoA production through ACC phosphorylation by AMPK is crucial for maintenance of metabolic homeostasis.

The activity states of AMPK and SIRT1 are hard to disentangle, partially due to their mutual regulation and the many target genes under both of their control [8, 13, 18]. Upon AMPK activation, processes increasing ATP are upregulated which results in the increased production of NAD<sup>+</sup>: the substrate required for SIRT1 function [8, 18]. Following ethanol administration, AMPK activation is decreased which leads to impaired mitochondrial  $\beta$ -oxidation as well as an increase in the activity of the pro-lipogenic transcription factor SREBP-1c, through inhibition of SIRT1 [8, 15, 17]. Though the resulting increase in lipogenesis is understood, the molecules responsible for ethanol's interaction with SIRT1 remain mysterious.

Regarding the early manifestations of ALD such as steatosis and steatohepatitis, it is currently believed that SIRT1 is one of the primary signaling molecules affected by acute or chronic ethanol consumption [13]. Murine studies have highlighted Sirt1's role in mitigating ethanol-induced hepatotoxicity [19-23], but the mechanisms by which ethanol works to inhibit Sirt1 function, aside from

decreasing the NAD<sup>+</sup>/NADH ratio, are not entirely understood. In homeostatic conditions, human cells tightly maintain the activity and expression of SIRT1 at the transcriptional, post-transcriptional and post-translational level by using positive and negative feedback mechanisms of regulation [24]. Following acute cellular stress, SIRT1 activation results in its increased positive transcriptional regulation, leading to an increase in SIRT1 mRNA levels [24]. Studies have shown that ethanol administration decreases both the mRNA and protein levels of murine Sirt1, correlating to worsening ALD pathology in mice [19, 25, 26].

In a study ultimately identifying novel upstream regulators of Sirt1 in ALD, a murine whole body knockout model for  $\alpha 4^*$ -nAChRs ( $\alpha 4$  KO; (where \* denotes pentamers containing the  $\alpha 4$  subunit)) were protected from the Sirt1 downregulation and hepatic steatosis that normally occurs in response to chronic ethanol exposure [10]. The protection of  $\alpha 4$  KOs from the decrease in Sirt1 protein expression extensively reported following chronic ethanol exposure implicates  $\alpha 4^*$ -nAChRs in ethanol's interaction with the deacetylase [2, 8, 10, 11, 26]. Other animal studies have reported the expression of  $\alpha 4^*$ -nAChRs in additional non-neuronal tissues including the pancreas, heart and kidney, indicating the potential for alternate functionality depending on organ [27-29].

### **Ethanol's Effects on Neuronal nAChRs**

nAChRs belong to a superfamily of Cys-loop ligand-gated ion channels and are responsible for the mediation of multiple biological processes [30, 31]. Primarily, these receptors respond to endogenous acetylcholine (ACh) binding by undergoing a conformational change which allows cations to flow into the cell. In

the brain, this cation flow results in signaling cascades aimed at stimulating neurotransmitter release and neuronal plasticity [31, 32]. Like all members of the Cys-loop superfamily, nAChRs are found complexed as pentamers of  $\beta(1-4)$  and/or  $\alpha(1-10)$  subunits, where subunit composition and stoichiometry determines ligand selectivity, affinity, and which ions are allowed to flow through the receptor upon ligand binding [30, 31, 33]. Ethanol has been shown to impact nAChRs differently depending on their subunit composition, going so far as to potentiate or inhibit the currents induced by other drugs [31, 34].

Along with ACh, neuronal nAChRs have a high affinity for certain xenobiotics, specifically nicotine. Nicotine binds orthosterically to nAChRs while studies show ethanol's ability to interact with nAChRs both orthosterically and allosterically [30, 31, 34]. Many nAChRs are expressed in the pre-synaptic region of neuronal synapses to mediate  $Ca^{2+}$  dependent neurotransmitter release although  $\alpha 7$ - and  $\alpha 4\beta 2$ -nAChRs are the most abundant [31]. Broadly, in order to exert the rewarding effects associated with their use, ethanol and nicotine exploit nAChR signaling mechanisms to increase dopamine release in the nucleus accumbens [31]. The nucleus accumbens is found in the ventral tegmental area of the brain which contains the mesocorticolimbic dopamine reward system, responsible for imparting the rewarding and reinforcing effects of various drugs, including ethanol.

Since it has not been confirmed whether ethanol binds directly at the ACh binding site on nAChRs [34, 35], ethanol modulation of these receptors is explained in the context of conformational states [31, 33]. Specifically, nAChRs



appear in three states depending on their exposure to ligand/agonist: active, when the channel permits free flow of cations down their concentration gradient; closed, when the receptor has the least binding affinity for ligand; desensitized, when the receptor is impeded, leaving it unable to respond to ligand activation [31]. Although it is not clear exactly how ethanol interacts with nAChRs, studies indicate intoxicating doses of ethanol (100 mM) may result in  $\alpha 4\beta 2$ -nAChR current potentiation by stabilizing the receptor in its active, open state [31, 34]. Studies utilizing site directed mutagenesis revealed that the M2 region of nAChRs, responsible for pore formation in the transmembrane domain, may be contributing to the binding pocket for ethanol and seems to be necessary for ethanol's potentiating effects on  $\alpha 4\beta 2$ -nAChRs [31, 36].

Because ethanol misuse is often concomitant with nicotine use, nAChRs have been investigated as potential targets for therapies designed for smoking cessation and ethanol abstinence [31, 37, 38]. Designing these therapies has proven challenging in part because the effects of ethanol on nAChR signaling are dependent on many factors, including dose and receptor subunit composition [30, 31]. nAChRs also exhibit specificity and preference for ligands. Neuronal  $\alpha 4\beta 2$ -nAChRs are known to have the highest affinity for nicotine binding and are abundantly expressed in the VTA [30, 31]. All of this together explains why these receptors have been studied extensively in neuronal regions for their interactions with nicotine and ethanol [31, 34, 35, 39].

## Non-Neuronal nAChRs

Originally, it was believed that nAChRs functioned only in the brain due to their breadth of action and expression here. Since then, there have been various studies completed that indicate nAChR expression and functionality in non-neuronal tissues, specifically lung epithelia and fibroblasts [40-43]. Additionally, through the extensive use of genomic databases, a comprehensive epigenetic landscape of nAChRs and their regulatory mechanisms revealed a high expression of nAChRs in various non-neuronal tissues [43]. The  $\alpha 4$  nAChR subunit is encoded by the *CHRNA4* gene which was shown to be highly expressed in human liver although with a different, non-neuronal expression pattern [43]. Ultimately, this meta-analysis identified a liver-specific *CHRNA4* promoter region with tissue specific epigenetic modifications upstream of the canonical neuronal *CHRNA4* promoter [43]. Along with these modifications, it was found that the transcription factors hepatocyte nuclear factor 4 alpha and retinoid x receptor alpha regulate the expression of *CHRNA4* in hepatocytes [43]. These transcription factors are not the same as those regulating *CHRNA4* expression in neural tissues, but they are abundantly expressed in hepatic tissues.

Liver specific transcription factors coupled with a non-canonical hepatic promoter region support the idea that the hepatic expression of nAChRs is regulated differently than those in the brain and their functions should be investigated. Aligning with the discovery of an alternate hepatic epigenetic and transcriptional activation profile of *CHRNA4* [43], the whole body lack of  $\alpha 4^*$ -

nAChRs resulting in protection from known ethanol associated hepatotoxicity suggests that these receptors are mediating the effects of ethanol in the liver.

### **Rationale for Study**

Chronic ethanol consumption is known to affect many biological systems, but many of the pathologies induced by ethanol misuse manifest in the liver since this is the primary site of ethanol metabolism in mammals [2, 4]. Further, ethanol metabolism primarily occurs in hepatocytes due to the abundant expression of metabolizing enzymes in these cells, making them specifically susceptible to ethanol's effects [2]. Although compelling, whole body knockout mice for  $\alpha 4^*$ -nAChRs cannot confirm the protective effects against ethanol are due to their lack of expression in hepatocytes, nor can they accurately delineate the mechanisms from which the phenotypic results arise.

It does appear that hepatic  $\alpha 4^*$ -nAChRs play a role, at least partially, in ethanol-induced hepatotoxicity but the mechanisms by which this effect occurs remain unclear. As previously stated, upon excessive ethanol consumption, lipid homeostasis is affected in several ways such that lipogenesis is increased while mitochondrial fatty acid  $\beta$ -oxidation is hindered. Along with altered lipid homeostasis, ethanol metabolism itself is an additional source of liver damage due to reactive oxygen species and acetaldehyde generation [2]. Despite widespread knowledge of the downstream effectors of ethanol exposure, the most proximal mediators of ethanol-induced hepatotoxicity remain elusive. There are differing theories about which receptors and upstream molecules may be involved in the hepatic signaling mechanisms mediated by ethanol, but the most recent and

promising of these is the  $\alpha 4^*$ -nAChR [10, 43]. The purpose of the present study was to probe the ethanol-induced  $\alpha 4^*$ -nAChR dependent pro-lipogenic signaling mechanisms in hepatocytes which are eliminated by whole-body knockout of this receptor [10].

## MATERIALS & METHODS

### **Primary Hepatocyte Isolation**

Male and female wild-type (WT) C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were housed in a pathogen-free facility with a 12-h light/dark cycle and access to food and water ad libitum. At 8 months of age, the mice were deeply anesthetized by ketamine/xylazine for hepatocyte isolation by collagenase perfusion. Briefly, a peristaltic pump was used to perfuse buffer containing calcium chloride dihydrate (560 mg/L) and Collagenase D (from *Clostridium histolyticum*; 125 U/L; Sigma Aldrich, Burlington, MA) at 5 mL/min through the inferior vena cava and to the liver for subsequent digestion. Once the organ began to swell, the hepatic portal vein was cut, and the digestion buffer was allowed to perfuse through the liver until digestion was complete. Next, the liver was excised and pulverized in growth medium for access to the now free liver cells in suspension. After a series of washes, a 10:1 iso-osmotic Percoll® solution was used to separate dead cells and non-hepatocytes from the desired fraction. Hepatocytes were analyzed for viability by Trypan Blue. Viable cells were plated at 400,000 cells/well in Waymouth's Growth medium supplemented with 10% fetal bovine serum, 1% antibiotic/antimycotic, 0.2% ITS (Gibco™, Grand Island, NY), and 100 nM dexamethasone on rat tail collagen type 1 (Gibco™, Grand Island, NY) coated 6-well plates (Corning™, Corning, NY).

## **Cell Culture**

*Human Hepatocytes:* Cryopreserved human hepatocytes (CHH) from BioIVT were a generous gift to us from the Hein Lab at the University of Louisville. CHH were plated in 12-well plates at 80% confluency in InVitroGRO HT medium (BioIVT) containing 1 mL TORPEDO™ Antibiotic Mix (BioIVT) per 45 mL media medium. Cells were allowed to adhere to Biocoat® collagen-coated plates (Corning™) overnight before treatment with 100 mM ethanol for an additional 24 h.

*Primary Mouse Hepatocytes:* WT primary mouse hepatocytes (PMH), isolated as previously described, were allowed to adhere to rat tail collagen type 1 (Gibco™, Grand Island, NY) coated 6-well plates (Corning™, Corning, NY) overnight before treatment with 100 mM ethanol for an additional 24 h.

*AML12 Cells:* AML12 cells obtained from ATCC (Manassas, VA) were plated at 200,000 cells/mL in 6-well cell culture plates and allowed to adhere overnight before treatment. Cells were maintained in DMEM:F12 (Gibco™) media supplemented with 10% fetal bovine serum, 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium (ITS, Gibco™), 1% penicillin/streptomycin and 100 nM dexamethasone, at 37 °C in a 5% CO<sup>2</sup> atmosphere. In some experiments, after adherence, AML12 cells were pre-treated for 2 h with 400 µM of the  $\alpha 4\beta 2$ -nAChR selective antagonist dihydro- $\beta$ -erythroidine (DH $\beta$ E; Tocris Bioscience, Bristol, UK) or 4 mM of the alcohol dehydrogenase (ADH) inhibitor 4-methylpyrazole (4-MP; Sigma Aldrich, Burlington, MA) before treatment with 100 mM ethanol for an additional 48 h.

## RT-qPCR

Following treatment and respective incubation, TRIzol® reagent (Invitrogen, Waltham, MA) was added to wells containing PMH or AML12 cells. Cell suspensions were collected, and RNA was separated into aqueous and organic phases by chloroform. Aqueous phase was collected; isopropanol was added to extract and precipitate the RNA; ethanol was used to wash and purify the RNA. Purified RNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham MA) then reverse transcribed using the qScript cDNA Synthesis Kit (Quanta Bio, Beverly, MA). Real-time quantitative PCR (RT-qPCR) was performed for the quantification of Chrna4, Sirt1, Srebp1c, Fasn, Dgat2, and  $\beta$ -actin mRNA expression using KiCqStart SYBR® Green Gene Expression Assays (human Chrna4, H\_CHRNA4\_2; mouse Chrna4, M\_Chrna4\_1; Sirt1, M\_Sirt1\_1; Srebp1c, M\_Srebf1\_1; Fasn, M\_Fasn\_2; Dgat2, M\_Dgat2\_1; Sigma Aldrich) according to the manufacturer's instructions. A StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA) was used with the following reaction settings: (holding stage: 95°C 30 s; cycling stage: 95°C 1 s; 60°C 30 s; melt curve stage: 95°C 15 s; 60°C 1 min; 95°C 15 s). Results were analyzed using Applied Biosystems (Waltham, MA) StepOnePlus™ Software version 2.0.2 and the Ct values obtained were normalized to  $\beta$ -actin  $\Delta$ Ct from untreated samples. Results were expressed as the fold increase in mRNA target compared to samples from untreated cell lysates using the  $2^{-\Delta\Delta C_t}$  method.

## **Statistical Analysis**

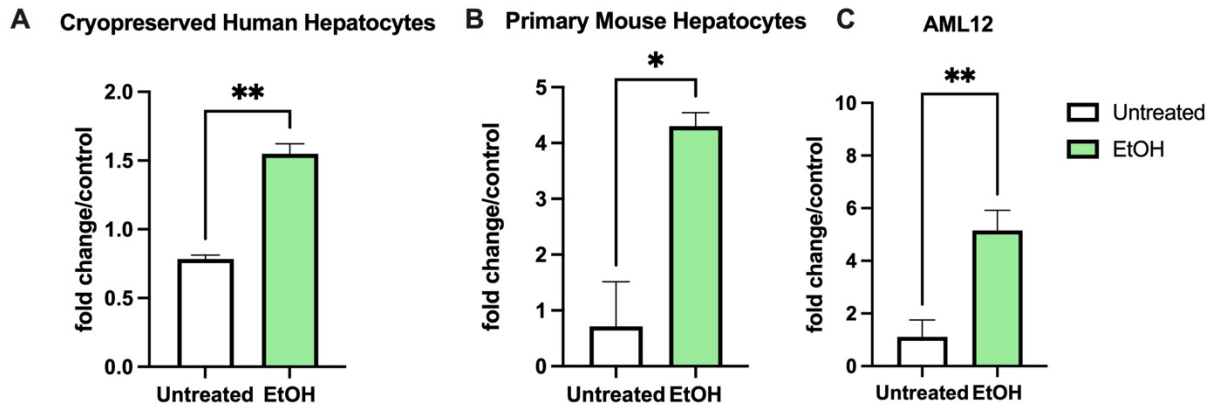
Each experiment was plated in triplicate; Data were analyzed using GraphPad Prism by Two-Way ANOVA followed by Fisher's exact post-hoc test for multiple comparisons or by Student's t-tests and represented as means + SD, with significance set at  $p < 0.05$ . The number of asterisks (\*) indicate the level of significance such that: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\* $p < 0.0001$ .



## RESULTS

***Chrna4* expression is increased after ethanol exposure in hepatocyte models.** We hypothesize that xenobiotic activation of nAChRs may be followed by increased transcription of the receptor's subunits due to the desensitization that occurs following repeated agonist exposure [31, 44]. Recurrent exposure of nAChRs to agonizing xenobiotics results in receptor upregulation, although the mechanisms underlying this process remain unclear [44]. In studies investigating the impact of ethanol on  $\alpha 4^*$ -nAChRs in the brain and liver of mice respectively, chronic ethanol exposure led to increased mRNA expression of the  $\alpha 4$  nAChR subunit, potentially signifying their sustained activation by ethanol [10, 45]. To confirm the presence of this phenomenon in and further solidify AML12 cells as an *in vitro* model of the endogenous murine liver, *Chrna4* expression levels were compared between CHH, PMH, and AML12 cells following ethanol exposure. CHH, PMH and AML12 cells were cultured in the presence of 100 mM ethanol for up to 48 h. Using RT-qPCR, it was found in all hepatocyte models acutely exposed to 100 mM ethanol that  $\alpha 4$  nAChR subunit mRNA expression was significantly increased (Figure 1). Each hepatocyte model revealing an increase in *Chrna4* mRNA expression after ethanol exposure indicates that  $\alpha 4^*$ -nAChRs are likely being stimulated in hepatocytes in response to acute ethanol challenge. The increase in  $\alpha 4$  nAChR subunit mRNA seen in AML12 cells following acute ethanol administration allows for further speculation that similar mechanisms are occurring

in this cell line as those seen in murine hepatocytes *ex vivo* following acute ethanol exposure.



**Figure 1.  $\alpha 4^*$ -nAChR mRNA (Chrna4) is expressed in mouse and human hepatocytes and its expression is increased by exposure to ethanol.**

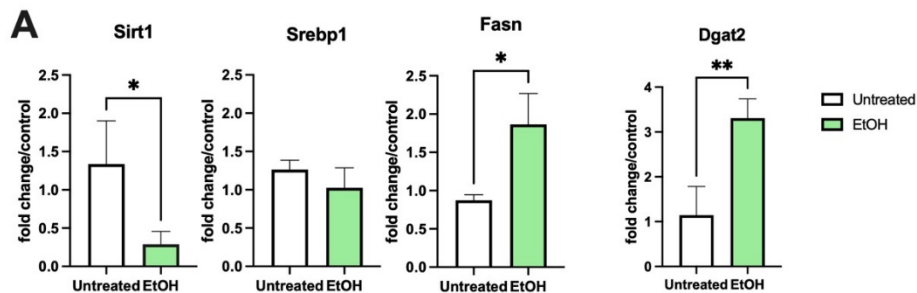
(A) Cryopreserved human hepatocytes were treated without or with (EtOH) 100 mM ethanol for 24 h. (B) Primary mouse hepatocytes were isolated from C57BL/6J male mice. Cells were left to adhere to collagen coated 6-well plates then treated without or with (EtOH) 100 mM ethanol for 24 h. (C) AML12 cells were treated without or with (EtOH) 100 mM ethanol for 48 h. RNA was isolated from the cells or tissue, and Chrna4 mRNA levels were measured by RT-qPCR. Results were normalized to  $\beta$ -actin and expressed relative to controls by the  $2^{-\Delta\Delta C_t}$  method. Each experiment (A-C) was plated in triplicate; Data are analyzed by Student's t-test and presented as means + SD. The number of asterisks (\*) indicate the level of significance.

***Ethanol exposure increases the expression of genes involved in Sirt1-mediated lipogenesis.*** To further probe the mechanism utilized by ethanol to induce lipogenesis, primary hepatocytes isolated from WT C57BL/6J mice and AML12 cells were isolated and allowed to adhere overnight before treatment. Both cell lines were treated with 100 mM ethanol for 24 h and 48 h respectively. TRIzol™ Reagent (Invitrogen, Waltham, Massachusetts, USA) was used to extract RNA from cells for quantification of Sirt1, Srebp-1c, Fasn, and Dgat2 mRNA by RT-qPCR. In both primary hepatocytes and AML12 cells, RT-qPCR of Sirt1 mRNA revealed a significant decrease in expression of the gene (Figure 2A, B). Decreased Sirt1 mRNA expression is in line with the known effects of chronic ethanol exposure on Sirt1 expression and activity [10, 19, 24], and led us to look downstream to view ethanol's effect on pro-lipogenic genes in this acute exposure model. RT-qPCR quantification of Srebp-1c, Fasn, and Dgat2 mRNA in PMH following ethanol challenge showed the expected decrease in Sirt1 followed by an increase in pro-lipogenic genes, although there was not a significant increase in Srebp-1c mRNA expression in this model (Figure 2A). AML12 cells showed a significant decrease in Sirt1 mRNA expression, coupled with a significant increase in the mRNA expression of its target genes when compared to untreated cells plated in parallel (Figure 2B).

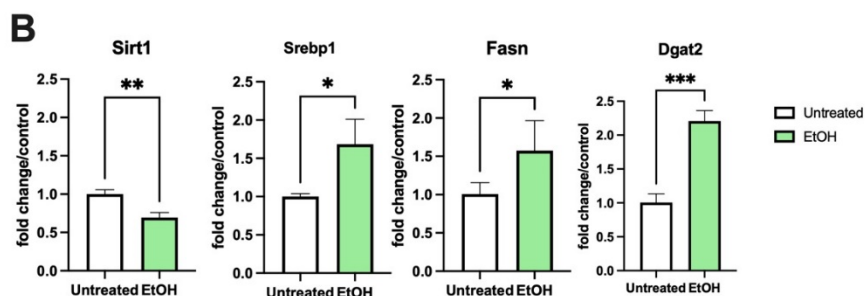
The absence of the Srebp-1c effect in primary hepatocytes could have been due to their loss of hepatocyte nature or their time in culture, but the repeated congruency between PMH and AML12 cells regarding the expression of pro-lipogenic endpoints after ethanol challenge further supports AML12 cells as a

sufficient model of the murine liver. Due to the previously reported similarities between AML12 cells and PMH shown here and previously reported (Figure 1, 2) [46], AML12 cells were used for the remainder of the study's mechanistic investigation.

## Relative mRNA Expression in Primary Mouse Hepatocytes



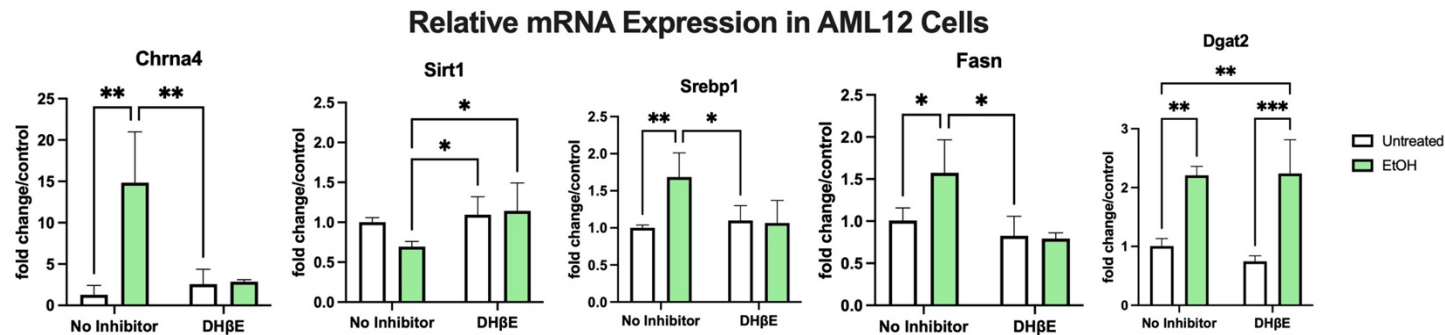
## Relative mRNA Expression in AML12 Cells



**Figure 2. Ethanol exposure initiates pathological Sirt1-mediated lipogenesis.** (A) Primary mouse hepatocytes and (B) AML12 cells were incubated for 24 and 48 h with 100 mM EtOH, respectively. TRIzol™ Reagent (Invitrogen, Waltham, MA) was used for RNA extraction and mRNA levels of components of the Sirt1-Srebp1-Fasn-Dgat2 pathway were measured by RT-qPCR. Each experiment (A-B) was plated in triplicate; Data are analyzed by Student's t test and presented as means + SD. The number of asterisks (\*) indicate the level of significance.

***Ethanol-dependent upregulation of lipogenesis through Sirt1 inhibition is  $\alpha 4^*$ -nAChR dependent.*** To confirm that the effects seen in Figure 2 after ethanol exposure were indeed  $\alpha 4^*$ -nAChR dependent, we pre-exposed cells for 2 h with an  $\alpha 4\beta 2$ -nAChR selective inhibitor, dihydro- $\beta$ -erythroidine (DH $\beta$ E). Due to their validity as an *in vitro* model of murine hepatocytes *ex vivo*, AML12 cells were pre-treated for 2 h with 400  $\mu$ M DH $\beta$ E before being exposed to 100 mM ethanol for another 48 h. RNA was collected for RT-qPCR quantification of Sirt1, Srebp-1c, Fasn, and Dgat2 mRNA as previously described. The pro-lipogenic effects seen in AML12 cells after exposure to ethanol appear to be dependent on  $\alpha 4^*$ -nAChR activation. This interpretation is supported by the fact that pre-treatment with Dh $\beta$ E was able to prevent the ethanol induced increase in mRNA expression of Srebp-1c and its transcriptional target, Fasn, by eliminating the dampened expression of Sirt1 mRNA (Figure 3).

Although Dgat2 is the rate limiting enzyme in triglyceride synthesis, its increased expression does not appear to be  $\alpha 4^*$ -nAChR dependent as evident by the uninhibited effect of ethanol exposure on its mRNA expression (Figure 3). This can be explained by the fact that ethanol administration triggers a fed state cellular environment which may induce carbohydrate response element binding protein (ChREBP) mediated transcription of *Dgat2*, regardless of  $\alpha 4^*$ -nAChR activation, resulting in Dgat2's increased expression. Further studies are warranted to determine the interaction between  $\alpha 4^*$ -nAChR and upstream regulators of Dgat2, or the lack thereof, as the pro-lipogenic pathways influenced by ethanol are elucidated.



1  
2

**Figure 3. Activation of the Sirt1-Srebp1-Fasn pathway by ethanol is  $\alpha 4^*$ -nAChR-dependent.** AML12 cells were incubated with 100 mM ethanol for 48 h with or without 2 h pre-treatment with the  $\alpha 4\beta 2$ -nAChR-selective antagonist, dihydro- $\beta$ -erythroidine (DH $\beta$ E). mRNA levels of components of the Sirt1-Srebp1-Fasn pathway and Dgat2 were measured by RT-qPCR. Note that Dgat2 is induced by alcohol independent of  $\alpha 4^*$ -nAChR activity. Experiment was plated in triplicate; Data are analyzed by Two-Way ANOVA followed by Fisher's exact post-hoc test for multiple comparisons and presented as means + SD. The number of asterisks (\*) indicate the level of significance.

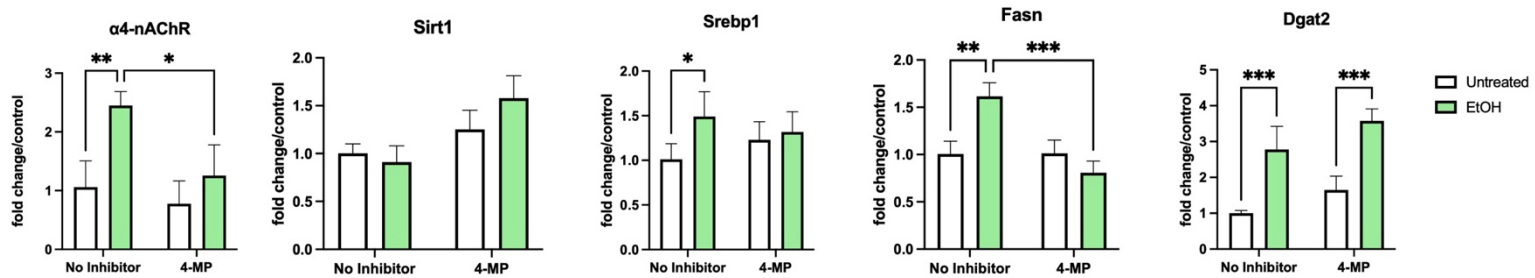


### **Ethanol metabolism plays a role in $\alpha 4^*$ -nAChR-dependent lipogenesis.**

Ethanol's dysregulation of critical processes involving lipid metabolism such as those regulating fatty acid synthesis is believed to be the primary cause of ethanol induced hepatotoxicity [2, 8]. The metabolism of ethanol itself poses a threat to the liver through the production of acetaldehyde after oxidation of ethanol by ADH [2]. Acetaldehyde itself is toxic to cells because of its ability to form protein, lipid, and DNA adducts which leads to cellular stress [47, 48]. To investigate the influence of ethanol metabolism on  $\alpha 4^*$ -nAChR-dependent lipogenesis following ethanol challenge, AML12 cells were pre-treated for 2 h with 4  $\mu$ M of the ADH inhibitor, 4-methylpyrazole (4-MP) before being exposed to 100 mM ethanol for another 48 h. RNA was collected for RT-qPCR quantification of Sirt1, Srebp-1c, Fasn, and Dgat2 mRNA as previously described. Similar to the effect seen following  $\alpha 4^*$ -nAChR inhibition with DH $\beta$ E, inhibition of ADH with 4-MP stopped the ethanol induced increase in Chrna4 mRNA expression observed following acute ethanol exposure (Figure 4). These results would suggest that ethanol metabolism may be sufficient or necessary for the  $\alpha 4^*$ -nAChR dependent activation of lipogenesis seen following ethanol exposure. Conversely, because the Sirt1 mRNA expression was not decreased in AML12 cells after 48 h of ethanol exposure (Figure 4), not much can be said about the effect of ethanol metabolism on Sirt1 mRNA levels. Instead, the decreased mRNA expression of Sirt1 may have been a missed observation as the experiment only viewed expression at one time point. Pre-treatment with 4-MP inhibited the ethanol-induced increase in Srebp-1c and Fasn while Dgat2

remained unaffected by 4-MP treatment (Figure 4), but further investigations are necessary to allocate the necessity of ethanol metabolism in inducing  $\alpha 4^*$ -dependent lipogenesis through Sirt1.

## Relative mRNA Expression in AML12 Cells



**Figure 4. Inhibition of ethanol metabolism attenuates the associated increase in  $\alpha 4^*$ -nAChR-dependent lipogenesis.** AML12 cells were incubated with or without 100 mM ethanol for 48 h, with or without pre-treatment with the alcohol dehydrogenase (ADH) inhibitor, 4-methylpyrazole (4-MP). mRNA levels of components of the Sirt1-Srebp1-Fasn pathway and Dgat2 were measured by RT-qPCR. Note that Dgat2 is induced by ethanol, independent of its metabolism. Experiment was plated in triplicate; Data are analyzed by Two-Way ANOVA followed by Fisher's exact post-hoc test for multiple comparisons and presented as means + SD. The number of asterisks (\*) indicate the level of significance.

## DISCUSSION

ALD presents as a spectrum of diseases ranging from steatosis to severe hepatic cirrhosis and carcinoma, none of which have any FDA approved therapies. Hallmarks of early-ALD include steatosis, or excessive triglyceride accumulation, accompanied by varying levels of inflammation and fibrosis. Though these steps are known, the precise etiology of early ALD pathologies, namely steatosis, is not fully understood. As stated previously, ethanol is known to alter the functionality of key metabolic regulator, SIRT1, but the molecular targets upstream and influencing the activity of SIRT1 remain elusive.

It has been shown phenotypically that mice lacking  $\alpha 4^*$ -nAChRs were protected from ethanol induced steatosis [10], but the work discussed here is the first to investigate the mechanisms contributing to this phenotype. We are the first to delineate  $\alpha 4^*$ -nAChRs as a novel target of ethanol, upstream of Sirt1, that is mediating the ethanol-dependent modulation of the Sirt1-Srebp-1c-Fasn pathway in mice. Herein, we have shown through pharmacological manipulation that the ethanol-induced steatosis seen in murine hepatocytes is regulated, at least at the transcriptional level, through  $\alpha 4^*$ -nAChR dependent changes in lipogenesis.

### **Ethanol-Induced Hepatic $\alpha 4^*$ -nAChR Signaling**

A commonly used *in vitro* model for the investigation of hepatic processes that underly various pathologies has been HepG2 cells due to their origin from human tissue. Although HepG2 cells originate from a human male, they originate

from a hepatocellular carcinoma and are genetically abnormal, containing anywhere from 50-100 chromosomes [49]. Also, HepG2 cells lack many hepatocyte-specific enzymes found in PMH, such as those involved in xenobiotic metabolism, which hinders the translatability of results obtained in HepG2 cells [46, 49]. It has been established that more genetically 'normal' cell lines, such as the immortalized-non-cancerous cell line AML12, phenotypically model PMH more accurately [46]. Because of this, we used AML12 cells as a model to probe the mechanisms involved in ethanol induced- $\alpha 4^*$ -nAChR dependent lipogenesis.

In another study investigating the functionality of  $\alpha 4^*$ -nAChRs in non-neuronal tissues, it was reported that  $\alpha 4^*$ -nAChR mRNA expression was significantly increased in the lungs of mice exposed to ethanol [41]. Coinciding with increased mRNA expression, the staining density of  $\alpha 4^*$ -nAChRs in the lung increased following ethanol exposure, potentially due to activation [41]. Since we are the first to investigate the validity of AML12 cells as an acute model of ethanol-induced  $\alpha 4^*$ -nAChR signaling in the liver, the  $\alpha 4^*$ -nAChR mRNA expression of CHH, PMH, and AML12 cells was quantified following ethanol exposure. Both primary cell lines showed a significant increase in  $\alpha 4^*$ -nAChR mRNA expression after ethanol exposure, coinciding with the perceived interaction between ethanol and peripheral  $\alpha 4^*$ -nAChRs based on established literature (Figure 1A-B) [31]. AML12 cells revealed a significant increase in  $\alpha 4^*$ -nAChR mRNA expression following ethanol exposure (Figure 1C) similarly to both human and murine primary hepatocytes, supporting the use of AML12 cells as a sufficient model for ethanol induced  $\alpha 4^*$ -nAChR-dependent lipogenesis.

AML12 cells have been widely used to model early ALD and have been repeatedly shown to have an increase in TG levels following ethanol exposure [50-53], aligning with the reported increase in TG levels seen *in vivo* following chronic ethanol exposure. In previous studies, AML12 cells have been used to investigate ethanol induced changes in hepatic signaling mechanisms, including those involving Sirt1/Ampk [51, 53]. Although these studies investigate Sirt1 in regard to ethanol induced hepatotoxicity, they fail to investigate and identify the interactions occurring *upstream* of Sirt1 as we have in the present study.

### **Sirt1-Srebp-1c-Fasn Pathway**

In line with the proposed hepatic function of  $\alpha 4^*$ -nAChRs being to promote lipogenic signaling cascades, acute exposure of both PMH and AML12 cells to ethanol led to a significant decrease in Sirt1 mRNA expression (Figure 2). Sirt1 regulates the activity of many downstream transcription factors, including Srebp-1c which promotes the transcription of various pro-lipogenic genes such as Fasn in a stepwise pathway (Figure 5). This pathway is known to be affected by ethanol exposure [7, 8, 12, 13], making it a reasonable point of investigation regarding ethanol's potential induction of  $\alpha 4^*$ -nAChR-dependent lipogenesis.

After ethanol administration, PMH showed a significant increase in Fasn mRNA expression although Srebp-1c mRNA was not induced upon exposure to ethanol (Figure 2A). In AML12 cells, ethanol administration led to a significant increase in Srebp-1c and Fasn mRNA expression, aligning with the significant ethanol-induced decrease in Sirt1 mRNA expression seen in this model (Figure 2B). Solidifying the necessity of  $\alpha 4^*$ -nAChRs to induce Sirt1-mediated lipogenesis

after ethanol exposure, pharmacological inhibition of  $\alpha 4^*$ -nAChRs with DH $\beta$ E protected murine hepatocytes from the mRNA induction of Srebp-1c and its downstream target, Fasn in AML12 cells (Figure 3).

DH $\beta$ E is a competitive antagonist of nAChRs with assumed selectivity for  $\alpha 4\beta 2$ - and  $\alpha 4\beta 4$ -nAChRs [54]. This selectivity comes from increased binding affinity at these nAChR pentamer combinations, but DH $\beta$ E has been shown to antagonize  $\alpha 3\beta 2$ - and  $\alpha 3\beta 4$ -nAChRs as well, albeit with much lower affinity [54]. Due to the low concentration required to antagonize  $\alpha 4\beta 2$ -nAChRs, DH $\beta$ E is often used to study signaling through this receptor [54] but investigation into the specific nAChR subunit(s) localizing with hepatic  $\alpha 4^*$ -nAChRs warrants further study. The downstream targets of Sirt1 were unaffected by ethanol exposure when cells were pre-treated with DH $\beta$ E and Sirt1 mRNA expression showed a trend downwards following ethanol exposure, although insignificant (Figure 3). Only one time point was observed in the experiment reported, meaning we may have missed the significant decrease in Sirt1 mRNA expression at the time point observed. Additionally, Figure 3 is representative of an experiment with technical replicates so repeated experimentation with biological replicates is needed to give insight into the effect of  $\alpha 4^*$ -nAChR inhibition on ethanol's impairment of Sirt1-mediated lipogenesis. Still, the results shown in Figure 3 support the role of  $\alpha 4^*$ -nAChRs in mediating the ethanol-induced induction of lipogenesis through Sirt1 targets Srebp-1c and Fasn.

## **Dgat2 is $\alpha 4^*$ -nAChR-Independent**

Based on the extensively reported result of hepatic TG accumulation following ethanol administration, it was hypothesized that the  $\alpha 4^*$ -nAChR mediated effects of ethanol on lipogenesis involve Dgat2. Contrary to this hypothesis, the pharmacological inhibition of  $\alpha 4^*$ -nAChRs did not hinder the increase in Dgat2 mRNA expression following ethanol administration (Figure 3). In human cells, DGAT2 works as the rate limiting enzyme in hepatic TG synthesis by covalently binding acyl-CoA and diacylglycerol molecules, resulting in TG formation [55]. Due to its necessity for the increased production of TG, DGAT2 has been studied for its role in the etiology of fatty liver diseases [55, 56]. Studies show that ethanol consumption increases the genetic induction and proteomic expression of murine Dgat2 [55], allowing for speculation that ethanol works in concert with Dgat2 to induce hepatic steatosis.

One explanation for Dgat2's lack of  $\alpha 4^*$ -nAChR dependence lies in the regulation of Dgat2 transcription. Contrary to many of the other key pro-lipogenic enzymes (i.e., Acc, Fasn) falling under the regulation of Srebp-1c, genetic regulation of Dgat2 is maintained by carbohydrate response element binding protein (Chrebp) (Figure 5) [56]. Chrebp is indeed a transcriptional regulator, but its activity is stimulated largely by carbohydrates or a cellular "fed-state" [57]. The known ethanol-induced inhibition of Sirt1/Ampk gives rise to a "pseudo-fed" state where the activation of their downstream targets results in enhanced energy storage cascades [58].



Although a “pseudo-fed” state seems to arise from the  $\alpha 4^*$ -nAChR dependent ethanol-induced decrease in Sirt1 (Figure 2, 3), Dgat2 expression is not mediated by the Sirt1-Srebp-1c-Fasn pathway (Figure 5) [56], which could be why pharmacological inhibition of  $\alpha 4^*$ -nAChRs did not diminish the ethanol-induced increase in Dgat2 mRNA expression (Figure 3). Future studies are required to delineate between ethanol-induced  $\alpha 4^*$ -nAChR-dependent and independent pro-lipogenic signaling pathways in human cells and *in vivo* as the current work was conducted only in murine cell culture models.

### **ADH in $\alpha 4^*$ -nAChR-Dependent Lipogenesis**

When the metabolism of ethanol by ADH was hindered pharmacologically, the ethanol induced increases in  $\alpha 4^*$ -nAChR, Srebp-1c, and Fasn mRNA were abolished in AML12 cells (Figure 4). Sirt1 mRNA expression was unchanged following ethanol exposure and pre-treatment with 4-MP (Figure 4) so there cannot be conclusions drawn about the effect of ethanol metabolism on Sirt1 mRNA expression. This outcome was incongruent with results found throughout the rest of the study which points to the need for further investigation into ethanol’s effects on Sirt1 and the source of NAD<sup>+</sup>/NADH ratio manipulation following ethanol exposure [59]. Future investigations should include metabolites of ethanol such as acetaldehyde since its oxidation requires NAD<sup>+</sup> as an electron acceptor and produces NADH, decreasing the NAD<sup>+</sup>/NADH ratio and exacerbating hepatotoxicity [2, 8, 11, 59]. Furthermore, acetaldehyde oxidation is slow which allows for acetaldehyde accumulation and subsequent toxicity due to acetaldehyde’s ability

to form adducts with various enzymes, structural proteins, lipids and, most damagingly, DNA [47, 48, 59].

Our results indicate that blocking the metabolism of ethanol in its entirety would give clearer insight into the role of ethanol metabolism in  $\alpha 4^*$ -nAChR-mediated lipogenesis. Still, it can be stated that blocking the activity of ADH and *production* of acetaldehyde protects hepatocytes from the ethanol-induced mRNA induction of the  $\alpha 4^*$ -nAChR-dependent pro-lipogenic genes Srebp-1c and Fasn.

### **Limitations and Future Directions**

Unfortunately, the in-depth probing of signaling pathways often involves the use of cell culture models which greatly diminishes translatability. To combat this hurdle in the present study, we made sure the signaling events in AML12 cells mostly coincided with those seen when *ex vivo* murine hepatocytes were analyzed (Figure 1, 2). Although confirmed in AML12 cells, the presence of  $\alpha 4^*$ -nAChR-dependent mediation of Sirt1 inhibition-induced lipogenesis after ethanol exposure must still be probed in PMH and CHH for validity and translatability. AML12 cells serve as a useful model of early ALD since they are able to recapitulate some of the hallmarks of the disease, including increased TG accumulation following ethanol exposure [51-53, 60]. The present study lacks the phenotypic confirmation of ethanol induced TG accumulation that is present in whole body animals and certain cell culture models, greatly diminishing the impact of some of the results reported here. Future studies including TG accumulation as an endpoint in AML12 cells and the theoretical inhibition of this accumulation by administration with DH $\beta$ E will support the claim that ethanol induced steatosis is dependent on  $\alpha 4^*$ -nAChRs.

In addition, the current study requires future validation with biological replicates to establish the reproducibility and subsequent validity of the reported results. This is imperative since the phenomena being studied could potentially serve as the foundation for mechanistic drug development studies for early-stage ALD. Many studies investigating ethanol's effects on hepatic homeostasis use Sirt1's dampened protein expression as an endpoint signifying ethanol-induced hepatotoxicity [19, 25, 26]. Studies performed previously in our lab included investigation of Sirt1's dampened protein expression by Western Blot [10] which is why mRNA expression was focused on in the present study. For completeness, though, and reproducibility, the study's lack of investigation into Sirt1's protein expression after ethanol exposure serves as another limitation.

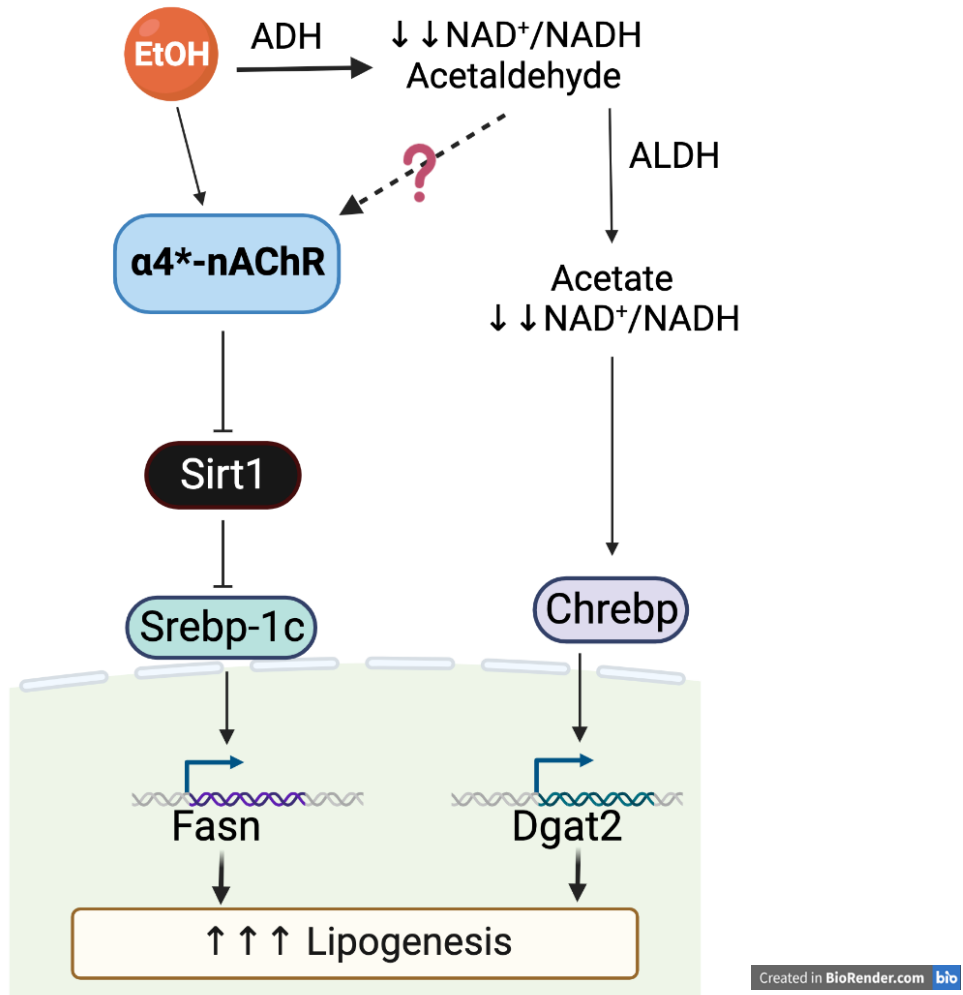
Moreover, the exact functionality of  $\alpha 4^*$ -nAChRs was not elucidated by the current study. It can be speculated that  $\alpha 4^*$ -nAChRs play a role in transcriptionally inducing downstream effectors of lipid biosynthesis pathways after ethanol stimulation, but *in situ* analysis of  $\alpha 4^*$ -nAChRs is required to determine the necessity of  $\alpha 4^*$ -nAChR-induced signaling in the development of hepatic steatosis.  $\alpha 4^*$ -nAChRs act as calcium channels in the brain, and the impairment of calcium signaling has been reported as a factor contributing to lipid droplet formation and disruption of hepatic metabolism [61, 62]. Maintenance of store operated calcium channels in the ER of hepatocytes is crucial to hepatic function since they promote the influx of calcium from extracellular stores following depletion of ER  $\text{Ca}^{2+}$  pools to tightly maintain intracellular  $\text{Ca}^{2+}$  at 0.1-2 $\mu\text{M}$  [61]. Deviation out of this range in either direction can be detrimental to cell function and viability. Parts of the ER

membrane associated with the mitochondria are recognized as hubs for various signaling pathways, including those regulating lipid homeostasis and dynamically regulating  $\text{Ca}^{2+}$  concentration depending on the cell's energy state [63]. It has been reported that ethanol exposure increases the number of mitochondria-associated ER membranes in mice, leading to dysregulated  $\text{Ca}^{2+}$  signaling and subsequent steatosis [63].

It was assumed in the present study that the  $\alpha 4^*$ -nAChRs of interest were acting at the plasma membrane, although the role of mitochondria associated ER membrane  $\text{Ca}^{2+}$  signaling and ethanol induced steatosis previously reported [63] points to investigation into the exact localization of  $\alpha 4^*$ -nAChRs in hepatocytes. Future studies using organelle fractions would prove useful in dissecting hepatic  $\alpha 4^*$ -nAChRs localization and may also give insight into the function of  $\alpha 4^*$ -nAChRs in hepatocytes based on this localization. Further, the molecules supporting the relationship between the ethanol induced alteration of  $\text{Ca}^{2+}$  homeostasis and steatosis have not been fully identified, adding an additional area of future investigation regarding the role of  $\alpha 4^*$ -nAChR in ethanol-induced lipogenesis and early ALD.

Since none of the many stages of ALD have any FDA approved therapies, it is imperative to fully understand ethanol-induced signaling mechanisms for the development of potential treatments. It has long been argued that there is minimal benefit to studying ethanol induced steatosis due to the potential for regression from this stage of ALD back to a healthier phenotype. However, steatosis is often viewed as the “first-hit” which ultimately primes the liver for a string of worsening

pathologies, highlighting the importance of targeting therapies here. Moreover, steatosis seems to have the most benign side effects of any ALD stage which allows for the assumption that halting ALD here can preserve the future quality of life for these patients.



**Figure 5.** Schematic of proposed mechanism for ethanol induced  $\alpha 4^*$ -nAChR-dependent lipogenesis in hepatocytes; created with BioRender.

## SUMMARY & CONCLUSIONS

The spectrum of ALD begins with steatosis which manifests as excessive triglyceride accumulation [4, 7], but the complete mechanisms by which steatosis results have not been fully determined. Experimental murine models of ALD allow for more extensive probing of the disease's etiology than clinical trials and are often used for pre-clinical assessment of ALD pathogenesis [60]. While generally preferred, *in vivo* models of ALD are limited in their ability to reproduce human ALD and they should be supplemented by *in vitro* data for validity [60].

Recently, the well-known Lieber–DeCarli model of ALD was used to induce the disease in WT and  $\alpha 4$  KO mice and the latter were protected from the steatotic effects of ethanol on the liver [10]. The purpose of the present study was to probe the ethanol-induced  $\alpha 4^*$ -nAChR dependent pro-lipogenic signaling mechanisms *in vitro* in hepatocytes, which are seemingly diminished by whole-body knockout of  $\alpha 4^*$ -nAChRs. AML12 cells were found to be a sufficient model of early ALD (Figure 1, 2), allowing for more in-depth inquiry into the mechanisms by which ethanol interacts with  $\alpha 4^*$ -nAChR to induce lipogenic hepatotoxicity. It was shown that ethanol's impairment of Sirt1-mediated lipogenesis through Srebp-1c's transcriptional induction of Fasn is dependent on the activity of  $\alpha 4^*$ -nAChRs (Figure 3). Additionally, inhibition of ADH activity was able to blunt  $\alpha 4^*$ -nAChR mRNA induction but the results reported were inconsistent and must be validated (Figure 4), indicating complete ethanol metabolism as a necessary future point of

investigation surrounding  $\alpha 4^*$ -nAChR dependent lipogenesis. All the data presented here taken together supports the assertion that ethanol interacts with  $\alpha 4^*$ -nAChRs in hepatocytes to inhibit Sirt1 activity and ultimately increase lipogenesis (Figure 5). Still, further work must be completed to ensure the translatability of AML12 cells as a model of early ALD, and for the future development of early ALD treatments potentially targeted at hepatic  $\alpha 4^*$ -nAChRs.



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## LIST OF ABBREVIATIONS

4-MP	4-methylpyrazole
ACC	Acyl-CoA carboxylase
ACh	Acetylcholine
ADH	Alcohol dehydrogenase
ALD	Alcohol-associated liver disease
AMPK	AMP-activated protein kinase
CHH	Cryopreserved human hepatocytes
ChREBP	Carbohydrate response element binding protein
CHRNA4	Gene coding for $\alpha 4^*$ subunit of nAChRs
CPT1	Carnitine palmitoyltransferase 1
DGAT2	Acyl-CoA:diacylglycerol acyltransferase 2
DH $\beta$ E	Dihydro- $\beta$ -erythroidine
EtOH	Ethanol
FASN	Fatty acid synthase
PMH	Primary mouse hepatocytes
SIRT1	Sirtuin 1
SREBP-1c	Sterol regulatory element binding protein 1
TG	Triglycerides
VLDL	Very low-density lipoprotein
WHO	World Health Organization

WT Wildtype  
 $\alpha 4$  KO Murine whole body knockout for  $\alpha 4^*$ -nAChRs  
 $\alpha 4^*$ -nAChRs Alpha 4 subunit containing nicotinic acetylcholine receptors

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