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A closer look at alcohol-induced changes in the ghrelin system: novel insights from preclinical and clinical data

Sara L. Deschaine[1], **Mehdi Farokhnia**[1,2,3], **Adriana Gregory-Flores**[1,4], **Lia J. Zallar**[1,4], **Zhi-Bing You**[5], **Hui Sun**[6], **Deon M. Harvey**[7], **Renata C. N. Marchette**[2,4], **Brendan J. Tunstall**[4], **Bharath K. Mani**[8], **Jacob E. Moose**[9,10], **Mary R. Lee**[1], **Eliot Gardner**[5], **Fatemeh Akhlaghi**[11], **Marisa Roberto**[12], **James L. Hougland**[9,10,13], **Jeffrey M. Zigman**[8,14,15], **George F. Koob**[4], **Leandro F. Vendruscolo**[4], **Lorenzo Leggio**[1,2,16,17,18,19]

[1]Clinical Psychoneuroendocrinology and Neuropsychopharmacology Section, Translational Addiction Medicine Branch, National Institute on Drug Abuse and National, Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Baltimore, Maryland, USA

[2]Center on Compulsive Behaviors, National Institutes of Health, Bethesda, Maryland, USA

[3]Johns Hopkins Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA

[4]Neurobiology of Addiction Section, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, Maryland, USA

[5]Neuropsychopharmacology Section, Molecular Targets and Medications Discovery Branch, National Institute on Drug Abuse, National Institutes of Health, Baltimore, Maryland, USA

[6]Clinical Core Laboratory, Office of the Clinical Director, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland, USA

[7]Office of the Scientific Director, National Institute on Drug Abuse, Baltimore, Maryland, USA

Correspondence Lorenzo Leggio and Leandro F. Vendruscolo, NIDA and NIAAA, National Institutes of Health, 251 Bayview Boulevard, Biomedical Research Center, Room 01A844, Baltimore, MD 21224, USA., lorenzo.leggio@nih.gov; leandro.vendruscolo@nih.gov.

Present address Brendan J. Tunstall, Department of Pharmacology, Addiction Science, and Toxicology, University of Tennessee Health Science Center, Memphis, TN, USA

AUTHOR CONTRIBUTIONS

Overall study basis, rationale and concept: LL. *Design of the experiments*: (1) current analyses from the human laboratory experiments (MF, LL), (2) *in vivo* rodent experiments (MF, AGF, LJZ, RCNM, BJT, GFK, LFV, LL); (3) *ex vivo* experiments in gastric mucosal cells (JMZ, BKM); (4) *in vitro* assays of GOAT enzyme activity (JEM, MR, JLH). *Acquisition and management of data*: (1) human laboratory experiments (MF, SLD, MRL, FA, LL), (2) post-mortem experiments (MF, SLD, HS, MRL), (3) *in vivo* rodent experiments (AGF, LJZ, RCNM, BJT, LFV); (4) *ex vivo* experiments in gastric mucosal cells (BKM, JMZ); (5) *in vitro* assays of GOAT enzyme activity (JEM); (6) human ghrelin assays (FA); and (7) rodent ghrelin assay (ZBY). *Analysis and interpretation of data*: (1) human laboratory experiments (MF, SLD, LL), (2) post-mortem experiments (MF, SLD, LL), (3) *in vivo* rodent experiments (MF, SLD, AGF, LJZ, RCNM, BJT, GFK, LFV, LL); (4) *ex vivo* experiments in gastric mucosal cells (BKM, MR, JMZ); (5) *in vitro* assays of GOAT enzyme activity (JEM, MR, JLH); (6) human ghrelin assays (MF, SLD, FA, LL); and (7) rodent ghrelin assay (MF, SLD, AGF, LJZ, RCNM, BJT, GFK, LFV, LL). *Clinical and safety monitoring for the human studies*: MRL, LL. *Provided funding*: FA, JLH, JMZ, GFK, LL. *Drafting the manuscript*: SLD. *Assisted with drafting the manuscript*: MF, DMH, LL. All authors have critically reviewed the manuscript for important intellectual content and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available upon reasonable request from the corresponding author.

SUPPORTING INFORMATION

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

CONFLICT OF INTEREST

The authors declare that they have no competing conflicts of interest.

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[8]Center for Hypothalamic Research, Department of Internal Medicine, UT Southwestern Medical Center, Dallas, Texas, USA

[9]Syracuse Biomaterials Institute, Syracuse University, Syracuse, New York, USA

[10]Department of Chemistry, Syracuse University, Syracuse, New York, USA

[11]Clinical Pharmacokinetics Research Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, Rhode Island, USA

[12]Department of Neuroscience, The Scripps Research Institute, La Jolla, California, USA

[13]BioInspired Syracuse, Syracuse University, Syracuse, New York, USA

[14]Division of Endocrinology, Department of Internal Medicine, UT Southwestern Medical Center, Dallas, Texas, USA

[15]Department of Psychiatry, UT Southwestern Medical Center, Dallas, Texas, USA

[16]Medication Development Program, National Institute on Drug Abuse Intramural Research Program, National Institutes of Health, Baltimore, Maryland, USA

[17]Center for Alcohol and Addiction Studies, Department of Behavioral and Social Sciences, School of Public Health, Brown University, Providence, Rhode Island, USA

[18]Division of Addiction Medicine, Department of Medicine, School of Medicine, Johns Hopkins University, Baltimore, Maryland, USA

[19]Department of Neuroscience, Georgetown University Medical Center, Washington, District of Columbia, USA

Abstract

Ghrelin is a gastric-derived peptide hormone with demonstrated impact on alcohol intake and craving, but the reverse side of this bidirectional link, that is, the effects of alcohol on the ghrelin system, remains to be fully established. To further characterize this relationship, we examined (1) ghrelin levels via secondary analysis of human laboratory alcohol administration experiments with heavy-drinking participants; (2) expression of ghrelin, ghrelin receptor, and ghrelin-O-acyltransferase (GOAT) genes (*GHRL*, *GHSR*, and *MBOAT4*, respectively) in post-mortem brain tissue from individuals with alcohol use disorder (AUD) versus controls; (3) ghrelin levels in *Ghsr* knockout and wild-type rats following intraperitoneal (i.p.) alcohol administration; (4) effect of alcohol on ghrelin secretion from gastric mucosa cells *ex vivo* and GOAT enzymatic activity *in vitro*; and (5) ghrelin levels in rats following i.p. alcohol administration versus a calorically equivalent non-alcoholic sucrose solution. Acyl- and totalghrelin levels decreased following acute alcohol administration in humans, but AUD was not associated with changes in central expression of ghrelin system genes in post-mortem tissue. In rats, alcohol decreased acyl-ghrelin, but not des-acyl-ghrelin, in both *Ghsr* knockout and wild-type rats. No dose-dependent effects of alcohol were observed on acyl-ghrelin secretion from gastric mucosa cells or on GOAT acylation activity. Lastly, alcohol and sucrose produced distinct effects on ghrelin in rats despite equivalent caloric value. Our findings suggest that alcohol acutely decreases peripheral ghrelin concentrations *in vivo*, but not in proportion to alcohol's caloric value or through direct interaction with ghrelin-secreting gastric mucosal cells, the ghrelin receptor, or the GOAT enzyme.

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Keywords

acyl-ghrelin; alcohol; calorie; des-acyl-ghrelin; ghrelin; GOAT

1 | INTRODUCTION

Alcohol use disorder (AUD) is a chronic relapsing disease characterized by consumption of alcohol to an extent that causes significant harm to the affected individual's health and overall quality of life. According to the 2018 National Survey on Drug Use and Health, 5.8% of individuals aged 18 and older in the United States had AUD in the past year, and an estimated 88,000 annual deaths are alcohol related.[1,2] Still, only three Food and Drug Administration (FDA)-approved medications are available for treatment of AUD, highlighting a significant need to develop novel pharmacotherapies for AUD. One such therapeutic strategy is based on the notion that harmful alcohol consumption can be alleviated by pharmacologically manipulating endocrine pathways that control both homeostatic and hedonic feeding, as well as stress-related pathways and reward processing.[3–6] The orexigenic peptide ghrelin is one hormone that has been shown to play a role in alcohol-related behavior across numerous studies.[7–9]

Ghrelin is a 28 amino acid hormone secreted primarily from P/D1 cells (X/A-like cells in rodents) located in the oxyntic glands of the fundus portion of the stomach. Encoded by the ghrelin gene (*GHRL*), ghrelin is post-translationally formed by cleavage of the 117 amino acid preproghrelin into proghrelin, which can then be acylated at the serine-3 residue by the membrane-bound enzyme, ghrelin-O-acyltransferase (GOAT).[10–13] Acylated proghrelin is then cleaved to form acyl-ghrelin—the endogenous ligand of the growth hormone secretagogue receptor 1a (GHSR1a). Acylation of ghrelin is essential for binding to GHSR1a, both centrally and peripherally, and mediates orexigenic effects.[10,14,15] Much research over the past decade demonstrates that the ghrelin system has a complex biology due to several factors: (1) circulating acyl-ghrelin can be de-acylated by plasma esterases to des-acyl-ghrelin[16]; (2) GOAT can acylate ghrelin in target tissues, both in the central nervous system and the periphery[17–19]; (3) plasma anti-ghrelin immunoglobulin Gs (IgGs) may bind and protect ghrelin from degradation in circulation[20]; (4) des-acyl-ghrelin may have effects seemingly opposite to acyl-ghrelin through GHSR1a-independent mechanisms[21]; and (5) GHSR1a has high constitutive, ligand-independent activity.[22,23] Moreover, an endogenous antagonist/inverse agonist for GHSR1a, known as liver-expressed antimicrobial peptide-2 (LEAP-2), was recently identified.[24–26] These different components of the ghrelin system help regulate and balance acyl-ghrelin's important effects on energy homeostasis to ensure survival of the organism.[27]

Central signaling of gastric-derived acyl-ghrelin occurs through activation of GHSR1a expressed on vagal afferent neurons in the stomach, as well as by acyl-ghrelin crossing the blood–brain barrier and binding to GHSR1a in the brain.[28] Acyl-ghrelin's central orexigenic signaling occurs directly through GHSR1a expressed on hypothalamic neuropeptide Y and agouti-related peptide-expressing neurons in the arcuate nucleus, and indirectly through activation of the lateral hypothalamus, hippocampus, amygdala, ventral tegmental area

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(VTA), and other regions.[28] These brain regions communicate with origins and terminal regions of the mesolimbic dopamine system, which affects motivational behaviors, including hedonic feeding and drug and alcohol seeking. Indeed, administration of acyl-ghrelin into the brain's reward circuitry increases extracellular dopamine via GHSR1a located in the mesolimbic pathway,[29–36] and stimulates food intake.[37,38] By communicating with these regions, the ghrelin system can regulate homeostatic and hedonic drives governing food-seeking behavior that seem to similarly affect alcohol-seeking behavior. In rodents, central or systemic ghrelin administration increases alcohol intake, whereas antagonism of GHSR1a and knockout (KO) of *Ghrl* or *Ghsr* decreases alcohol preference and consumption and blunts both conditioned place preference and dopamine release in the nucleus accumbens (NAc) induced by alcohol.[39–44] Moreover, higher peripheral ghrelin concentrations are positively correlated with alcohol craving and risk of relapse in humans,[41,45–49] and exogenous ghrelin administration increases cue-induced craving[46] and intravenous self-administration of alcohol in heavy-drinking individuals with alcohol dependence.[50] Collectively, these studies demonstrate a clear relationship between ghrelin and alcohol-related behaviors, wherein ghrelin appears to potentiate alcohol seeking and consumption, and partly regulate its reinforcing effects.

To obtain a better understanding of the crosstalk between ghrelin and alcohol, further research into how alcohol itself affects the ghrelin system is critically needed. To date, only a few studies have examined the effect of alcohol on peripheral ghrelin concentrations. In rodents, alcohol acutely decreased both acyl-ghrelin and total-ghrelin concentrations in plasma,[51,52] and in humans, acute administration of alcohol decreased plasma ghrelin concentrations.[53–58] Among individuals with AUD, abstainers had higher peripheral ghrelin concentrations compared to current drinkers.[45,48,49,59–64] Ghrelin concentrations were significantly lower in individuals with AUD compared to matched, non-AUD controls.[45] However, mean daily alcohol consumption over the past 12 months was positively correlated with plasma ghrelin concentrations among individuals without an AUD diagnosis.[65] Collectively, these studies suggest that acute and chronic exposure to alcohol differentially affect the ghrelin system, with effects from chronic alcohol use likely reflecting compensatory mechanisms dependent on the extent and duration of alcohol use. Although the literature to date demonstrates an interplay between alcohol and ghrelin, it is unclear how these effects are occurring—whether through direct action on ghrelin-producing cells, modification of GOAT activity, and/or through other mechanism(s). Understanding the mechanism by which these effects are occurring is important for designing and understanding the clinical translation of inhibitors of the ghrelin system as a potential pharmacotherapy for AUD. The objective of the present body of work was therefore to further understand the effect of alcohol on the ghrelin system by first performing secondary analysis of data from alcohol administration in humans[50,66,67] and conducting follow up experiments to probe for direct interactions between alcohol and the ghrelin system.

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2 | MATERIALS AND METHODS

2.1 | Effects of alcohol on peripheral ghrelin levels in humans

To examine the effect of alcohol on endogenous ghrelin levels, we performed separate analyses of four human laboratory experiments conducted by our team at the National Institutes of Health (NIH) Clinical Center in Bethesda, Maryland. These experiments were originally performed as part of three placebo-controlled trials[50,66,67] (ClinicalTrials.gov: NCT02039349, NCT01779024, NCT01751386) and included the administration of alcohol to non-treatment-seeking, heavy-drinking individuals, as well as measurement of plasma ghrelin levels. Here, we only included data from the placebo conditions of these experiments (Figure S1–4). Participants provided informed consent and were compensated for participating in each study. The eligibility criteria of each parent study can be found in appendices (Appendix S1A, S2A, S3A) and baseline characteristics of each sample analyzed here can be found in Table S1. We analyzed data separately for each of the following experiments: (1) oral alcohol self-administration (ASA),[66] (2) oral fixed-dose alcohol administration,[67] (3) intravenous (IV) ASA,[50] and (4) IV fixed-dose alcohol administration.[50] Detailed descriptions of these studies and their primary outcomes have been previously reported.[50,66,67] Descriptions of standardized meals for each study can be found in the appendices (Appendix S1B, S2B, S3B). An overview of these experiments, including information about times of blood draws, meals, and alcohol administration can be found in Figure 1 and Table S2. Below we provide a brief description of each experiment.

2.1.1 | Oral alcohol self-administration—The main aim of the parent study was to test the role of baclofen on alcohol drinking, using a randomized, between-subjects, double-blind, placebo-controlled human laboratory design.[66] Here, we analyzed data from the placebo group only. Participants received their assigned study medication (placebo only in this analysis) for approximately a week before returning to complete the experimental session. Participants were instructed to abstain from alcohol 24 h prior to the experiment (verified by breath alcohol concentration [BrAC] = 0 g/dl) and to take their first medication dose before arriving at the clinic. The experimental session consisted of alcohol cue reactivity followed by alcohol priming and ASA (for full details, see:[66]). Briefly, during alcohol priming, participants were provided with their preferred choice of alcohol and mixer (S1C, S2C). The amount of alcohol in the priming drink was calculated to raise each participant's blood alcohol concentration (BAC) to 0.03 g/dl.[68] Participants were asked to consume the entire drink within 5 min. The ASA session began 40 min after consumption of the priming drink. At the beginning of the ASA session, four mini-drinks were offered. Each mini-drink had half the amount of alcohol as the priming drink (BAC increase of 0.015 g/dl), and participants were allowed to drink as many of the mini-drinks as they chose, with the knowledge that they would receive $3 for each mini-drink not consumed. An additional four mini-drinks were offered 60 min after the beginning of the ASA session. The total ASA session lasted for 120 min, during which participants were monitored to not exceed a BrAC of 0.12 g/dl. Following completion of the ASA session, participants were escorted to an inpatient unit where they were monitored until BrAC reached 0 g/dl, and they were discharged the next morning.

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2.1.2 | Oral fixed-dose alcohol administration—The main aim of the parent study was to test the safety of a ghrelin receptor blocker (PF-5190457), co-administered with alcohol, using a Phase 1b, within-subjects, dose-escalating, single-blind, placebo-controlled human laboratory design.[67] Here, we analyzed data from the placebo condition only. The alcohol administration experiment was held on the third day of an inpatient visit, after taking five doses of the study drug (placebo only in this analysis). A standardized alcoholic beverage (Smirnoff vodka, 40% alcohol by volume; 80% proof) was administered, and participants were instructed to drink the beverage within 15 min. Alcohol was provided as a mixed drink containing the participants' choice from a list of common mixers (S1C). Alcohol administration was designed to bring each participant's BAC to a target level of 0.06 g/dl.[68]

2.1.3 | IV alcohol administration—The parent study under which both IV alcohol experiments were performed was a cross-over, randomized, double-blind, placebo-controlled study testing the effects of exogenous ghrelin administration and consisting of four experimental sessions: two IV ASA (one ghrelin, one placebo) sessions and two brain fMRI sessions (one ghrelin, one placebo). We analyzed data from the placebo sessions only. Participants were admitted to the NIAAA inpatient unit at the NIH Clinical Center on the evening before each experiment day. Before each experiment, an IV catheter was inserted into each arm (one for ghrelin/placebo and one for alcohol infusion/blood sampling). For the placebo conditions (which are the only ones considered in this analysis), saline solution was infused during the entire experiment. IV alcohol was given as 100% dehydrated alcohol diluted by saline to 6.0% (v/v).

IV ASA experiment: For the IV ASA experiment, participants were given the opportunity to press a button to receive IV alcohol infusions using a Computerized Alcohol Infusion System (CAIS) during a 120-min session. A progressive-ratio schedule for self-administration was applied, which required the participants to press the button an increasing number of times to receive the subsequent alcohol infusion. Incremental infusion rates were calculated individually to raise each participant's BAC by 0.0075 g/dl within 2 min.[68] BrAC measurements were taken every 15 min throughout the procedure and entered in the CAIS software for model-based algorithm adjustments and BAC prediction. Participants were not allowed to exceed a BrAC of 0.12 g/dl during the ASA session. For additional details, see the literature.[50,69,70]

IV fixed-dose alcohol administration experiment: The IV alcohol administration was conducted as part of a brain fMRI experiment in which subjects completed an alcohol–food incentive delay (AFID) task that exposed participants to food, alcohol, and neutral symbols. As part of this session, participants received an IV alcohol infusion calculated to raise each participant's BAC linearly to 0.08 g/dl,[68] within 20 min, and clamp the BAC at this target value until the end of the experiment. The total duration of the IV alcohol infusion was 35 min. For additional details, see the literature.[50,69,70]

2.1.4 | Clinical blood collection, processing, and assay of ghrelin levels—For each experiment listed above, blood was collected at multiple time points throughout each

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experimental session to allow for repeated measures of plasma acyl- and total-ghrelin levels (Figure 1 and Table S2). The full technical details for blood collection, plasma extraction, and acyl- and total-ghrelin assays can be found in the appendices (S1D, S2D, S3C).

2.2 | *GHSR*, *GHRL*, and *MBOAT4* gene expression in human post-mortem brain tissue

Expression levels of the ghrelin receptor gene (*GHSR*), ghrelin gene (*GHRL*), and GOAT gene (*MBOAT4*) were analyzed in post-mortem brain samples from male subjects diagnosed with severe AUD (DSM-5) who also smoked, and controls who did not have a diagnosis of AUD. Human post-mortem brain tissue was obtained from the New South Wales Tissue Resource Centre (NSWBTRC) at the University of Sydney, Australia.[71] *GHSR*, *GHRL*, and *MBOAT4* RNA extraction, reverse transcription, and qPCR analysis were performed using procedures previously reported.[72] mRNA was extracted from the five available brain regions, including amygdala, hippocampus, ventral tegmental area (VTA), nucleus accumbens (NAc), and prefrontal cortex (PFC; superior frontal Brodmann areas 8 and 9). Full technical details can be found in Appendix S4.

2.3 | Effects of alcohol administration on peripheral ghrelin levels in Ghsr knockout and wild-type rats

Male wild-type (WT) and *Ghsr* knockout (KO; Wistar background) rats 18–20 weeks of age from date of birth were obtained from the Transgenic Breeding Facility at the National Institutes on Drug Abuse (NIDA) Intramural Research Program (IRP) (Baltimore, MD, USA). The development and characterization of the *Ghsr* KO rat has been previously described.[73] All procedures in rats adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition) and were approved by the Institutional Animal Care and Use Committee of the National Institute on Drug Abuse Intramural Research Program. Animals were single-housed and maintained in temperature-controlled facilities on a 12 h/12 h light cycle with standard chow and water available *ad libitum*. At the time of the experiment, rats weighed 350–850 g. Rats from both genotype groups randomly received either ethanol (20% w/v) and saline: (1) *Ghsr* KO–alcohol (n = 8), (2) *Ghsr* KO–saline (n = 9), (3) WT–alcohol (n = 9), and (4) WT–saline (n = 8). Rats were given an intraperitoneal (i.p.) injection of either alcohol (1.5 g/kg, 7.5 ml/kg) or saline (7.5 ml/kg) 15 min before collection of trunk blood into EDTA coated tubes containing inhibitors appropriate for acyl-ghrelin/des-acyl-ghrelin measurement. The dose of 1.5 g/kg of alcohol in rats can be considered moderate as it typically reduces anxiety-like behaviors and produces moderate motor incoordination, but does not cause sedation (i.e., hypnosis).[74] Full technical details of processing and assays can be found in Appendix S5.

2.4 | The effect of alcohol on ghrelin secretion from gastric mucosal cells

Gastric mucosal cells were isolated and established from 8–12 week-old male C57BL/6 N mice, as reported previously,[75,76] and then supplemented with sodium octanoate-bovine serum albumin (BSA) before they were treated with medium containing different ethanol concentrations. After incubation, mediums were assayed for acyl-ghrelin by ELISA. Full technical details can be found in Appendix S6.

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2.5 | Effect of alcohol on human GOAT ghrelin acylation activity

Assays were performed with 70 mg membrane protein from Sf9 cells expressing human GOAT (hGOAT), as determined by Bradford assay. Each ethanol concentration was tested by adding an ethanol stock to a mix of HEPES, membrane protein, and MAFP before initiating reactions with octanoyl-coA and $GSSFLC_{AcDan}$ peptide. Reactions were stopped after 1 hr with acetic acid, and the medium was analyzed using reverse-phase HPLC, as described previously.[77,78] GOAT acylation activity was determined by substrate and product peak integration in the presence of either ethanol or water (vehicle). Percent activity for each reaction was calculated using Equations 1 and 2.[79] Full technical details can be found in Appendix S7.

$$\%\text{peptide octanoylation} = \frac{\text{fluorescence of octanoylated peptide}}{\text{total peptide fluorescence (substrate and product)}} \tag{1}$$

$$\%\text{activity} = \frac{\%\text{ peptide octanoylation in presence of inhibitor}}{\%\text{ peptide octanoylation in absence of inhibitor}} \tag{2}$$

2.6 | Effects of alcohol versus sucrose administration on peripheral ghrelin levels in rats

Male Wistar rats 18–20 weeks from date of birth were obtained from Charles River Laboratory (Wilmington, MA). Animals were single-housed and maintained in temperature-controlled facilities on a 12 h/12 h light cycle with standard chow and water available *ad libitum*. At the time of the experiment, rats weighed 400–700 g, and were randomized by weight to alcohol or sucrose groups. Prior to the experiment, rats were habituated to i.p. injections for 3 days by performing daily saline injections. On the day before the experiment, rats were also habituated to the testing room for 1 h. The following day, baseline measures for each rat were collected via tail bleed 6–7 h into the light cycle, at 15 and 60 min, following saline injection (7.5 ml/kg, i.p.). Rats were returned to their home cages in between injection and blood draws. Food and water remained accessible to the rats in the home cages. The following day, rats were divided into two groups and received either 20% w/v ethanol (1.5 g/kg, 7.5 ml/kg, i.p., 10.8 kcal/kg) or 35% w/v sucrose (2.8 g/kg, 8 ml/kg, i.p., 11.2 kcal/kg). Tail blood was again drawn at the 0, 15, and 60 min time points, and processed as described in Experiment 3.3 (Appendix S5).

2.7 | Statistics

Human laboratory experiments: outliers (defined as outside of ±1.5 interquartile range per hormone per time point) were removed. Data were analyzed using Linear Mixed Effects Models in SPSS 25 (IBM Corporation, Armonk, NY) and were evaluated for random effect of subject, main effect of time point, and covariates (age, gender, BMI, and race) on acyl- or total-ghrelin. Random effects were described with a scaled identity covariance structure. Covariates that were not significant in the initial run of each model were removed from the final model. Post hoc analyses were performed using pairwise comparisons of group means at each time point within an experiment, and Bonferroni correction was used to conservatively control for multiple comparisons. *Human post-mortem experiment*: Human

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post-mortem data were analyzed using Linear Mixed Effects Models in SPSS 25 and were evaluated for random effect of subject, fixed effect of group (AUD, Non-AUD), and covariates (post-mortem interval [PMI], age, brain weight, brain pH, BMI, and cigarette pack years) on fold change ($2^{- \quad Ct}$) mRNA expression in each brain region. Covariates that were not significant in the initial run of each model were removed from the final model. A variance components covariance structure was used to describe random effects. To conservatively control for multiple comparisons, Bonferroni correction was applied to correct for the number of brain regions tested (5 brain regions). *Alcohol experiments with KO and WT rats*: A two-way ANOVA was used to evaluate genotype (KO vs. WT), treatment (alcohol vs. saline), and genotype x treatment interaction main effects among saline- and alcohol-treated *Ghsr* KO and WT rats using GraphPad Prism 8 software (San Diego, CA, USA). Tukey's multiple comparison test was used when appropriate. *Gastric mucosal cell experiments*: For ghrelin secretion studies in gastric mucosal cells, a one-way ANOVA followed by Dunnet's test was used to analyze the effect of different concentrations of alcohol on acylghrelin secretion in Graph Pad Prism. *Alcohol and sucrose experiment with rats*: Lastly, two-way repeated measures ANOVA was used to evaluate treatment (alcohol vs. saline and sucrose vs. saline), time (0, 15, and 60 min), and treatment x time interaction main effects on acyl-ghrelin and des-acyl-ghrelin in rats using GraphPad Prism. Sidak's multiple comparison test was used for post hoc analyses when appropriate. For all analyses, significance was set at $P < 0.05$.

2.8 | Approvals

Human laboratory experiments were approved by the NIH Addictions Institutional Review Board, registered at ClinicalTrials.gov (NCT02039349, NCT01779024, NCT01751386), reviewed by the FDA if applicable under Investigational New Drug (IND) applications, and conducted in accordance with Declaration of Helsinki principles. All participants provided written informed consent before any protocol-specific research procedure took place. The human postmortem brain project was approved by the NIAAA Scientific Advisory Board and the NIH Office of Human Subjects Research Protections and was exempt from review by the NIH Institutional Review Board. Animal studies performed at the NIH IRP adhered to the National Research Council *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of the NIDA IRP. All animal procedures and use of mice at UT Southwestern Medical Center (UTSW) were approved by the Institutional Animal Care and Use Committee of UTSW.

3 | RESULTS

3.1 | Plasma ghrelin levels are reduced after alcohol administration in humans

Overall, alcohol administration led to a reduction in ghrelin levels, regardless of the route of alcohol administration, within a time window ranging from 45–165 min post-alcohol administration. Using linear mixed effects modeling, we found that acyl-ghrelin [$F(3, 27.5) = 6.6$, $P = 0.002$] and total-ghrelin [$F(3, 37.7) = 4.5$, $P = 0.009$] were significantly reduced during the oral ASA session (Figure 2A). Moreover, there was a significant reduction in acyl-ghrelin [$F(5, 53.8) = 10.5$, $P < 0.001$] and total-ghrelin [$F(5, 52) = 13.6$, $P < 0.001$] during the oral fixed-dose alcohol administration session (Figure 2B). Analysis of peripheral

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ghrelin during the IV ASA session also revealed a significant reduction in both acyl-ghrelin [F(4,37.7) = 7.5, P < 0.001], and total-ghrelin [Covariate: Gender, F(4, 38.7) = 5.6, P = 0.001] (Figure 2C). Lastly, we observed a reduction in acyl-ghrelin [F (4,19) = 2.0, P = 0.134) and total-ghrelin [F(4,17.1) = 2.7, P = 0.067] during the IV fixed-dose alcohol administration session, but this change did not reach statistical significance (Figure 2D). Pairwise comparisons corrected for multiple testing were conducted between all time points for experiments where significant overall effects were found and are presented in Figure 2.

3.2 | *GHSR, GHRL*, and *MBOAT4* expression levels in select brain regions are not significantly altered by chronic alcohol consumption

Fold changes of *GHSR* mRNA, *GHRL* mRNA, and *MBOAT4* mRNA expression levels in the hippocampus, VTA, amygdala, PFC, and NAc were compared between smoking AUD individuals (N = 11) and controls (N = 15–16) (Table 1). Baseline characteristics of the sample are provided in Table S3; the differences between the two groups were controlled for in the analyses. Using linear mixed effects modeling, we found no significant effect of group (AUD vs. control) on *GAPDH*-corrected *GHRL*, *GHSR*, or *MBOAT4* expression in any of these brain regions tested (Figure 3 and Table 1).

3.3 | Acyl-ghrelin levels are reduced by alcohol in rats, independent of ghrelin receptor knockout

Rats given alcohol (n = 17, 625.3 g) or saline (n = 17, 619.5 g) had no differences in body weight (paired *t* test, P = 0.91) at the time of the experiment (Table S4). Using two-way ANOVA, we observed a significant main effect of treatment (alcohol vs. saline) on acyl-ghrelin levels [F(1,29) = 6.212, P = 0.019], where levels of acyl-ghrelin in alcohol-treated rats were reduced compared with saline-treated rats. There was no main effect of genotype (WT vs. KO) [F(1,29) = 0.2309, P = 0.63] or treatment × genotype interaction [F(1,29) = 0.01, P = 0.92] (Figure 4A). Moreover, there was no effect of treatment [F (1,29) = 0.013, P = 0.91], genotype [F(1,29) = 0.3245, P = 0.57], or treatment x genotype interaction [F(1,29) = 0.003, P = 0.95] on plasma des-acyl-ghrelin levels (Figure 4B).

3.4 | Ghrelin secretion from gastric mucosa cells is not altered by alcohol

We evaluated ghrelin release from gastric mucosal cells in the presence of different concentrations of alcohol. We cultured cells in both 5 mM glucose environment and 0 mM glucose environments. A 5 mM glucose condition was used to study the effect of alcohol in settings simulating physiological blood glucose concentrations. A 0 mM glucose condition was used to study the effect of alcohol without any interference of glucose as an energy source, and is known to be associated with higher ghrelin secretion as compared to 5 mM glucose.[80] Because it has been shown to stimulate ghrelin secretion from primary cultures of gastric mucosal cells, norepinrephine (10 μM) was used as a positive control for ghrelin secretion.[76] As observed previously,[80] absence of glucose (0 vs. 5 mM) increased acyl-ghrelin secretion from primary cultrues of gastric mucosal cells. However, alcohol did not change acyl-ghrelin secretion at any of the concentrations tested (Figure 5A–C).

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3.5 | hGOAT acylation activity is not affected by alcohol

We assayed GOAT activity *in vitro* in increasing concentrations of ethanol, using membrane protein from Sf9 cells expressing hGOAT. Ethanol was tested at concentrations representing intracellular ethanol levels ranging from sub-intoxicating (1 mM) to grossly intoxicating, lethal (87 mM) doses.[81,82] We found that ghrelin acylation by GOAT was not dose-dependently inhibited by ethanol over this physiologically relevant concentration range, with less than 20% inhibition observed at the highest concentration tested (Figure S5).

3.6 | Plasma ghrelin levels in rats are differentially affected by alcohol and sucrose

The body weights of both alcohol (n = 10, 596.3 g) and sucrose (n = 10, 600 g) treated rats were not significantly different at the time of the experiment (Student *t* test, $P = 0.91$). As for alcohol, we observed a significant main effect of treatment (alcohol vs. saline) on acyl-ghrelin levels [$F(1, 18) = 7.83$, $P = 0.02$], and a treatment × time interaction effect [$F(2,36) = 18.09$, $P < 0.0001$], but no main effect of time. Post hoc testing revealed a decrease in acyl-ghrelin levels following alcohol treatment compared to baseline and to saline (Figure 6A, left). For des-acyl-ghrelin, significant main effects of treatment [$F(1,18) = 5.253$, $P = 0.034$] and time [$F(1.673, 30.12) = 3.799$, $P = 0.041$], as well as treatment × time interaction [$F(2, 36) = 3.301$, $P = 0.048$] were observed. Post hoc testing revealed significant increase in des-acyl-ghrelin levels following saline treatment, but no changes following alcohol treatment (Figure 6A, right). There was also a significant main effect of treatment [$F(1, 18) = 10.68$, $P = 0.0043$], time [$F(2, 36) = 59.03$, $P < 0.0001$], and interaction [$F(2, 36) = 50.02$, $P < 0.0001$] on the acyl- to des-acyl-ghrelin ratio (AG:DAG ratio). Post hoc testing revealed significant reduction in AG:DAG ratio following alcohol treatment, but no changes following saline treatment (Figure S6).

As for sucrose, we observed an overall significant main effect of treatment (sucrose vs. saline) [$F(1, 16) = 7.705$, $P = 0.01$] on acyl-ghrelin levels, but no effect of time or interaction effect, indicating lower levels of acyl-ghrelin under sucrose treatment, compared to saline, regardless of time (Figure 6B, left). There was no effect of treatment or treatment × time interaction on des-acyl-ghrelin levels, but a significant effect of time [$F(1.839, 29.43) = 20.11$, $P < 0.0001$] was observed, with post hoc testing indicating an overall increase in des-acyl-ghrelin over time (Figure 6B, right). Lastly, we observed a significant main effect of treatment [$F(1, 16) = 4.93$, $P = 0.041$], time [$F(1.966, 31.45) = 26.38$, $P < 0.0001$], and treatment × time interaction [$F(2, 32) = 19.18$, $P < 0.0001$] on the AG:DAG ratio. Post hoc testing revealed significant reduction in AG:DAG ratio following sucrose treatment, but no, or less robust, changes following saline treatment (Figure S6).

4 | DISCUSSION

The results presented herein demonstrate that alcohol administration acutely suppresses plasma acyl-ghrelin levels in humans and rats, while the effects on total-ghrelin (acyl-ghrelin + des-acyl-ghrelin + c-terminal ghrelin fragments) and des-acyl-ghrelin are more variable. We have additionally addressed several important questions regarding this effect by demonstrating that alcohol-induced suppression of acyl-ghrelin does not occur through direct interaction between alcohol and gastric mucosal cells or the GOAT enzyme, and

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by showing that alcohol does not suppress acyl-ghrelin in proportion to caloric load, as previously hypothesized.[83] Moreover, we have further characterized the alcohol–ghrelin relationship by presenting preliminary results on the potential effect of long-term alcohol consumption on central ghrelin system gene expression in humans.

Our findings from analyzing four different human laboratory experiments demonstrate a consistent direction of an alcohol-induced change in ghrelin, where alcohol appears to decrease ghrelin despite each session employing a different duration, dose of alcohol, and type of alcoholic beverage. While a reduction in acyl- and total-ghrelin was observed during the fixed-dose administration of IV alcohol, this change did not reach statistical significance. Given that IV fixed alcohol administration only lasted 40 minutes while the other paradigms had time points available over a longer time period (120–165 min), it is likely that this smaller time window did not fully capture the extent of alcohol's effect on ghrelin. For all experiments, the change in acyl-ghrelin was more robust and appeared to occur on a faster time scale than total-ghrelin. We observed a peak decrease in acyl-ghrelin at 165 minutes (−60% acyl-ghrelin from −15 min) during oral ASA, 90 min during oral fixed alcohol administration (−69% acyl-ghrelin from 0 min), and 60 min during IV ASA (−52% acyl-ghrelin from −30 min). For total-ghrelin, peak decreases occurred again at 120 min for oral fixed alcohol administration (−49% total-ghrelin from 0 min), at 120 min during IV ASA (−39% total-ghrelin from −30 min), and at 165 min during oral ASA (−17% total-ghrelin from −15 min). Pairwise comparisons revealed less significant decreases in total-ghrelin from time points just prior to alcohol administration only for IV ASA and oral fixed alcohol. For oral ASA, later time points may have revealed a larger change in total ghrelin from −15 minutes, given that alcohol was again provided at +40 and +80 minutes into the session. These data suggest that alcohol more potently affects acyl-ghrelin, and it is unclear whether the effects of alcohol on total-ghrelin are simply reflective of a decrease in acyl-ghrelin or also represent a change in des-acyl-ghrelin.

Our results are supported by data from previous publications. In humans, oral alcohol (0.55 g/kg), in comparison to a water beverage, has previously been found to significantly decrease acyl- and total-ghrelin levels in young, fasting, healthy males and females within an hour.[54,55] In another study using heathy male participants, alcohol given orally with juice (0.6 g/kg) decreased total-ghrelin levels, in comparison to juice alone. This experiment found a significant decrease in total-ghrelin 15 minutes after alcohol administration that lasted for 120 min.[56] Later, the effect of IV alcohol on ghrelin was examined by showing that continuous alcohol infusion (50 mg%) for 180 min significantly suppressed acyl-ghrelin levels in fed, male and female participants. Here, acyl-ghrelin was not significantly suppressed in comparison to baseline (0 min), but significantly blunted a fasting-induced rise in acyl-ghrelin levels observed under the placebo condition in healthy males and females.[57] This was later expanded by data showing that both moderate (100 mg%) and low doses (40 mg%) of 1 h continuous IV alcohol infusion significantly decreased acyl, but not total-ghrelin, relative to a placebo condition, in fed, healthy male and female participants.[58] While the present results are limited due to the secondary nature of the analysis and lack of a control for alcohol (i.e., placebo infusion/caloric beverage), they are strengthened by showing a consistent direction of effect with previous reports.

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To address the limitation stated above, we followed up our findings from secondary analysis of human laboratory paradigms with a saline-controlled experiment in rats. Intraperitoneal injection of alcohol in *Ghsr* KO and WT rats had no acute effect on desacyl-ghrelin and only reduced acyl-ghrelin 15 min post-injection. Moreover, we found that this effect of alcohol on acyl-ghrelin occurred independently of the presence or absence of the ghrelin receptor, as we found no genotype or genotype interaction effect. While a direct interaction between alcohol and the ghrelin receptor does not likely underlie the effect of acute alcohol on reducing peripheral ghrelin, previous work has shown that rats with differing alcohol preference have differences in both GHSR1a expression levels in select brain regions and in alcohol-induced suppression of plasma ghrelin concentrations.[51] Likewise, the ghrelin receptor is able to form heteromers with receptors for peptides known to regulate ghrelin secretion, such as the somatostatin receptor, oxytocin receptor, and dopamine receptors.[84,85] This raises the possibility that ghrelin receptor heteromers could modulate downstream effects of alcohol-induced changes in heteromer ligands (i.e., dopamine, oxytocin, etc.) on ghrelin secretion. Our findings from our experiment with KO rats suggest that this is not the case. Still our data from this experiment add to the existing literature by demonstrating acute suppression of acyl-ghrelin, but not des-acyl-ghrelin, in rodents.

It has previously been hypothesized that ghrelin suppression induced by acute alcohol might be due to direct suppression of acyl-ghrelin secretion. Here we report that *in vitro* assay with the hGOAT enzyme, a ghrelin mimetic peptide, and octanoyl-CoA revealed no dose-dependent effects of alcohol on GOAT acylation activity within a physiologically relevant concentration range. These data indicate that alcohol does not mediate its effects on ghrelin secretion by allosteric modification or direct inhibition of GOAT acylation activity by interfering with GOAT substrate binding. Moreover, murine gastric mucosal cell secretion of acyl-ghrelin was unaffected by incubation with alcohol either alone or in the presence of glucose. Cells were also tested in the presence of glucose to eliminate the presence or absence of an energy source as a confounding variable in acyl-ghrelin secretion. As reported previously, absence of glucose increased ghrelin secretion,[75,80] and alcohol had no effect on ghrelin in either condition. The concentrations of alcohol that were applied to the GOAT enzyme and murine gastric mucosal cells encompass BAC levels reached after alcohol administration in the human laboratory experiments (14–28 mM) and approximate those in rats (30–97 mM). It should be taken into consideration that we only evaluated secretion of acyl-ghrelin from gastric mucosal cells and did not evaluate any changes in des-acyl-ghrelin for these experiments. Taken together, our *in vitro* and *ex vivo* data point toward an indirect mechanism by which alcohol suppresses peripheral ghrelin levels.

Previous studies have suggested that post-prandial ghrelin suppression occurs in proportion to caloric load and that this may underlie alcohol-induced ghrelin suppression.[83,86–88] Here, we show that acyl-ghrelin was significantly decreased as a result of i.p. alcohol administration in rats both 15 and 60 min following injection compared to baseline, whereas there was no significant change in des-acyl-ghrelin following alcohol treatment between each time point. Following sucrose treatment, there was no change in acyl-ghrelin relative to baseline, but des-acyl-ghrelin was significantly increased relative to baseline at 15 and 60 min. Interestingly, given these effects, alcohol and sucrose had similar effects on the AG:DAG ratio, where a decrease of acyl-ghrelin by alcohol, and an increase of des-acyl-

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ghrelin after sucrose both decreased the plasma AG:DAG ratio significantly at 15 and 60 min post-injection. Both acyl- and des-acyl-ghrelin increased over time among saline-treated controls. Relative to saline, acyl-ghrelin was decreased by alcohol, and the increase in des-acyl-ghrelin observed following saline treatment was blunted by alcohol. Sucrose treatment, however, blunted an increase in acyl-ghrelin compared to saline-treated controls and did not change the increase in des-acyl-ghrelin relative to saline. It is possible that the increase in acyl- and des-acyl-ghrelin following saline treatment represents a fasting-induced increase in ghrelin that is differentially affected by alcohol and sucrose. Given that calorically equivalent injections of alcohol and sucrose produced markedly different effects on acyl- and des-acyl-ghrelin, our data suggest that ghrelin is not suppressed in proportion to caloric value alone. This observation is supported by studies in humans demonstrating that administration of one type of macronutrient differentially affects ghrelin secretion when compared to a different macronutrient of equivalent caloric value.[89,90] Moreover, acyl-ghrelin is not dose-dependently decreased by higher doses of IV alcohol (associated with higher caloric value).[58] Our data also suggest that ghrelin acylation and ghrelin peptide secretion are regulated by separate mechanisms, given the markedly different effects of alcohol and sucrose on these different forms of ghrelin. Acyl-ghrelin plays an important role in relaying meal-related information,[83] and it is likely that differences in post-prandial (or post-alcohol) acyl-ghrelin secretion are not simply reflective of calorie content, but represent a more complicated summation of the metabolic effects resulting from a meal or alcohol on energy homeostasis, which can vary according to macronutrients, meal status, and size of meal.

Lastly, we also show no change in central ghrelin, GOAT, or ghrelin receptor expression as a result of chronic exposure to alcohol in individuals with severe AUD (human post-mortem sample). More specifically, *GHRL*, *GHSR*, and *MBOAT4* mRNA expression levels were not statistically different (after correcting for multiple comparisons) between post-mortem brain samples from individuals with AUD and non-AUD controls, suggesting that chronic alcohol exposure does not directly affect central ghrelin system expression in these brain regions. The results for GHSR are in disagreement with available preclinical literature, where alcohol preferring rats demonstrated increases in GHSR1a in the PFC, hippocampus, VTA, NAc, and amygdala in comparison to non-alcohol preferring rats depending on an alcohol access model used.[51] However, it should also be considered that the expression of *GHSR* produces two transcripts, GHSR1a and GHSR1b, the latter of which heterodimerizes with and attenuates GHSR1a.[91] Likewise, expression of *GHRL* can also produce products other than proghrelin.[91] Therefore, these findings are not restricted to ghrelin and its receptor. Given that *GHSR* is not highly expressed in the hippocampus, amygdala, PFC, VTA, or NAc, and that our sample size was relatively small, we may not have been able to precisely capture any effect of long-term alcohol use on the ghrelin receptor that was statistically meaningful. Furthermore, the significance of central *GHRL* mRNA expression remains to be determined. While some *GHRL* mRNA levels are found in the brain, and central *GHRL* mRNA translation has been demonstrated in rodents, it remains to be demonstrated whether *GHRL* is centrally translated in humans, with the more significant sites of *GHRL* expression being the stomach and duodenum.[92] Still, our results from human post-mortem samples

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provide preliminary data on the lack of effect of alcohol on central ghrelin system gene expression.

Overall, this set of results contributes to a better understanding of the complex interactions between alcohol and the ghrelin system. Nevertheless, these results should be interpreted in light of the study's limitations. Data presented from human laboratory experiments are the result of secondary analyses and were therefore not designed a priori to evaluate the effect of alcohol on peripheral ghrelin (e.g., no control infusion/oral saline administration was performed to compare to alcohol). Additionally, the sample sizes from our human and post-mortem experiments are small. Our findings on the central expression of *GHRL*, *GHSR*, and *MBOAT4* in humans should be further investigated in larger samples. We were unable to evaluate expression of these genes in the hypothalamus, a prominent site of central acyl-ghrelin action that might be more significantly altered by chronic exposure to alcohol. Moreover, while we have included BMI and pack years of cigarettes as covariates in our analysis of gene expression data, it should be noted that the majority of individuals with AUD were also smokers while controls were not, and that the control group had a relatively higher average BMI compared to the AUD group. Lastly, it should be noted that our results may only be generalized to males, given that the human laboratory experiment samples (all > 70% male) were largely male, and human post-mortem samples and animals, used in alcohol administration experiments and for gastric mucosal experiments, were all male. It is also important to note that all our studies were conducted in adult humans and adult rats. A recent study suggests that alcohol may actually increase peripheral ghrelin levels in adolescent rats,[93] therefore posing the question whether alcohol may differently affect the ghrelin system during development, a critical question but outside the scope of this work.

We report here novel insights into the potential underlying mechanism(s) linking alcohol and the ghrelin system. Understanding both acute and long-term effects of alcohol on the ghrelin system is important to inform the use and clinical translation of ghrelin inhibitors as a potential pharmacotherapy for AUD. It is also important to examine a potential bidirectional interplay between alcohol use and ghrelin's effects on alcohol consumption. Irrespective of how these effects occur, acute suppression of ghrelin by alcohol may produce compensatory changes whereby long-term alcohol use leading to an upregulation of ghrelin secretion over time. Acute increases in ghrelin secretion have been demonstrated to be a learned, anticipatory response that increases mesolimbic dopamine release in response to food and alcohol,[94] and it is possible that long-term alcohol consumption results in an upregulation of this response. Indeed, increased levels of ghrelin have been demonstrated in abstinent participants with AUD.[45,48,49,59,60,62–64] Our results suggest that an indirect mechanism underlies alcohol-induced suppression of acyl-ghrelin that is unique to alcohol. To date, models of acyl-ghrelin secretion have identified insulin, glucagon, long chain fatty acids, oxytocin, dopamine, norepinephrine, epinephrine, endocannabinoids, somatostatin, glutamate, and glucose as direct regulators of acyl-ghrelin secretion,[75,95–101] and it is possible that alcohol may decrease acyl-ghrelin indirectly by affecting these targets. Another possibility is that alcohol-induced suppression of acyl-ghrelin results from alcohol's marked acute inhibition of fatty acid β-oxidation.,[102] as it has been suggested that ghrelin acylation can be supported by β-oxidation of long chain fatty acids to produce medium chain fatty acids able to act as GOAT substrates.[97,100,103] Alternatively, alcohol may suppress ghrelin

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through its effects on vagal signaling.[104,105] Further work should focus on identifying indirect mediators by which alcohol affects ghrelin.

In conclusion, our data collectively demonstrate that alcohol affects the ghrelin system by acutely decreasing acyl-ghrelin concentration in the circulation and by blunting fasting-induced increase of plasma des-acyl-ghrelin concentrations. This effect appears to occur independently of the ghrelin receptor, and without direct action on the GOAT enzyme or acyl-ghrelin secretion from gastric mucosal cells. Additionally, alcohol and sucrose in equivalent caloric amounts do not have the same effect on peripheral ghrelin, differentially affecting acyl- and des-acyl-ghrelin relative to baseline and saline-treated controls. Therefore, this study suggests that alcohol acutely suppresses ghrelin without directly interacting with the ghrelin system and not simply according to calorie content of alcohol. While further studies are needed to uncover this mechanism of alcohol-induced ghrelin suppression, our data provide new insight into how these effects occur.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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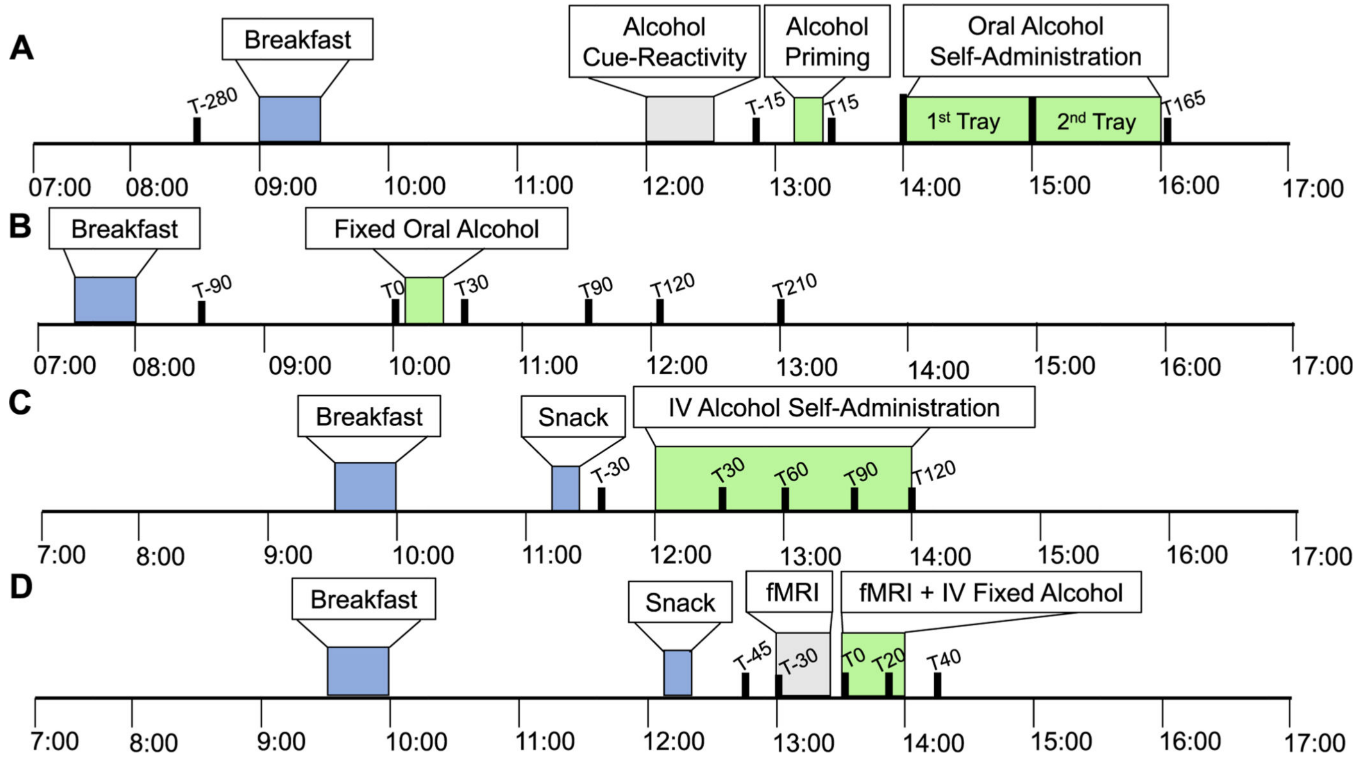

FIGURE 1.

Schematic overview of human laboratory alcohol administration experiments. Overview of human laboratory experiments depicting meals (blue), duration of alcohol intake (green), and blood draw times (black). Other study procedures not involving alcohol administration are also outlined (gray). (A) Oral priming and alcohol self-administration (ASA) experiment. Blood draw time points relative to 0 = alcohol administration at 13:15 are T-280, T-15, T15, and T165 min. (B) Oral fixed alcohol administration experiment. Blood draw time points relative to 0 = alcohol administration at 10:15 are T-90, T0, T30, T90, T120, and T210 min. (C) Intravenous alcohol self-administration experiment. Blood draw time points relative to 0 = alcohol administration at 12:00 are T-30, T30, T60, T90, and T120 min. (D) Intravenous fixed alcohol administration experiment. Blood draw time points relative to 0 = alcohol administration at 13:00 are T-45, T0, T20, T30, and T40 min

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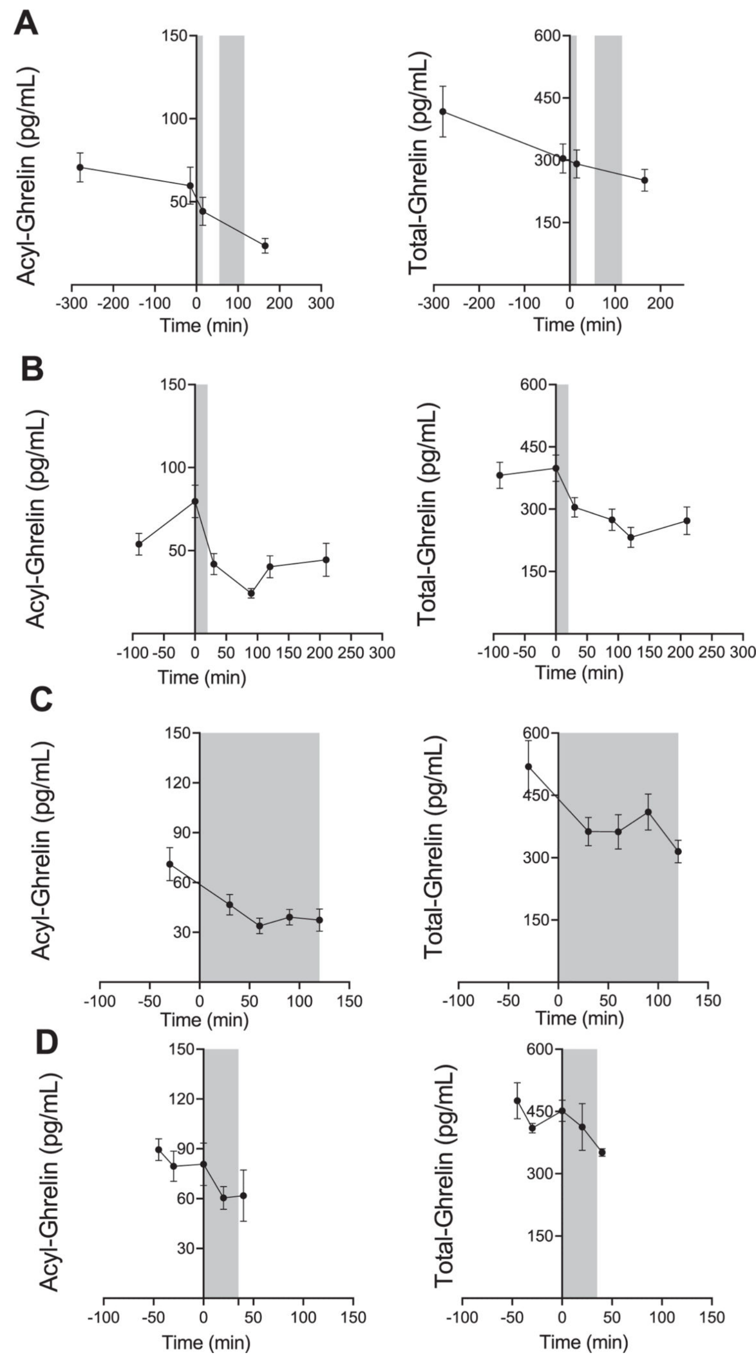

FIGURE 2.

Effect of alcohol administration on peripheral ghrelin levels in humans. Plasma ghrelin levels over the course of different alcohol administration experiments in participants with heavy drinking. Gray zones indicate time periods of alcohol administration. For all data, 0 min = beginning of alcohol administration session. Data are presented as mean ± SEM. (A) Oral alcohol self-administration analysis (N = 16); fixed effect of time (−280, −15, 15, 165) on acyl-ghrelin (left: $P < 0.002$) and total-ghrelin (right: $P = 0.009$); pairwise comparisons: Acyl-ghrelin (−280 vs. 165, −15 vs. 165; $P < 0.05$), total ghrelin (−280 vs. 165; $P < 0.05$).

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(B) Oral fixed-dose alcohol administration analysis (N = 12); fixed effect of time (−90, 0, 30, 90, 120, and 210 min) on acyl-ghrelin (left: P < 0.0001) and total-ghrelin (right: P < 0.0001); pairwise comparisons: Acyl-ghrelin (0 vs. −90; P < 0.05, 0 vs. 30, 90, 120, 210; P < 0.001), total-ghrelin (0 vs. −90, 30, 210; P < 0.05, 0 vs. 120, P < 0.001). (C) IV alcohol self-administration analysis (N = 11); fixed effect of time (–30, 30, 60, 90, and 120 min) on acyl-ghrelin (left: P < 0.0001), and total-ghrelin (right: P < 0.0001); pairwise comparisons: Acylghrelin (–30 vs. 30; P < 0.05, –30 vs. 60, 90, 120; P < 0.001), totalghrelin (–30 vs. 30, 60; P < 0.05, –30 vs. 120; P < 0.01). (D) IV fixed-dose alcohol administration analysis (N = 6) evaluated a fixed effect of time point (–45, 0, 20, 30, and 40 min) on acyl-ghrelin (left: P = *NS*), and total-ghrelin (right: P = *NS*). P values presented for pairwise comparisons are Bonferroni corrected

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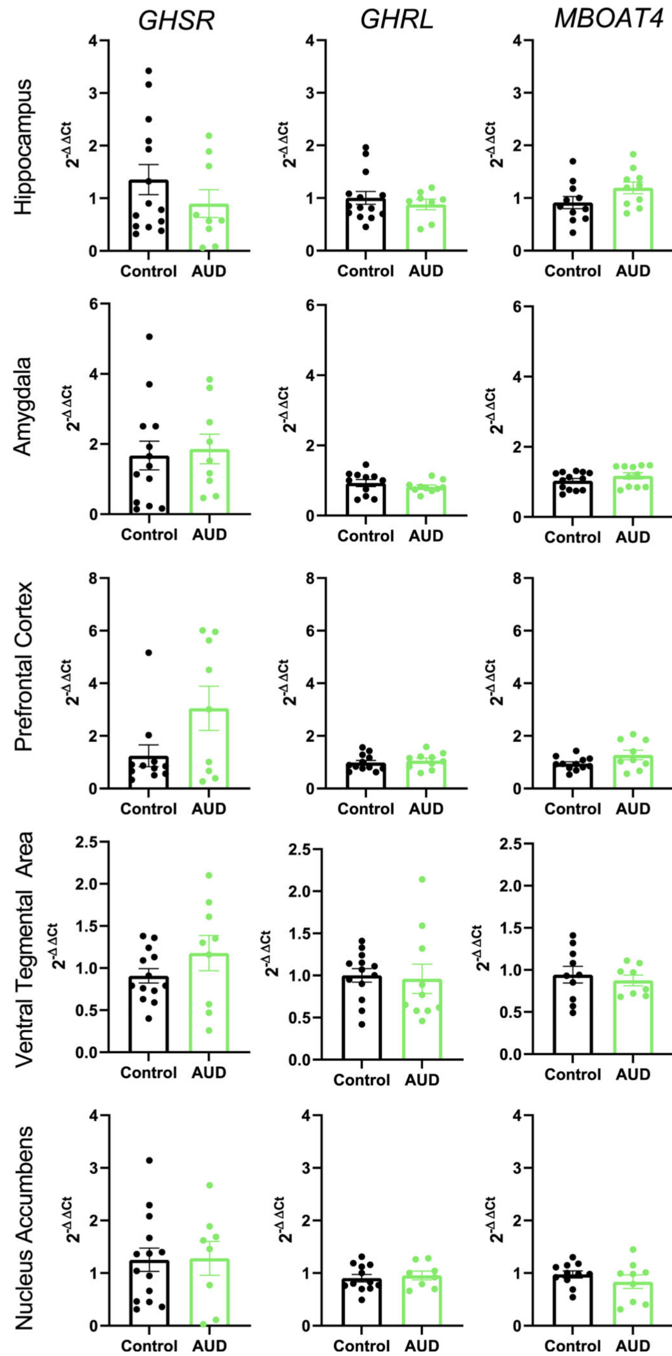

FIGURE 3.

Central post-mortem expression of *GHSR*, *GHRL*, and *MBOAT4* in AUD individuals and controls. Fold expression of *GHSR*, *GHRL*, and *MBOAT4* in five selected brain regions examined in post-mortem brain tissue from individuals with AUD and controls. Fold expression change is expressed as $2^{-\ \ Ct}$ where Ct is the difference in Ct between AUD and control samples and Ct is the difference in cycle threshold (Ct) for the gene of interest—*GADPH* in the same sample. No regions are significantly different from each other after controlling for multiple comparisons. AMG = amygdala, AUD = alcohol

use disorder, GHRL = growth hormone receptor ligand (ghrelin gene), GHSR = growth hormone secretagogue receptor (ghrelin receptor gene), HIPP = hippocampus, MBOAT4 = membrane-bound o-acyl transferase 4 (GOAT gene), NA = nucleus accumbens, PFC = prefrontal cortex, VTA = ventral tegmental area

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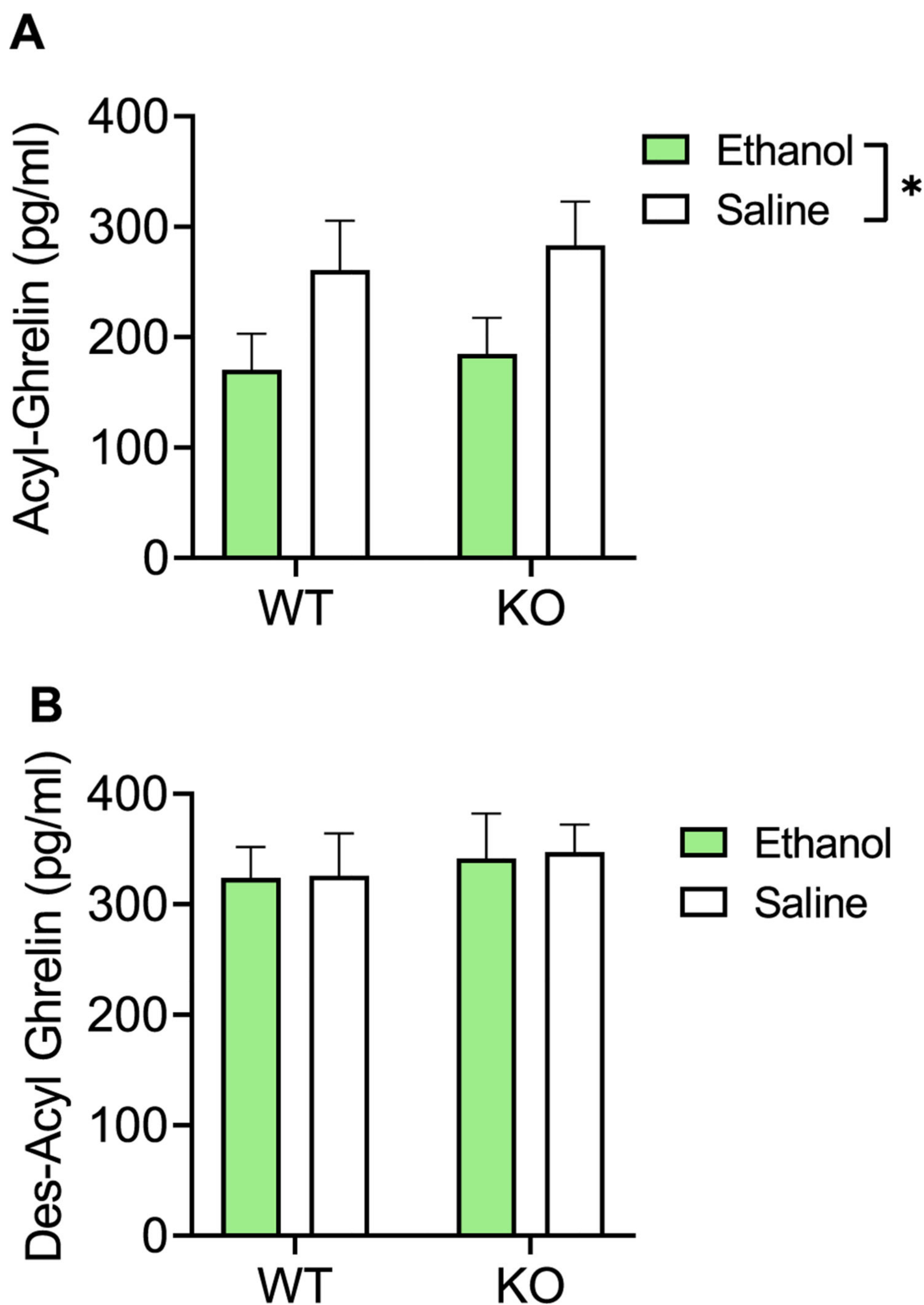

FIGURE 4.

Effect of alcohol on peripheral ghrelin levels in *Ghsr* KO and WT rats. (A) Effect of alcohol (1.5 g/kg, i.p.) on plasma acyl-ghrelin levels in *Ghsr* KO and WT rats. Data represent change in ghrelin secretion with alcohol treatment versus saline. N = 8–9/group. Treatment effect: $P < 0.019$, genotype effect: P = *NS*, and interaction effect: P = *NS*. (B) Effect of alcohol (1.5 g/kg, i.p.) on plasma des-acyl-ghrelin levels in *Ghsr* KO and WT rats. Data represent change in ghrelin secretion with alcohol versus saline treatment. N = 8–9/group. Treatment effect: P

= *NS*, genotype effect: P = *NS*, and interaction effect: P = *NS*. Data are presented as mean ± SEM. *P < 0.05

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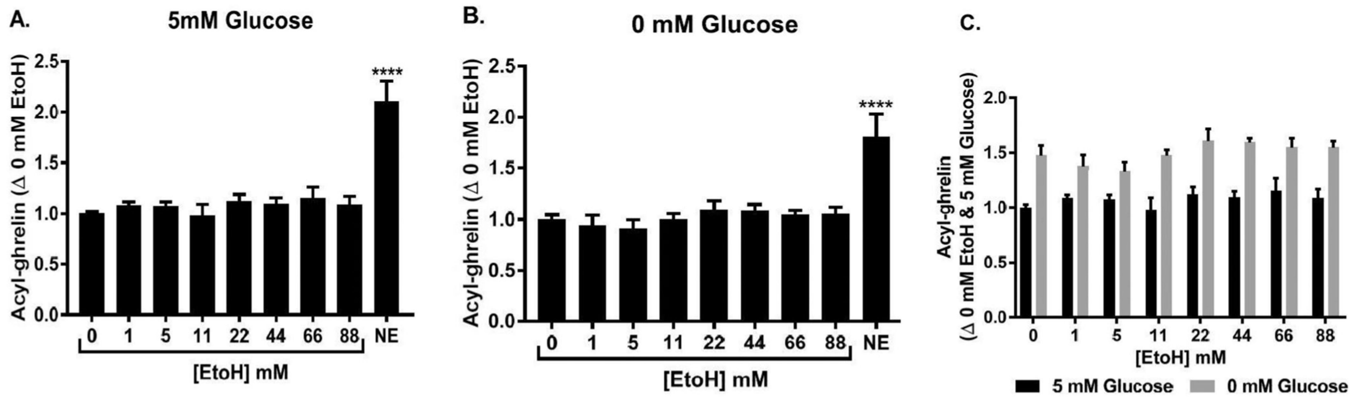

FIGURE 5.

Effect of alcohol on ghrelin secretion from gastric mucosal cells. (A) Effect of increasing concentrations of alcohol on acyl-ghrelin secretion in mouse primary gastric mucosal cells incubated with medium containing 5 mM glucose. Data represent change in ghrelin secretion with alcohol (EtoH) treatment compared to untreated control (0 mM EtoH). N = 4 for each condition. ****P < 0.0001, one-way ANOVA followed by Dunnett's test. (B) Effect of increasing concentrations of alcohol on acyl-ghrelin secretion in mouse primary gastric mucosal cells incubated with medium containing 0 mM glucose. Data represent change in ghrelin secretion with EtoH treatment compared to untreated control (0 mM ethanol). N = 4 for each condition. ***P < 0.0001, one-way ANOVA followed by Dunnett's test. (C) Effect of increasing concentrations of alcohol on acyl-ghrelin secretion in mouse primary gastric mucosal cells incubated with medium containing either 5 or 0 mM glucose (for reference only). Data represent change in ghrelin secretion with treatment compared to untreated control (0 mM ethanol in 5 mM glucose). N = 4 for each condition

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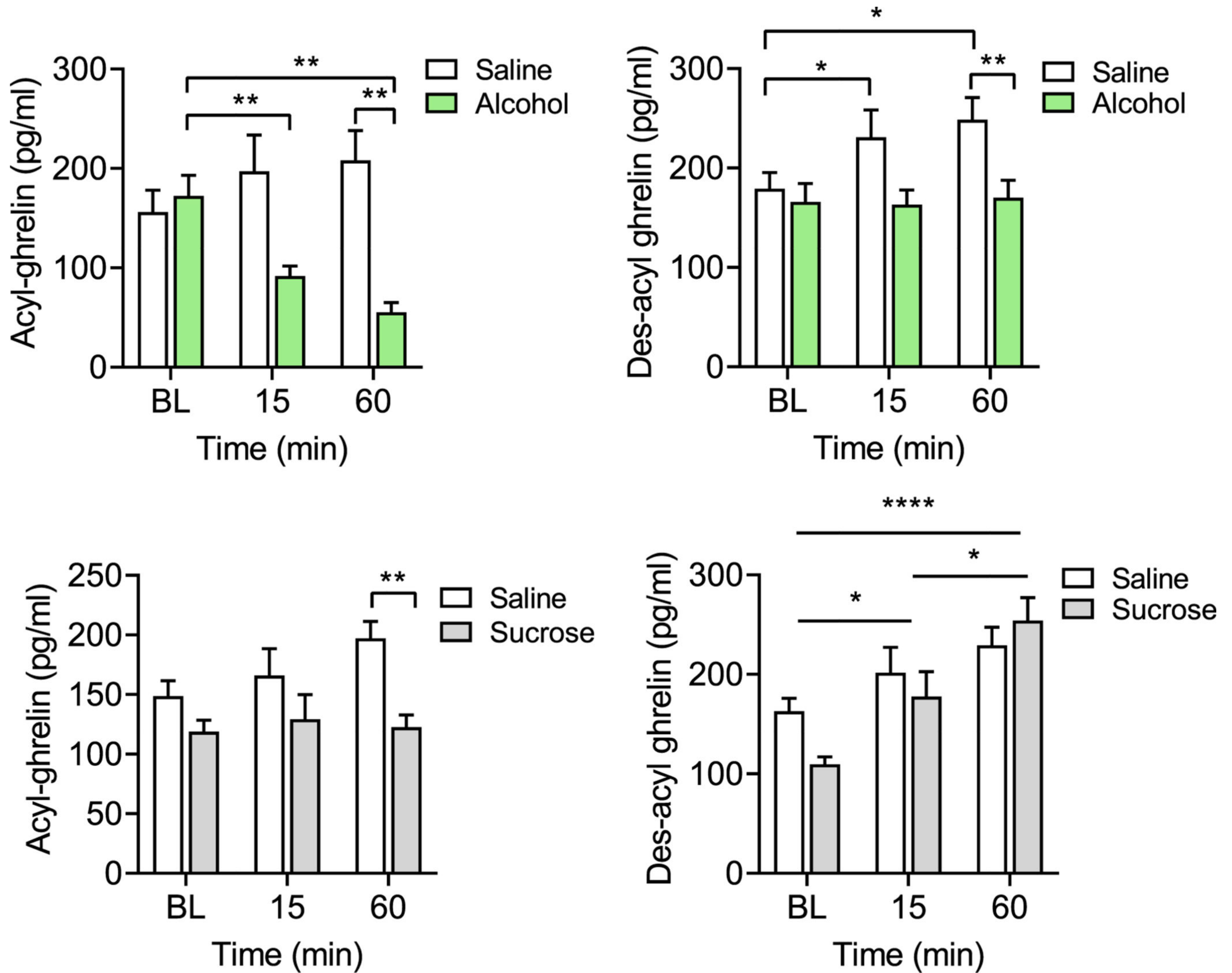

FIGURE 6.

Change in peripheral ghrelin as a result of alcohol or sucrose. (A) Plasma acyl-ghrelin and des-acyl-ghrelin resulting from alcohol and saline at baseline (BL) and 15 and 60 min post-injection. Acyl-ghrelin (left): Two-way repeated measures (RM) ANOVA: Overall treatment ($P < 0.05$) and interaction ($P < 0.0001$) effect. Post hoc testing revealed a decrease in acyl-ghrelin levels at 15 min ($P = 0.003$) and 60 min ($P = 0.004$) following alcohol treatment, compared with the pre-treatment baseline. When compared to saline injections, acyl-ghrelin levels were significantly lower in alcohol-treated rats at 60 min post-treatment ($P = 0.002$). Des-acyl-ghrelin (right): Two-way RM ANOVA: Overall time ($P < 0.05$), treatment ($P < 0.05$), and interaction ($P < 0.05$). Post hoc testing revealed significant increases in des-acyl-ghrelin at 15 min ($P = 0.047$) and 60 min ($P = 0.018$) following saline treatment, whereas no changes in des-acyl-ghrelin levels were observed following alcohol treatment. Des-acyl-ghrelin levels following saline treatment were significantly higher compared to alcohol treatment at 60 min ($P = 0.038$). (B) Plasma acylghrelin and des-acyl-ghrelin resulting from sucrose and saline treatment at baseline and 15 and 60 min post-injection. Acyl-ghrelin

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(left): Two-way RM ANOVA: Overall treatment ($P < 0.05$) effect. Post hoc testing revealed significantly lower acyl-ghrelin levels at 60 min following sucrose treatment, compared to saline treatment ($P = 0.002$). Des-acyl-ghrelin (right): Two-way ANOVA: Overall time ($P < 0.0001$) effect. Post hoc testing revealed a significant increase in des-acyl-ghrelin at 15 min ($P = 0.031$) and 60 min ($P < 0.001$), compared to baseline, and at 60 min ($P = 0.0190$), compared to 15 min. Post hoc Sidak's multiple comparison tests: *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

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TABLE 1

Comparison of *GHRL*, *GHSR*, and *MBOAT4* expression changes in post-mortem brain tissue between AUD and controls

	AUD Ct[a]	Control Ct[a]	AUD 2− Ct[b]	Control 2− Ct[b]	Statistic	Adjusted P value
GHRL						
HIPP	12.5	12.3	0.80	1.02	F(1,17) = 5.311, P = 0.034 Brain pH, PMI, Age[c]	0.17
AMY	10.3	10.2	0.85	0.93	F(1,19) = 0.846, P = 0.369	1
PFC	12.9	13.0	1.04	0.98	F (1,20) = 0.354, P = 0.559	1
NA	11.3	11.4	0.93	0.90	F(1,18) = 0.218, P = 0.646	1
VTA	11.5	11.4	0.96	1.00	F(1,19) = 3.900, P = 0.063 Pack years[c]	0.315
GHSR						
HIPP	10.1	9.1	0.90	1.35	F(1,21) = 1.206, P = 0.284	1
AMY	12.6	13.1	1.77	1.67	F(1,20) = 0.098, P = 0.758	1
PFC	14.4	15.5	2.95	1.24	F(1,18) = 4.195, P = 0.055	0.275
NA	10.4	9.9	1.49	1.25	F(1,18) = 1.126, P = 0.303 Brain pH, PMI[c]	1
VTA	12.9	13.1	1.18	0.91	F(1,20) = 1.829, P = 0.191	0.96
MBOAT4						
HIPP	14.3	14.8	0.91	1.19	F(1,19) = 3.036, P = 0.098	0.49
AMY	11.7	11.5	1.17	1.03	F(1,22) = 1.731, P = 0.202	1
PFC	13.0	13.4	1.28	0.94	F(1,19) = 3.564, P = 0.074	0.37
NA	12	11.6	0.84	0.97	F(1,15) = 4.693, P = 0.047 Pack years, BMI[c]	0.235
VTA	15.2	15.3	0.88	0.94	F(1,14) = 3.502, P = 0.082 Pack years[c]	0.41

Abbreviations: AMY = amygdala, AUD = alcohol use disorder, *GHRL* = growth hormone receptor ligand, *GHSR* = growth hormone secretagogue receptor, HIPP = hippocampus, *MBOAT4* = membrane-bound o-acyl-transferase 4, NAc = nucleus accumbens, PFC = prefrontal cortex, PMI = post-mortem interval, VTA = ventral tegmental area.

[a] Ct = Cycle threshold (Ct) of gene of interest (*MBOAT4*, *GHSR*, or *GHRL*)—cycle threshold for *GAPDH* (housekeeping gene used as endogenous control).

[b] 2− Ct calculated based on Ct = Ct AUD − Ct controls.

[c] Covariates in final model.