Protocol Title: **Effects of Ghrelin on Alcohol Administration in non-treatment seeking heavy drinkers**
Abbreviated Title: **Ghrelin and Alcoholism**

Protocol Number: **13-AA-0043**
Date of This Submission: June 24, 2016 /Version 5.0_CR 2016

Principal Investigator

Name, Degree	Branch/Institute	Bldg/Rm	Phone	E-mail
Lorenzo Leggio, M.D., Ph.D., M.Sc.	SCPN/LCTS/ NIAAA	10 (CRC)/ Room 1-5429	301-435-9398	lorenzo.leggio@nih.gov

Lead AI

Mehdi Farokhnia, M.D.	CPN/LCTS/NIAAA	10/(CRC)/ 1-5413	(301) 827-1488	mehdi.farokhnia@nih.gov

Associate Investigators

Name, Degree	Branch/Institute	Bldg/Rm	Phone	E-mail
Vijay A. Ramchandani, Ph.D.	SHP/LCTS/ NIAAA	10CRC/ Rm 2-2352	301-402-8527	vijayr@mail.nih.gov
David Ted George, M.D.	CATE/LCTS/ NIAAA	10CRC/ Rm 2-2352	301-435-9385	tedg@mail.nih.gov
Melanie Schwandt, Ph.D.	LCTS/NIAAA	10-CRC/ 1-5330 SE	301-451-6960	Melanie.Schwandt@mail.nih.gov
Reza Momenan, Ph.D.	SBEI/LCTS/ NIAAA	10-CRC/ 1-5435	301-451-6972	rezam@nih.gov
Mary R. Lee, M.D.	SCPN/LCTS/ NIAAA	10(CRC)/1-5330 SE	(301) 827-0545	leemary@mail.nih.gov
LaToya S. Sewell, CRNP-Family	LCTS/NIAAA	10(CRC)/1-5417	(301) 496-1103	Latoya.sewell@nih.gov

Fatemeh Akhlaghi, PhD, PharmD	Department of Biomedical & Pharmaceutical Sciences, University of Rhode Island	495A College of Pharmacy	401-874-9205	Fatemeh@uri.edu

Research Contact

Name, Degree	Branch/Institute	Bldg/Rm	Phone	E-mail
Lorenzo Leggio, M.D., Ph.D., M.Sc.	SCPN/LCTS/ NIAAA	10CRC/ Room 1-5429	301-435-9398	lorenzo.leggio@nih.gov

Medical Advisory Investigator

Name, Degree	Branch/Institute	Bldg/Rm	Phone	E-mail
Mary R. Lee, M.D.	SCPN/LCTS/NIAAA	10(CRC)/1-5330 SE	(301) 827-0545	leemary@mail.nih.gov

Total requested accrual
(no. 00) Patients
(no. 62) Volunteers

Project Uses Ionizing Radiation: ☒ No ☐ Yes (*attach RSC/RDRC documentation)*
- ☐ Medically-indicated only
- ☐ Research-related only
- ☐ Both

IND/IDE ☐ No ☒Yes *(attach FDA documentation)*
Drug/Device/# 117,778

Durable Power of Attorney ☒ No ☐ Yes

Multi-institutional Project ☒ No ☐ Yes
Institution#1_____ FWA #_____
Date of IRB approval _____*(attach IRB documentation)*

Institution#2_____ FWA #_____
Date of IRB approval _____*(attach IRB documentation)*

Data and Safety Monitoring Board ☒ No ☐ Yes

Technology Transfer Agreement ☐ No ☒ Yes
Agreement type and number MTA between NIAAA and URI Expiration Date N/A

Samples are being stored ☐ No ☒ Yes

Flesch-Kincaid reading level of consent form: 9.1 grade
(exclude boilerplate in assessing reading level)

Precis:

Objective. Ghrelin is a 28-amino acid peptide acting as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R). Ghrelin stimulates appetite by acting on the hypothalamic arcuate nucleus (ARC), a region that controls the intake of food and other substances, including alcohol. In addition to the ARC, GHS receptors (GHS-Rs) are also highly expressed in the caudal brain stem, the ventral tegmental area (VTA), hippocampus, substantia nigra, and dorsal and medial raphe nuclei. The expression of the GHS-R in the mesolimbic dopamine (DA) pathway suggests that ghrelin could play a role in reward processing. The role of ghrelin in the DA reward processing and the role of the DA reward system in alcoholism suggest a role of ghrelin in alcoholism. Consistent with this hypothesis, preclinical studies demonstrate that both ghrelin and ethanol activate the cholinergic-dopaminergic reward link, implying neurochemical analogies between ghrelin and ethanol. This supports the hypothesis that ghrelin is involved in mediating the rewarding properties of ethanol. Additional animal experiments demonstrate that the central ghrelin action not only stimulates the reward processing but is also required for stimulation of that system by alcohol. Human studies show reduced ghrelin levels in actively drinking alcoholics; increased ghrelin levels during alcohol abstinence; and a positive correlation between ghrelin level and alcohol craving scores. More recently, a study conducted at Brown University by the PI demonstrated the safety of the administration IV of human ghrelin to non-treatment seeking alcohol-dependent heavy drinking individuals. Furthermore, a preliminary interim analysis shows that IV ghrelin administration may lead to a temporary significant increase in alcohol craving.

The primary objective of this protocol is to investigate whether IV ghrelin, as compared to placebo, will increase motivation for alcohol reward, as measured by a progressive ratio (PR) schedule paradigm with IV alcohol self-infusion (primary aim). We will also assess a number of secondary aims. Specifically, we will also assess whether IV ghrelin, as compared to placebo, will also alter urges to drink and the subjective response to IV alcohol. Adverse events will also be assessed to ensure the safety of the IV co-administration of ghrelin and alcohol. During an "fMRI/alcohol clamp" session, fMRI will be used to see whether ghrelin affects the activation of the ventral striatum induced by acute IV alcohol administration and the incentive salience of cues associated with alcohol administration.

Study Population. Male and female participants will be non-treatment seeking heavy drinking volunteers.

Design. The study is designed as a within-subject, double-blind, placebo-controlled study of ghrelin. The first visit will be the initial screening visit. The second and third visits will be PR sessions with IV ethanol, during which each participant will also receive IV ghrelin (or matched placebo). The fourth and fifth visits will combine an fMRI session with an 'alcohol clamp' session (i.e. a fixed dose of alcohol will be administered) and subjects will participate in a modified version of the monetary incentive delay (MID) task in which they will respond to cues that indicate the opportunity to press a button to gain a reward. On some trials the reward will be points that can be exchanged for snack foods while on other trials the reward will be points that

will determine how much intravenous alcohol a subject will be given during the "alcohol clamp" procedure which will immediately follow the modified MID task. After the modified MID task is complete subjects will have a short break and then begin the alcohol IV infusion.
Outcome measures. The primary measure of this study will be the breakpoint, which is the schedule (number of button presses) at which the individual stops to work for more alcohol. Also, the BrAC exposure measures will be determined. Alcohol craving in response to ghrelin will be measured using the Alcohol Visual Analogue Scale (A-VAS) and the Alcohol Urge Questionnaire (AUQ) during the PR sessions. Sensitivity to alcohol will be measured using the Drug Effects Questionnaire (DEQ), Biphasic Alcohol Effects Scale (BAES), and the Profile of Mood States (POMS), repeatedly during the PR sessions and the CASE Experience Questionnaire (CEQ) at the end of all sessions. fMRI BOLD signal in brain areas associated with incentive salience and areas associated with reward circuitry (including the ventral striatum) will be measured during the "fMRI/alcohol clamp" session. This study may facilitate the identification of a novel neuropharmacological target, thus facilitating the development of novel pharmacological treatments for alcoholism.

- **Table of Contents**

- **List of Abbreviations**

AE	Adverse Events
AD	Alcohol Dependence
AEFQ	Alcohol Effects Questionnaire
A-VAS	Alcohol Visual Analogue Scale
ARC	Arcuate Nucleus
AUDIT	Alcohol Use Disorders Identification Test
AUQ	Alcohol Urge Questionnaire
BAES	Biphasic Alcohol Effects Scale
BAS	Behavioral Approach System
BIS	Behavioral Inhibition System
BMI	Body Mass Index
BP	Blood Pressure
BASDA	Brief Addictive Behavior Social Density Assessment
BOLD	Blood Oxygen Level Dependent
BAC	Blood Alcohol Content
BrAC	Breath Alcohol Concentration
BrCO	breath carbon monoxide
CASE	Computer-Assisted Self-infusion of Ethanol
CBC	Complete Blood Count
CEQ	CASE Experience Questionnaire
CIWA	Clinical Institute Withdrawal Assessment
CeA	central nucleus of the amygdala
CPP	Conditioned Place Preference
CPRS	Comprehensive Psychopathology Rating Scale
CR	Cue-Reactivity
CRF	Corticotropin-Releasing Factor
C-SSRS	Columbia Suicide Severity Rating Scale
DA	Dopamine
DEQ	Drug Effects Questionnaire
FDA	Food and Drug Administration
fMRI	functional Magnetic Resonance Imaging
GHS-R	Growth Hormone Secretagogue Receptor
HPA	hypothalamic-pituitary-adrenal
HR	Heart Rate
ICV	Intracerebral Ventricular
IP	Intraperitoneal
IV	Intravenous
LDTg	Laterodorsal Tegmental Area
MCR	Metabolic Clearance Rate
MID	Monetary Incentive Delay
NAc	Nucleus Accumbens
nAChR	Nicotinic Acetylcholine Receptor
NPY	Neuropeptide Y
OCDS	Obsessive-Compulsive Drinking Scale
OFC	Orbitofrontal Cortex
P	Preferring

PACS	Penn Alcohol Craving Score
PDS	Pharmaceutical Development Service
POMS	Profile of Mood States
PR	Progressive Ratio
QA	Quality Assurance
ROI's	Regions-of-Interest
RYGB	Roux-en-Y Gastric Bypass
SAEs	Serious Adverse Events
SCID	Structured Clinical Interview for DSM-IV
TLFB	Timeline Followback
VTA	Ventral Tegmental Area
UDT	Urine Drug Test

1. Introduction

There exist commonalities over-eating and over-consumption of alcohol.[1-21] Like alcoholism, obesity and binge eating are complex genetic traits determined by several genes, and interacting with the environment. It is also notable that medications used to treat alcohol dependence (AD) often result in weight loss in other populations. Naltrexone, topiramate and ondansetron have all been utilized to treat obesity and/or eating disorders[22]. In recent years there has been an interest in the role of ghrelin in food-seeking behavior. Animal studies have shown that ghrelin is involved in the central dopaminergic reward processing and human studies have shown a direct relationship between ghrelin and alcohol craving. The overall conclusion is that the ghrelin system deserves human investigations in alcoholism, as detailed next.

Ghrelin.

Ghrelin is a 28-amino acid peptide acting as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R), a G-protein coupled receptor that induces growth hormone (GH) release from the pituitary.[1] The n-octanoyl bearing ghrelin is known as active ghrelin (acylated), although the des-acylated ghrelin is not totally inactive.[23] Ghrelin was first isolated from the stomach in 1999.[1] A hypothalamic production of ghrelin was suggested.[24] Ghrelin activates hypothalamic orexigenic neurons and inhibits anorectic neurons to induce hunger[25,26]. Intracranial administration of ghrelin stimulates feeding in mammals[27,28] and non-mammals[29,30]. Human studies have demonstrated the role of ghrelin in stimulating appetite, vasodilatation and gastrointestinal motility.[31] Several studies have already used ghrelin intravenous (IV) both in healthy controls (Table 1) and in subjects with chronic diseases (Table 2). All of these studies have demonstrated the safety of IV ghrelin in humans as detailed next.

Table 1. Studies in healthy controls

#	N	IV Ghrelin dose	AEs
32	6	10 mcg/Kg (B)	decreased BP
33	18	1 or 5 pmol/kg/min (I)	somnolence, mild glucosuria, fever, diarrhoea
34	17	5 pmol/Kg/min (I)	None
35	8	1 or 3 mcg/kg (B)	None

36	4	1 mcg/kg (B)	None
37	9	5 pmol/kg/min (I)	None
38	7	1 mcg/kg (B)	None
39	11	1 mcg/kg (B)	None
40	7	50 mcg X 4 (B)	None
41	7	1 mcg/kg (B)	None
42	34	1 mcg/kg (B)	None
43	7	1 mcg/kg (B)	None
44	7	1 mcg/kg (B)	Flushing
45	6	1 mcg/kg (B)	None
46	12	1 or 5 pmol/kg/min (I)	None
47	9	100 mcg (B)	fatigue; elevated mood; vertigo; emesis; flush
48	9	1 mcg/kg (B)	None
49	8	10 pmol/kg/min(I)	None
50	9	40 mcg (I)	None
51 52	10	5 pmol/Kg/min (I)	None
53	21	0.3 mcg/kg (B)	facial warmth
54	21	0.3 mcg/kg (B)	facial and/or neck flushing
55	15	5 pmol/Kg/min (I)	None
56	12	50 mcg X 4 (B)	sweating
57	10	50 mcg X 4 (B)	None
58	8	5 pmol/Kg/min (I)	None
59	7	1 mcg/kg (B)	None
60	12	1 mcg/kg (B)	None
61	9	1 mcg/kg (I)	None
62	20	0.5 – 2.4 µg/kg (I)	flushing, facial warmth
63	8	0.5 mug/kg/h (I)	None
64	6	1 mcg/kg (B)	facial flushing
65	9	5 pmol/Kg/min (I)	None
66	20	50 mcg X 4 (B)	None
67	10	1 mcg/kg (B)	None
68	10	40 mcg (I)	None
69	5	2.5, 5 & 10 pmol/kg/min (I)	Warmth
Legend for Table 1: #: reference number; **N**: number of subjects; **AEs**: adverse events; **B**: bolus; **I**: Infusion; **BP**: blood pressure; **PCOS**: polycystic ovary syndrome			

Pharmacokinetic Profile. Because ghrelin is degraded in the digestive tract, ghrelin cannot be administered orally. Studies in healthy controls[32-35] have used bolus IV ghrelin doses range from 1 to 10 µg/kg, and continuous ghrelin infusion rates from 3.4 to 16.9 ng/kg/min.
The concentration-dependence of ghrelin kinetics may contribute to some disparities in results, as suggested by Paulo et al[35]. Mean terminal half-life estimates for total ghrelin vary from as low as 10 min to as high as 47 min after bolus injection and 146 min after constant infusion. After constant infusion, the peak plasma concentrations (Cmax) are 1058.7 (882.6 - 1234.8) fmol/ml

when 1 mcg/kg ghrelin is infused and 6597.9 (5919 - 7276.6) fmol/ml when 5 mcg/kg total ghrelin is infused[33]. After bolus injection, Cmax is of 4438 ± 407pg/mL after 1 mcg/kg ghrelin is injected, and of 8176 ± 273 after 3 mcg/kg ghrelin in injected[35]. Bolus and steady-state infusions of ghrelin yield concentration-dependent (median) estimates of the metabolic clearance rate (MCR) of blood ghrelin[35].

IV Ghrelin in healthy subjects (Table 1 above).[32-69]
Studies testing IV ghrelin in healthy subjects demonstrate that IV ghrelin reproduces physiological effects similar to that of endogenous ghrelin, e.g.: increased appetite, gastrointestinal motility. All the studies clearly demonstrate the safety of IV ghrelin either infused over time or given as a bolus. Described adverse events (AEs, e.g.: hypotension, diarrhoea, flushing, somnolence) were sporadic and transitory with a rapid and spontaneous resolution. There were no serious adverse events (SAEs) after IV ghrelin, nor AEs requiring medical intervention or the cessation of IV ghrelin infusion.

IV Ghrelin in non-healthy subjects (Table 2 below)[44-46;48,49,51,52,67,69,70-89].
Based on the physiological actions of ghrelin and its safety in healthy controls, a growing number of studies have tested IV ghrelin in subjects with severe chronic diseases, including patients afflicted with cancer cachexia. For example, IV ghrelin significantly decreased blood pressure (BP) without significant changes in heart rate (HR) in patients with chronic heart failure[70]. IV Ghrelin also reverses endothelium dysfunction in patients with metabolic syndrome[77] and increases gastric emptying in patients with diabetic gastroparesis[78]. Studies on the role of IV ghrelin in appetite are described below.

Table 2. Studies in non-healthy subjects				
#	**N**	**chronic disease(s)**	**Ghrelin dose i.v.**	**AEs**
44	9	Obesity	1 mcg/kg (B)	flushing
45	6	Obesity	1 mcg/kg (B)	none
46	12	Obesity	1 or 5 pmol/kg/min (I)	none
48	7	Bulimia nervosa	1 mcg/kg (B)	none
49	6	GH-deficiency	10 pmol/kg/min (I)	none
51,52	10	Anorexia nervosa	5 pmol/Kg/min (I)	none
67	8	Gastrectomy	5 pmol/Kg/min (I)	none
67	9	Obesity	5 pmol/Kg/min (I)	none
69	5	Geriatric frailty	2.5, 5 & 10 pmol/kg/min (I)	warmth
70	6	Chronic heart failure	0.1 mcg/kg (I)	warmth; sleepness
71	6I	Idiopathic GH-deficiency	1 mcg/kg (B)	none
72	6	GH-deficiency	1 mcg/kg (B)	none
73	8	GH-deficiency	1 mcg/kg (B)	none
74	9	Anorexia nervosa	1 mcg/kg (B)	none
75	7	Cancer	5 pmol/kg/min (I)	none
76	6	Idiopathic gastroparesis	40 mcg (I)	none
77	18	Metabolic syndrome	200 mcg/min (I)	none
78	10	Diabetic gastroparesis	5 pmol/kg/min (I)	none
79	9	Malnourished peritoneal dialysis patients	3.6 nmol/kg (SC)	decreased BP
80	10	Gastrectomy	5 pmol/Kg/min (I)	none
81	25	Obesity	0.33 mcg/kg (B)	none
82	15	Obesity & PCOS	1 mcg/Kg (B)	none
83	6	Dyspepsia	3 mcg/Kg (I)	abdominal pain; flushing; somnolence; hyperhidrosis
84	35	Obesity & PCOS	1 mcg/Kg (B)	none
85	15	Primary hyperparathyroidism	1 mcg/kg (B)	none
86	7	Obesity & PCOS	1 mcg/kg (B)	none
87	12	Malnourished dialysis patients	12 mcg/kg (SC)	abdominal discomfort, decreased, BP, lethargy, sleepiness
88	8	Obesity	100 and 200 mcg (B)	flushing, dizziness
89	5	Anorexia nervosa	3 mcg/kg b.i.d. for 14 days	loose stools, warmth, sweating
Legend for Table 2: #: reference number; **N**: number of subjects; **AEs**: adverse events; **B**: bolus; **I**: Infusion; **SC**: subcutaneous, **BP**: blood pressure; **PCOS**: polycystic ovary syndrome.				

Rationale for studying the ghrelin system in alcohol dependence.

Ghrelin stimulates appetite by acting on the hypothalamic arcuate nucleus (ARC), a region that controls the intake of food and other substances, including alcohol. Opioidergic neurons, which play a role in the reinforcing effects of alcohol, also are located in the ARC[90]. Plasma levels of ghrelin are higher under fasting conditions and prior to meals, indicating that ghrelin transmits a hunger signal from the periphery to the brain and participates in meal initiation[91]. Plasma ghrelin levels then fall to minimum levels within 1 hour after eating[92]. In addition to the ARC, GHS receptors (GHS-Rs) are also highly expressed in the caudal brain stem, the ventral tegmental area (VTA), hippocampus, substantia nigra, and dorsal and medial raphe nuclei[93-95]. The expression of the GHS-R in the mesolimbic dopamine (DA) pathway suggests that ghrelin could play a role in the reward processing.[3,4]

Feeding behaviour, DA release and locomotor stimulation are triggered following intra-VTA microinfusion of ghrelin into either the VTA or nucleus accumbens (NAc)[96] and intracerebral ventricular (ICV) administration[97]. Kawahara and colleagues[98] demonstrated that ghrelin: i) administered to rats into the VTA induced DA release in the NAc and food consumption; and ii) administered to rats (without food deprivation) peripherally induced a robust increase of DA levels in the NAc and this stimulatory effect of ghrelin primarily required activation of GHS-Rs in the VTA. Furthermore, subcutaneous administration of ghrelin in rats produced a significant increase in extracellular levels of DA in the NAc shell, but not in the NAc core[99]. These results suggest that the mesolimbic DA processing is implicated in the effects of ghrelin on food-seeking behaviour[2]. The role of the DA processing in the neurobiology of alcohol and other drug dependence is well known. In particular, cortico-mesolimbic DA pathways may mediate

alcohol's rewarding effects (including craving) associated with its abuse liability[20,100-102]. In vivo microdialysis studies have shown that ethanol administration in non-dependent rats is associated with increased NAc DA concentrations, and these DA rises are greater in ethanol-preferring compared with wild-strain animals[103-105]. Increased extracellular DA levels in the NAc shell have been typically associated with the acute reinforcing effects of alcohol and addictive drugs[102]. Indeed, baseline extracellular DA concentrations and the rate of rise following ethanol self-administration may predict alcohol preference in animals[106]. Clinical studies further support the preclinical evidence reported above. Increased appetite is the most common effect in studies testing IV ghrelin. For example, Schmid et al.[47] demonstrated that a single bolus injection of ghrelin acutely increases appetite, as well as ideas about food in healthy subjects. IV Ghrelin also increases appetite in patients with chronic diseases, such as cancer-related anorexia[**75**], dyspepsia-related anorexia[**83**], obesity[**46**] and geriatric frailty[69].

In summary: i) in rats, ghrelin increases food consumption and in humans, IV ghrelin increases appetite; ii) in rats, ghrelin acts centrally modulating the DA reward processing; and iii) the DA reward processing regulates appetite.[107] Therefore, in humans ghrelin could increase appetite via the DA reward processing. Moreover: i) alcohol and food-seeking behaviour share the same neurobiological mechanisms;[108] and ii) both alcohol and food exert their reinforcing effects in part by increasing DA in limbic regions. Thus, the role of ghrelin in the DA reward processing and the role of the DA reward processing in alcoholism suggest a role of ghrelin in alcoholism. It is important to keep in mind that the mechanisms regulating alcohol-seeking behaviours are multiple and complex. In fact, alcohol and drugs of abuse activate the mesolimbic DA processing, but much evidence suggests that dopamine-independent reinforcement occurs at the level of the nucleus accumbens, suggesting multiple inputs to the activation of critical reinforcement circuitry in these brain regions.[109] Brain arousal/stress systems in the extended amygdala may be key components of the negative emotional states that drive dependence on alcohol and drugs of abuse. Furthermore, the central nucleus of the amygdala (CeA) also plays a key role in the acute reinforcing actions of drugs of abuse; in fact, compulsive alcohol and drug use is mediated by not only loss of function of reward systems but also recruitment of brain stress systems such as corticotropin-releasing factor (CRF) and neuropeptide Y (NPY) in areas such as the amygdala. [110,111] CRF is associated with an increased stress response and negative affect, and NPY with anxiolytic properties. As widely described by Koob's lab,[109,112] dysregulation of the CRF and NPY systems plays an important role in the motivational basis of continued alcohol-seeking behavior. In particular, NPY regulates the anxiolytic effects of ethanol, thus the role of NPY is crucial in the motivational basis to alcohol intake. Interestingly, Skibicka and colleagues[113] performed a series of experiments with rats trained in a progressive ratio sucrose-induced operant schedule to measure food reward/motivation behavior and reported that the ventricular ghrelin-induced increase in sucrose-motivated behavior and chow intake were suppressed by ICV pretreatment with either an NPY-Y1R antagonist or naltrexone. Furthermore, ghrelin infusion was associated with elevated VTA-opioid receptor expression. As such, this study provided evidence of the role of central NPY and opioid signaling as key mediators of food intake and reward effects of ghrelin. On the other and, Naleid and colleagues [114] demonstrated that ghrelin had a significant, short-term effect on reward-based food intake when injected into the VTA and that this effect on feeding behavior was independent from opioid signaling in the Acb. Additional evidence was provided by Spencer and colleagues[115] who used ghrelin knockout mice to investigate anxious behavior and hypothalamic-pituitary-adrenal axis (HPA) responses to acute stress. In this study, the authors found that ghrelin reduces anxiety

after acute stress by stimulating the HPA axis at the level of the anterior pituitary.[116] This additional evidence is of interest given that HPA dysregulation may contribute to alcohol consumption. In summary, the neurobiological mechanisms of alcohol dependence are complex and while there is robust evidence to support the role of the DA system in the mechanisms of ghrelin modulation of alcohol reward, on the other hand alternative hypotheses cannot rule out and deserve further investigations.

Preclinical Studies.

Ghrelin activates the cholinergic-dopaminergic reward link.

Ghrelin administration into the VTA increases extracellular concentrations of accumbal DA in mice[4]. The non-selective nicotinic acetylcholine receptor (nAChR) antagonist mecamylamine attenuates the stimulatory and DA-enhancing effects of ghrelin infused into the third ventricle[97], indicating that nAChRs mediates these neurochemical properties of ghrelin; nAChRs also mediate the locomotor stimulatory and DA-enhancing properties of ghrelin administered into the VTA[117]. Taken together, these studies suggest that ghrelin activates the cholinergic-dopaminergic reward link and that ventral tegmental nAChRs have a central role for the DA-enhancing properties of ghrelin. Only some specific nAChR subtypes (alpha3-beta2, beta3, alpha6) are implicated in the central rewarding actions of ghrelin[117]. Interestingly, the same nAchRs (alpha3-beta2, beta3) mediate an increase in ventral tegmental ACh and accumbal DA levels after voluntary ethanol consumption[118-119]. Ghrelin administered into the lateral hypothalamus or paraventricular nucleus has no effects on ethanol consumption[120], confirming that ghrelin works in specific brain reward nodes (i.e., VTA). Peripherally injected ghrelin also activates brain reward parameters, such as locomotor activity, accumbal-DA release and conditioned place preference (CPP)[121].

In summary, these preclinical studies demonstrate that both ghrelin and ethanol activate the cholinergic-dopaminergic reward link, supporting the hypothesis that ghrelin is involved in mediating the rewarding properties of ethanol.

Requirement of central ghrelin signaling for alcohol reward.

Another set of experiments[122] demonstrated that: i) alcohol-induced brain reward parameters, such as enhanced extracellular accumbal DA overflow (a measure reflecting synaptic DA release), locomotor stimulation and CPP were consistently abolished or attenuated by two GHS-R1A antagonists in wild-type mice and were abolished in GHS-R1A knockout mice; ii) ICV administration of ghrelin to mice significantly increased alcohol consumption compared to vehicle treatment in a 2-bottle (alcohol/water) free choice limited access paradigm. Bilateral administration of ghrelin into either the VTA or the laterodorsal tegmental area (LDTg) also increased alcohol consumption in comparison to vehicle. The percentage increase in alcohol consumption was significantly greater following administration to the VTA or the LDTg compared to the ICV route. Food intake (normal chow) was increased by ICV ghrelin administration in comparison to vehicle but was not affected by bilateral ghrelin administration into either the VTA or the LDTg. This observation is particularly relevant as it may shed light on the specific role of ghrelin in alcohol-seeking behavior, as opposed to a non-specific role of ghrelin in appetitive behaviors. In fact, ICV administration of ghrelin significantly increased alcohol consumption as well as food intake. On the other hand, when ghrelin was administered directly into either the VTA or the LDTg, then only alcohol consumption was increased, while food intake was not affected. Interestingly, the percentage increase in alcohol consumption was

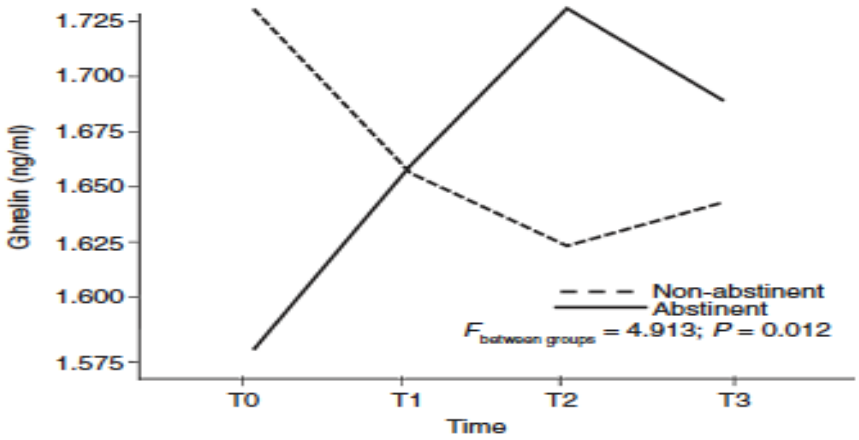
significantly greater following administration to the VTA or the LDTg compared to the ICV route, thus suggesting that ghrelin's actions via reward areas (VTA, LDTg) are somewhat specifically related to alcohol reward and not simply secondary to its effect on appetite. Alcohol intake in the 2-bottle (alcohol/water) free choice limited access paradigm was suppressed in mice by both the GHS-R1A antagonists [delivered either ICV or intraperitoneal (IP)]. The effects of ICV ghrelin on alcohol intake were absent in GHS-R1A knockout mice. In summary, using genetic and pharmacological models of suppressed ghrelin signaling, these studies demonstrate that the central ghrelin action not only stimulates the reward system but is also required for stimulation of that system by alcohol.

Human Studies on Ghrelin in alcoholic individuals:

Ghrelin, alcohol intake and craving.

Alcohol, compared to water, significantly reduces ghrelin levels in healthy subjects[123-124]. Zimmermann et al.[125] showed a significant reduction of blood ghrelin levels after alcohol ingestion in healthy subjects but unchanged ghrelin levels after a laboratory stressor exposure. Preliminary studies reported that actively drinking alcohol-dependent patients (with an alcohol intake within 24 hrs) show lower plasma ghrelin levels compared to controls[5,126]. On the other hand, other studies demonstrated a significant increase of ghrelin in abstinent alcoholics[127-128]. All together, these studies suggest that ghrelin is suppressed by acute alcohol intake and increases during abstinence. One of these studies also reported a significant positive correlation between plasma ghrelin levels and the Obsessive-Compulsive Drinking Scale (OCDS) craving scores in actively drinking alcoholics[5]. Interestingly, ghrelin was positively correlated with the Compulsive but not with the Obsessive subscore of the OCDS[5]. This last finding is consistent with the role of ghrelin in modulating food intake and with the observation that compulsion reflects the behavioral reward mechanisms of addictive disorders like eating disorders[129]. Successive human studies have confirmed, at least partially, the relationship between plasma ghrelin levels and alcohol craving. In particular, Hillemacher et al.[130] reported a significant relationship between ghrelin and OCDS score in an alcoholic subtype (Lesch type 1) characterized by a positive family history of alcoholism. Wurst et al.[131] found a relationship between ghrelin levels and the OCDS compulsive subscore in female alcoholics. Recently, the PI completed a study with alcohol-dependent individuals[132], where plasma ghrelin levels were determined several times – the first longitudinal study testing repeated blood ghrelin determinations in a sample of alcohol-dependent individuals. Baseline ghrelin (T0) was taken after 72 hrs of alcohol abstinence, in order to avoid an acute effect of alcohol on ghrelin levels as a possible confounding factor. Then, ghrelin was re-tested after 2 weeks (T1), 6 weeks (T2) and 12 weeks (T3). Results showed a relationship between ghrelin, alcohol intake and craving.[132] In fact, a comparison of non-abstinent vs. abstinent (during the 12 weeks) subjects showed that: i) non-abstinent subjects had higher ghrelin levels, which went down during the 12-week period (consistent with the inhibitory effect of alcohol on ghrelin[5,123-126]); ii) in abstinent subjects ghrelin levels increased during the 12-week period, as previously shown[127-128]; iii) there was a significant difference in the changes of ghrelin between the two groups ($F_{between\ groups}$=4.913;p=0.012; Figure 1 below) and the difference in baseline ghrelin levels was statistically significant (p=0.035).

Figure 1



Legend: a comparison of non-abstinent alcohol-dependent subjects (n = 13) and abstinent alcohol-dependent subjects (n = 19) indicated a significant difference in the changes of ghrelin between the two groups (p=0.012) and a significant difference in baseline ghrelin levels (p=0.035).

These findings suggest that baseline ghrelin levels play an important role in alcoholic individuals; those patients with higher ghrelin levels at T0, in fact, were those who relapsed during the 12-week period. We also found a statistically significant positive correlation between ghrelin at baseline (T0) and craving during the 12-week period (T1-3), measured by the OCDS and the Penn Alcohol Craving Score (PACS).

Additional translational studies.

Consistent with the preclinical and clinical data presented above and with our overall hypothesis that the ghrelin plays a key role in alcohol reward and consumption, Davis and colleagues[133] at the University of Cincinnati analyzed self-report of ethanol intake in 6,165 bariatric patients before and following a Roux-en-Y gastric bypass (RYGB) surgery. Patients that reported frequent consumption of ethanol before RYGB reported decreased consumption following RYGB surgery. The same group also examined the hypothesis that RYGB surgery attenuates ethanol intake and reward in the context of frequent ethanol consumption utilizing a rodent model of RYGB and examined ethanol consumption and ethanol reward in male ethanol-preferring (P) rats. Interestingly, the RYGB procedure decreased ethanol intake and the reinforcing properties of ethanol in P rats. Of special interest for this protocol, pharmacologic replacement of the gut hormone ghrelin restored drinking behavior in P rats following RYGB.

Genetic polymorphisms of the ghrelin gene.

In another study[132], our group explored two polymorphisms of the ghrelin gene (i.e. G152A (Arg51Gln) and C214A (Leu72Met); these two polymorphisms were investigated previously in patients with binge eating disorders) in alcoholics and controls. Statistical comparisons of BMI values by means of two-way ANOVA revealed a main effect of genotype ($F = 7.013$, $P = 0.009$) with no main effect of diagnosis ($F = 0.705$, $P = 0.402$) and a trend toward a significant diagnosis–genotype interaction ($F = 0.096$, $P = 0.0757$). Furthermore, Landgren and colleagues[134] reported that SNP rs2232165 of the GHS-R1A gene was associated with heavy alcohol consumption and SNP rs2948694 of the same gene as well as haplotypes of both the pro-ghrelin and the GHS-R1A genes were associated with body

mass in heavy alcohol consuming individuals. In summary, very preliminary data suggest a possible role of the genes encoding ghrelin and/or its receptor in alcohol use, but these findings are not conclusive and suggest the need to further explore this aspect.

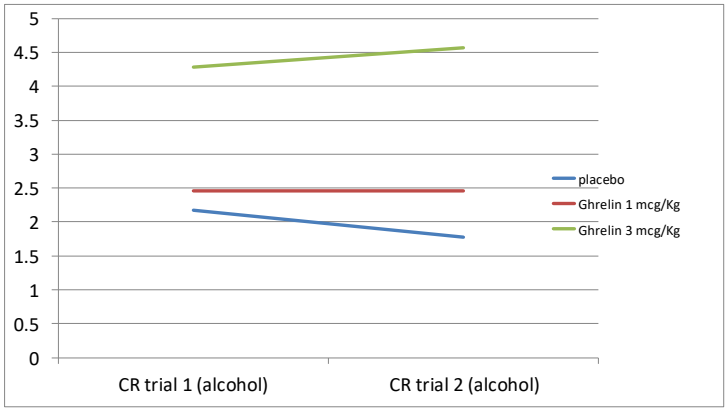
Summary.
In summary, human studies show: i) reduced ghrelin levels in actively drinking alcoholics; ii) increased ghrelin levels during alcohol abstinence; iii) a positive correlation between ghrelin level and alcohol craving scores. In keeping with the pre-clinical studies, the human studies suggest that ghrelin represents a potential new target for treatment of alcoholism.

Additional clinical data: preliminary findings with IV Ghrelin in alcohol-dependent individuals
While at Brown University, Dr. Leggio received funding from NIAAA (AA019709) to conduct the first study ever administering IV human ghrelin to non-treatment seeking heavy drinking alcohol-dependent individuals. The study – clinicaltrials.gov NCT01190085 – was also reviewed by the FDA (IND# 109242; sponsor and holder: Dr. Leggio). The experimental design is a between-subject 3-arm randomized double-blind placebo-controlled laboratory. Human ghrelin or saline solution (placebo) represents the between subjects factor. Each participant is randomly assigned to receive IV ghrelin 1 microg/kg or ghrelin 3 microg/kg or saline solution and then participates in a cue-reactivity (CR) experiment. Cue reactivity has demonstrated utility in eliciting urge to drink in alcoholics[135-137]. Exposure to alcohol cues (e.g.: the sight and smell of an individual's preferred beverage), reliably elicits increased urge to drink alcohol, increased salivation, and increased attention to the cues[135-137] when compared with control cues.
The two main endpoints of the trial performed at Brown University were: (1) whether IV ghrelin, as compared to IV saline, dose-dependently results in increased CR responses to alcohol cues in terms of urge to drink; and (2) whether IV ghrelin, as compared to IV saline, does not significantly increase frequency and intensity of Adverse Events (AEs).
The main findings of this trial were:

- Forty-five (n = 45) non-treatment seeking alcohol-dependent heavy drinking individuals were randomized and received administration of either IV ghrelin 1 microg/kg, IV ghrelin 3 microg/kg or placebo (IV saline). There were no serious adverse events (SAEs), unexpected AEs or severe AEs. There were only a few AEs, all were in the mild-moderate range and they were all expected. AEs were only transitory, and were all resolved without sequelae. It should be noted that the most common AE, i.e. increase in appetite, is in actuality a highly expected effect of ghrelin, given that ghrelin physiologically increases appetite (indeed, current studies are investigating the therapeutic role of ghrelin to increase appetite, for example, in patients with cancer). The increase in appetite was only transitory and resolved quickly and without sequelae. Notably, although this study enrolled heavy drinkers, none of them reported flushing after the infusion IV of ghrelin.
- We found no significant differences ($p > 0.5$) in the frequency AEs among the 3 arms of the study. In a repeated measures ANCOVA with the baseline SAFTEE score entered as covariate, dose did not significantly predict adverse events for the 3 post-injection assessments [$F(2,40) = 0.97$, $p = .39$]. Due to the short half-life of ghrelin, we also ran a parallel ANCOVA on just the first two post-injection assessments and this was also non-signficant [$F(2,40) = 1.37$, $p = .27$].
- Repeated measures ANCOVAs were conducted for the increase in alcohol urge during the CR procedure relative to the pre-medication urge level. Covariates included age, weight,

gender, and ethnicity/race. Pairwise comparisons revealed that alcohol urge was significantly greater for high dose ghrelin (3 mcg/kg) than for the placebo [p = .04, Least Significant Difference procedure (low vs. High dose: p = .19, placebo vs. Low dose: p = .52)] (Figure 2). The effect size (d) for the increase in alcohol urge for the high dose ghrelin versus placebo was 0.77. Notably, while CR studies usually use water as a neutral cue, here we used juice as a non-alcoholic appetitive control cue, and we didn't find a significant effect of ghrelin in increasing urge to juice, thus suggesting the specificity of ghrelin's effects on urge to alcohol.

Figure 2
A-VAS score for alcohol craving



Legend: significant increase in A-VAS score for craving in subjects receiving IV ghrelin 3 mcg/kg (n = 14) vs. those receiving IV ghrelin 1 mcg/kg (n = 13) or placebo (n = 18).

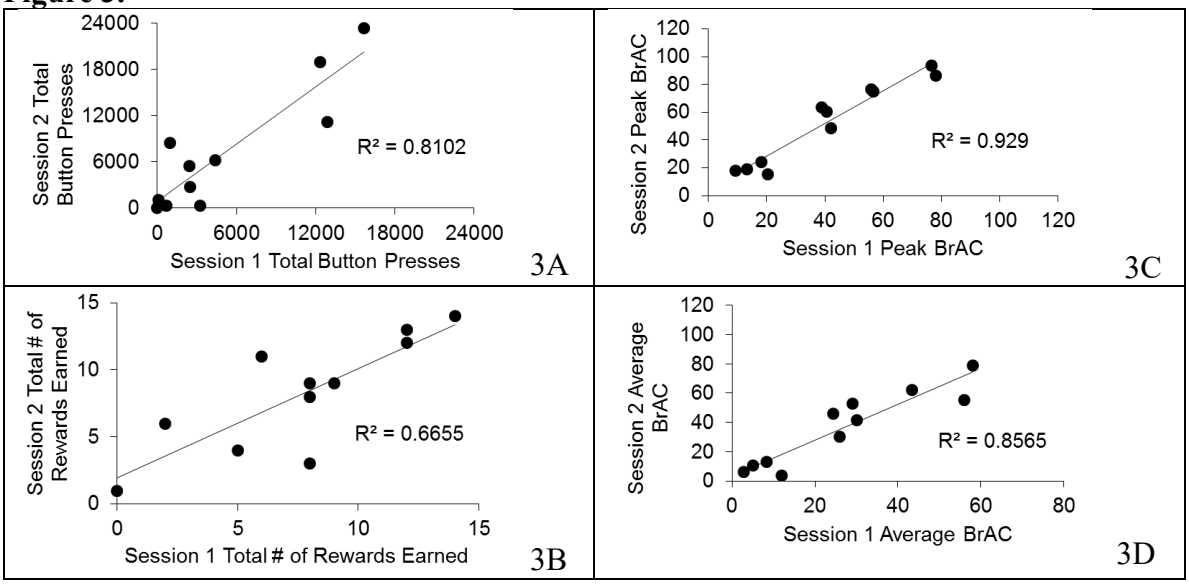
Summary of studies of ghrelin in alcoholism and questions that need to be answered.
Both animal and human studies provide strong evidence that the ghrelin system influences not only hunger but also has a clear role in the search for rewarding substances such as alcohol. Taken together, the preclinical studies provide strong evidence that ghrelin works not only on homeostatic energy-regulatory centers (e.g., hypothalamus and hindbrain), thus increasing the research of food, but also on reward centers (i.e., mesolimbic DA pathways), thus playing a role on the reward value of substances, such as food and alcohol. As reported above, animal studies suggest that ghrelin activates the cholinergic-dopaminergic reward link and that ventral tegmental nAChRs have a central role for the DA-enhancing properties of ghrelin.
Similarly, human studies have provided important information, showing that alcoholic individuals with higher plasma ghrelin levels have higher alcohol craving. These experiments suggest a model wherein elevated ghrelin levels might contribute to craving for substances such as alcohol. While the previous human studies have only tested plasma ghrelin levels, the study NCT01190085 conducted at Brown University (PI: Leggio) represents a translational research investigating the direct effect of exogenous ghrelin administration IV on alcohol-seeking behaviors. In addition to showing the safety of ghrelin administered IV to alcohol-dependent heavy drinkers, study NCT01190085 provides preliminary evidence that, as demonstrated in animal models, ghrelin may represent a novel pharmacological target to treat alcoholism. While ghrelin antagonists are not available (but their development for other medical diseases is in progress), there is a crucial need to provide additional human data using IV ghrelin

administration that can corroborate the animal studies performed to date and can provide evidence that ghrelin plays an important role in alcohol-seeking behavior. While study NCT01190085 provides safety data and a preliminary evidence that ghrelin may increase acutely alcohol craving, a logical next step of this research program is to test the effects of IV ghrelin administration on alcohol administration and on the behavioral effects of alcohol. Furthermore, it is important to investigate the CNS areas involved with alcohol reward via imaging tools. In the proposed study, we wish to provide direct, controlled clinical evidence that ghrelin is involved in the behavioral and central effects of alcohol administration. We propose to assess this using 1) a progressive ratio (PR) schedule paradigm with IV alcohol infusion, a paradigm that this research team has already used and validated (as detailed next), for example in protocol 08-AA-0178; and then 2) an "fMRI/alcohol clamp" paradigm that this research group has already validated and currently using, for example, in a protocol assessing varenicline (08-AA-0137).

PR IV Alcohol Procedure

The goal of the first paradigm is to investigate whether ghrelin alters alcohol's pharmacologic effects, including motivation for alcohol reward, as well as subjective measures of craving, and the biphasic effects of alcohol. The progressive ratio (PR) schedule paradigm requires participants to press the button an increasing number of times for each subsequent alcohol exposure (for e.g., 10, 20, 40, 80, etc., button presses for the 1st, 2nd, 3rd, 4th, etc. infusions). Outcome measures of this paradigm are the "breakpoint", i.e., the point at which the individual stops to work for more alcohol, as well as the average and peak BrACs achieved during the study. The PR schedule paradigm is an operant paradigm that is expected to assess motivation for reward (in this case, alcohol), and are based on the principle that people will work harder for greater rewards. There is much animal work to suggest the utility of operant paradigms in characterizing motivation for reward.[18] These operant paradigms have been used extensively in animal models and in a human studies to evaluate self-administration behavior for other drugs of abuse such as heroin and cocaine. Our research team at LCTS has experience using the PR paradigm, e.g. 08-AA-0178. The PR schedule paradigm represents an interesting and novel extension of the current development of IV alcohol self-administration methods extensively used by our research team (e.g.[138,139]). Notably, since the alcohol administration is not oral, it avoids the cues associated with oral alcohol administration that can confound the interpretation of laboratory studies employing drinking paradigms. Finally, during the PR sessions, the safety of ghrelin and alcohol, when co-administered IV will be evaluated, therefore the PR sessions will assure the safety of alcohol and ghrelin co-administered before performing the second paradigm ("fMRI/alcohol clamp") with the same volunteers (in fact, we will employ a within-subject design, as detailed after).Our team has recently demonstrated (PI: Vijay Ramchandani, Ph.D.; unpublished) the validity and utility of this procedure in a study whose primary objective was to examine alcohol intake behavior and the motivation for reward using subjective measures of alcohol effects and personality measures in a PR schedule of IV alcohol self-administration in 28 social drinkers. In this study, we estimated the correlations between sessions for number of button presses (Fig. 3A), total rewards earned (Fig. 3B), peak BrAC (Fig. 3C), and average BrAC (Fig. 3D); the correlation coefficients were high, demonstrating that this method has good test-retest reliability.

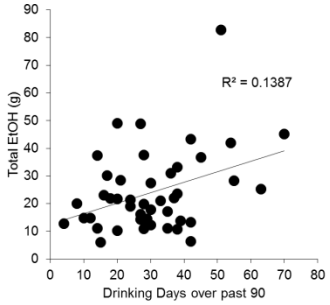
Figure 3.



Legend: between-sessions correlations for number of button presses (3A), total rewards earned (3B), peak BrAC (3C), and average BrAC (3D).

Furthermore, total ethanol self-administered during the PR schedule was significantly associated with drinking days measured with the TimeLine Follow-Back (TLFB) (Figure 4), thus indicating the clinical relevance of this procedure given its ability to reproduce in the human lab setting, the alcohol use in the naturalistic milieu.

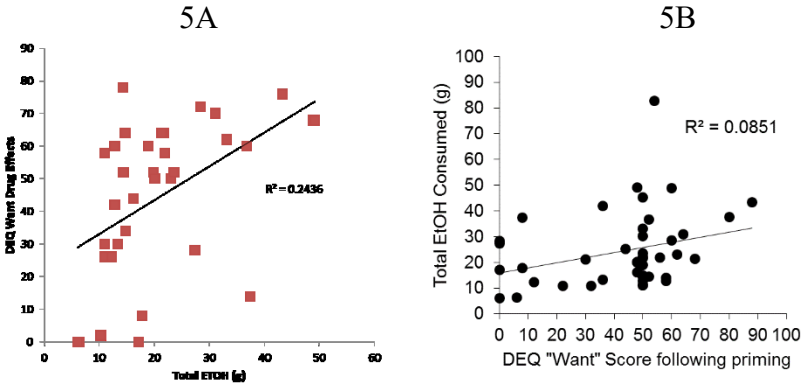
Figure 4



Legend: significant association between total ethanol self-administered during the PR schedule and drinking days.

Additionally, in this study we also found that total ethanol self-administered during the whole PR schedule was significantly associated with the Drug Effects Questionnaire (DEQ) score (considering its peak value) for measures of "wanting" (Fig. 5A), as well as with the DEQ score for "wanting" following the priming (Fig. 5B).

Figure 5



Legend: significant association between total ethanol self-administered and both DEQ "Want" score during the whole PR procedure (A) and following the priming (B).

Additional interesting results of this study were that total ethanol self-administered during the PR schedule was significantly associated with DEQ scores for measures of "feeling" and "liking" drug effects, as well as "high" and "intoxication" were positively associated with number of button presses. Alcohol Urge Questionnaire (AUQ) maximum measures were significantly associated with total ethanol and number of button presses. Biphasic Alcohol Effects Scale sedation measure after priming was strongly associated with peak BrAc, average BrAC, and total rewards earned.

In summary, these results demonstrate that the PR IV alcohol infusion procedure has good test-retest reliability and demonstrate that this method is sensitive to the rewarding and motivational properties of alcohol.

fMRI/alcohol clamp

The goal of the second paradigm is to investigate possible mechanisms of action of ghrelin though the use of two slightly different but related fMRI procedures. Specifically:

a) We will determine if ghrelin increases the incentive salience of alcohol predicting cues by using a modified version of the monetary incentive delay (MID) task. In this task subjects will button press to earn credits associated with subsequent alcohol administration, instead of monetary, reward. The MID task has been used extensively in neuroeconomic imaging research and the difference in BOLD signal in the ventral striatum (VS) between anticipation of responding for $0 and anticipation of responding for $5 is thought to measure the incentive salience of cues associated with monetary reward.[140,141] We propose that the difference in striatal BOLD signal between anticipation of responding for a cue predicting no alcohol delivery and cues predicting delivery of alcohol will provide an objective measure of the incentive salience of cues associated with alcohol reward. Our hypothesis is that ghrelin increases the motivational value of alcohol cues. In addition to the VS, other fMRI regions-of-interest (ROI's) for this study are the amygdala, insula and orbitofrontal cortex (OFC), as IV ghrelin has already shown its ability to activate these areas,[142] and all these areas play an important role in the neural systems model of alcoholism. [20,101,109,112,143]

b) In addition to using fMRI to examine ghrelin's effect on the incentive salience of alcohol cues, we will also use fMRI procedures we have developed[144] to examine the effect of ghrelin on the brain's response to IV alcohol. Our previous work has shown that IV

alcohol administration using the "alcohol clamp" procedure (NIAAA protocol 04-AA-0060) results in a robust increase in BOLD signal in the human ventral striatum. Thus, by administering alcohol during functional imaging we should be able to determine if ghrelin enhances the direct pharmacological effect of alcohol on the brain's reward circuitry.

Our team has validated the procedures we propose here for the fMRI/clamp sessions. Dr. Hommer of the Section of Brain Electrophysiology and Imaging has used and validated different versions of the MID task in the last decade and during the last years these procedures have been widely used in the functional neuroimaging imaging community to study reward processing. In the basic version of the task subjects see a cue indicating that on the current trial they are working for money (e.g., $5.00, $1.00, or $.20) or for no money; next they wait for a variable delay, and then press a button in response to presentation of a target. If they respond before the target disappears, they win the money indicated at the start of the trial. If they are too slow they win nothing. The interval between appearance of the initial cue and the target cue signaling the need to make a response is considered to manifest the brain state underlying the motivation for the specific action and involves both elements of motor preparation as well as anticipation of gaining reward. Striatal activation during this interval can also be considered a measure of incentive salience. Finally the subjects get visual feedback indicating if they successfully responded, how much they won or lost, and their total earnings. One of the major goals has been to develop ways of using a version of MID task to directly measure motivation to experience alcohol's effects in humans and to use this model to study pharmacotherapies. In fact, Dr. Ramchandani of the Section on Human Psychopharmacology is currently using this new approach in an experimental medicine study to evaluate the efficacy of varenicline for the reduction of excessive alcohol use in non-treatment seeking heavy drinkers. In this study, subjects are initially trained to button press to obtain intravenously administered ethanol. Then they are scanned while they perform a MID task during which they can button press to obtain points that determine how much intravenous alcohol or snack food they will be given at the conclusion of the scan. Thus, instead of measuring brain activation while anticipating working for money, we can measure brain activation while anticipating working for alcohol or food. Our group has recently conducted preliminary analysis (PI: Vijay Ramchandani, PhD; unpublished) of the data obtained from subjects on placebo (n=7); this analysis indicated significantly higher BOLD activation in striatal regions during anticipation of working for alcohol reward under placebo conditions. Preliminary analysis of data from subjects receiving varenicline (n=8) compared to placebo (n=7) show lower BOLD activation in striatum during anticipation of working for alcohol ($p<0.01$, uncorrected, $k>10$) (Figure 6). These results suggest that the Alcohol-MID task can be used to measure motivation or desire for alcohol as well as the effect of a drug on this measure.

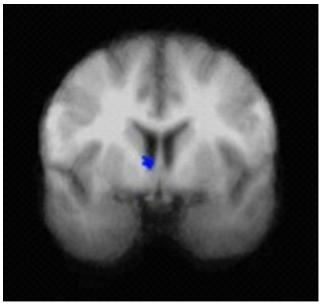


Figure 6: decreased BOLD activation in striatum (blue region) seen in patients on varenicline compared to placebo during anticipation for alcohol reward in Alcohol-MID task.

Innovation of the study

This protocol is not only a logical 'next step' of Dr. Leggio's NIAAA-funded study with ghrelin performed at Brown University, but also there are several new and innovative aspects, i.e. 1) the

study at Brown University only assessed alcohol craving (i.e. alcohol was not administered), while this study will test for the first time the role of IV ghrelin on alcohol administration. Using a model already validated by our team (the PR paradigm with IV alcohol administration; 08-AA-0178), this protocol represents a direct translational study of the preclinical studies conducted by Jerlhag and colleagues.[3,4,117,121,122] In fact, the use of the PR administration will provide novel information, that is if IV ghrelin alters motivation for alcohol reward; 2) this study will provide, for the first time, information on the effect of ghrelin on the brain's response to alcohol; 3) the study at Brown University only assessed the effects of a bolus on alcohol craving, while this protocol will assess the effects of ghrelin for a longer period of time. In fact, after a bolus of ghrelin, then an IV infusion of ghrelin will take place during the entire duration of the experiment; 4) there are only a few and preliminary data on the role of genetic polymorphisms of the ghrelin and ghrelin receptor genes on alcohol consumption; also, none of the previous studies was conducted in subjects receiving an IV administration of ghrelin, thus this protocol will test genetic polymorphisms related to the ghrelin system in heavy drinkers receiving IV ghrelin; and 5) although the well-known role of ghrelin in appetite and food intake, and while several data suggest that sweet preference may represent an important phenotype in alcoholic individuals, this protocol will explore if and how sweet-liking status (a sweet preference test will be performed, as detailed below) may affect ghrelin levels and behavioral responses in heavy drinkers receiving IV ghrelin.

In summary, this project proposes to use validated approaches (i.e. IV alcohol infusion and fMRI) to provide a direct and objective evidence of the central role of ghrelin in alcohol reward and administration. The overall goal is to provide evidence of a novel neuropharmacological target to treat alcoholism, i.e. the ghrelin system. If our hypothesis will be demonstrated, then this study will serve as the platform to test ghrelin antagonists/inverse agonists as new pharmacotherapies for alcoholism. Additionally, this study may provide evidence of the use of IV ghrelin as a 'challenge/provocative test' for future behavioral and pharmacological studies.

2. Study objectives

The primary objective of this protocol is to investigate whether ghrelin alters alcohol's pharmacologic effects, including motivation for alcohol reward, as well as subjective measures of craving, and the subjective responses to alcohol in heavy drinkers receiving IV administrations of ghrelin and alcohol.

a. Primary objectives

The primary measure of this study will be the breakpoint, which is the schedule (number of button presses) at which the individual stops to work for more alcohol.

b. Secondary objectives

Secondarily, the BrAC exposure measures will also be determined, that is the highest (max) BrAC, average BrAC and the area under the BrAC-time curve determined for each subject. Urge to drink and alcohol sensitivity will be measured during the PR session. Adverse events will also be assessed in order to assess the safety of the IV co-administration of ghrelin and alcohol. Also, during the "fMRI/alcohol clamp" session, fMRI will be used to see whether ghrelin affects the activation of the ventral striatum (VS) in anticipation to work for alcohol reward and the incentive salience of cues in subjects who will subsequently receive IV alcohol administration. In

addition to the VS, other fMRI ROI's are the amygdala, insula and OFC. Adverse events will also be assessed during the fMRI/alcohol clamp sessions.
Furthermore, an additional secondary aim will be an exploratory analysis of the role of genetic polymorphisms of both ghrelin and ghrelin receptor genes – detailed in page 14 – as possible moderators of ghrelin's effects on the primary aim ('breakpoint', i.e. number of button presses in the IV alcohol PR procedure).

3. Subjects:

a. Description of study populations

Male and female adults will be eligible to participate in this study. Participants will be non-treatment seeking heavy drinking volunteers. Our goal is to screen 124 subjects and recruit 62 individuals. Assuming a 20% drop-out rate, we will reach 50 completers.

b. Inclusion criteria

1. Male and female participants between 21-60 years of age.
2. Good health as determined by medical history, physical exam, ECG and lab tests.
3. Creatinine ≤ 2 mg/dl.
4. Female must have a negative urine pregnancy (hCG) test at the start of each study session. Females of childbearing potential who are sexually active and have not been surgically sterilized must agree to use an adequate method of birth control during the study. Adequate methods of contraception for sexually active women are having a male sexual partner(s) who is surgically sterilized prior to inclusion; having a sexual partner(s) who is/are exclusively female; using oral contraceptives (either combined or progestrogen only) with a single-barrier method of contraception consisting of spermicide and condom or diaphragm; using double-barrier contraception, specifically, a condom plus spermicide and a female diaphragm or cervical cap plus spermicide; or using an approved intrauterine device (IUD) with established efficacy.
5. Participants must drink alcohol regularly at a heavy level, on average greater than 20 drinks per week for men, and greater than 15 drinks per week for women, and not be seeking help for alcohol-related problems.
6. Participant must be willing to receive two IV lines.

c. Exclusion criteria

1) Current or prior history of any clinically significant disease, including CNS, cardiovascular, respiratory, gastrointestinal, hepatic, renal, endocrine, or reproductive disorders.
2) Specific exclusion criteria related to the administration of ghrelin, are chronic inflammatory diseases (e.g., Crohn's disease, ulcerative colitis, celiac disease), diabetes, obesity (BMI ≥ 30 kg/m^2), weight ≥ 120 Kg, high triglycerides level (> 350 mg/dL), history of clinically significant hypotension (e.g.: history of fainting and/or syncopal attacks) and/or resting systolic BP < 100 mmHg.
3) Positive hepatitis or HIV test at screening.
4) Current clinically significant major depression or anxiety; or prior clinically significant psychiatric problems, including eating disorders, schizophrenia, bipolar disorder, obsessive compulsive disorder.
5) Current diagnosis of substance dependence (other than alcohol or nicotine).

6) Currently seeking treatment for alcohol use disorder.
7) History of significant withdrawal symptoms or presence of clinically significant withdrawal symptoms (Clinical Institute Withdrawal Assessment (CIWA) score > 8) at screening.
8) Non-drinkers (alcohol-naïve individuals or current abstainers) or no experience drinking 5 or more drinks on one occasion.
9) Unable to provide a negative urine drug screen.
10) Pregnancy or intention to become pregnant for women. Female participants will undergo a urine beta-hCG test to ensure they are not pregnant.
11) Use of prescription or OTC medications known to interact with alcohol within 2 weeks of the study. These include, but may not be limited to: isosorbide, nitroglycerine, benzodiazepines, warfarin, anti-depressants such as amitriptyline, clomipramine and nefazodone, anti-diabetes medications such as glyburide, metformin and tolbutamide, H2-antagonists for heartburn such as cimetidine and ranitidine, muscle relaxants, anti-epileptics including phenytoin and phenobarbital codeine, and narcotics including darvocet, percocet and hydrocodone. Drugs known to inhibit or induce enzymes that metabolize alcohol should not be used for 4 weeks prior to the study. These include chlorzoxazone, isoniazid, metronidazole and disulfiram. Cough-and-cold preparations, which contain anti-histamines, pain medicines and anti-inflammatories such as aspirin, ibuprofen, acetaminophen, celecoxib and naproxen, should be withheld for at least 72 hours prior to each study session.
12) Current or prior history of alcohol-induced flushing reactions.
13) Contraindications for MRI scanning, including metal in body that are contraindicated for MRI (such as implants, pacemaker, prostheses, shrapnel, irremovable piercings), left-handedness, and claustrophobia.

Please also see eligibility checklist (Attachment).

Clearance for eligibility to this protocol will be consistent with the recent "*NIAAA/LCTS Patient Eligibility Checklist Process"* policy implemented by the NIAAA Clinical Director. As such, eligibility criteria will be reviewed by a team of three individuals: research nurse coordinator (or designee), a nurse practitioner and PI or AI designated by the PI. All three signatures will be required in order to establish participants' eligibility to this protocol.

4. Study Design and Methods:

a. Study overview

The study is designed as a within-subject, double-blind, placebo-controlled randomized clinical trial of ghrelin with non-treatment seeking heavy drinking volunteers. Including screening and follow-up evaluations, the study lasts about 8 weeks and will require five visits to the NIH CRC. The five visits will be as follow and performed in a counter-balanced order across participants:

Visit #1 - Initial screening visit (**under the NIAAA 98-AA-0009 or 14-AA-0181 protocol**);
Visit #2 - Alcohol PR + ghrelin/placebo;
Visit #3 - Alcohol PR + placebo/ghrelin;
Visit #4 - fMRI/alcohol clamp + ghrelin/placebo;
Visit #5 - fMRI/alcohol clamp + placebo/ghrelin;

Visit #6 - Post-study follow-up.

b. Recruitment

Participants will be recruited through referrals from the NIH Volunteer Office, and from the Patient Recruitment and Public Liaison (PRPL) Office as well as through ResearchMatch.org (facilitated through PRPL). ResearchMatch is a voluntary service that matches people interested in being research participants with researchers conducting a wide range of studies. ResearchMatch is currently used for other protocols of our Clinical Program at NIAAA, as well as for many other NIH protocols. ResearchMatch is a Clinical and Translational Science Awards (CTSA) initiative funded by the National Center for Advancing Translational Sciences, part of the National Institutes of Health.

Furthermore, participants will also be recruited by word of mouth and through local advertisements. Advertisement language will be used as flyers with tear off tabs, posted on the NIH campus, campuses of universities and colleges in the greater Washington DC area, as well as in billboards of public places and public transportation services in the greater Washington DC area. Advertisements will also be posted in electronic and printed local media, including newsletters, websites and local newspapers in the greater Washington DC area (for example, the 'Express'). Listserv ad will be used on craigslist.org, under the volunteers' section, as well as in NIH and other local email distribution lists, that are moderated, and following approval from the managers of these lists. 'ClinicalTrials.gov' may also represent a source of recruitment.

Finally, participants will also be recruited via the NIAAA Screening Protocol (98-AA-0009, PI: Reza Momenan, Ph.D.), or 14-AA-0181 as the 98-AA-0009 and 14-AA-0181 protocols serve as the screening platform not only for this study but also for any other NIAAA protocol..

c. Screening

Candidates will first be evaluated over the phone. Qualified candidates will come to the NIH CRC for a screening visit (**Visit #1**) during which they will be enrolled in the NIAAA screening protocol (98-AA-0009 or 14-AA-0181). If a clinical procedure is repeated (e.g. blood/urine lab work, EKG) under the screening protocol, exclusion/inclusion criteria will be based on the most recent clinical values. The screening procedures covered under the NIAAA screening protocol include (details of the procedures are in the 98-AA-0009 protocol or 14-AA-0181 protocol) (a) medical history, (b) drinking history, using the timeline followback method (TLFB), (c) family history of alcoholism, using the Family Tree Questionnaire, (d) Structured Clinical Interview for DSM-IV (SCID) psychiatric diagnoses including alcohol or drug dependence, (e) physical examination, including ECG, (f) blood tests for routine blood chemistry, complete blood count (CBC), liver enzymes, hepatitis and HIV screen, (g) urine tests to screen for illicit drugs, (h) the NEO-PI personality inventory, and (i) an Alcohol Flushing Questionnaire[145-146] to identify individuals who might have a flushing response (including facial flushing, palpitations, drowsiness and other unpleasant symptoms) when drinking alcoholic beverages. The visit may last up to 4 hours.

Normally, the results of this evaluation, including possible diagnoses of alcohol use disorders, will be discussed with the subject after the study is completed. However, if an alcohol-related problem is found that requires immediate clinical attention, or if the subject requests it, the diagnostic information will be given immediately and the subject will have an additional opportunity to consider whether they would like to receive treatment.

Given that the 98-AA-0009 or 14-AA-0181 protocol serves as the screening platform for this study, identified data obtained under this protocol may be shared with the data collected under the AA-98-0009 or 14-AA-0181 protocol and may be combined for analysis. The 98-AA-0009 and 14-AA-0181 protocol are also protected by Certificate of Confidentiality.
Eligible participants will be randomized to 1 of 2 counterbalanced arms to receive first either ghrelin or placebo during the first PR session. Participant then will receive the alternate condition during the second PR session. Similarly, participants will receive ghrelin first and then placebo (or vice versa) during the third and fourth sessions, namely the two "fMRI/alcohol clamp" sessions. Eligible participants will be randomized to receive either ghrelin or placebo during the study according to gender, based on a stratified block design. The Pharmaceutical Development Service (PDS) of the NIH CRC will develop the randomization schedule and maintain the blind; they will also be responsible for determining subject allocation. The allocation code will be kept in a safe location at the pharmacy, with access only for the responsible pharmacist. In emergency medical situations, which may require premature breaking of the code, the PI will be able to obtain access to the code table through the pharmacist on duty; this will then be documented, and reported to the IRB.

d. Study Procedures

Visits and procedures

IV Ghrelin infusion: dose justification

Overview.

After a 10 minute bolus of IV ghrelin or matched placebo (saline solution), IV ghrelin or placebo infusion will last 120 min during visits #2 and #3 (120 minutes is the duration of the alcohol PR session) and 65 min during visits #4 and #5 (the total duration of the fMRI/alcohol clamp is 95 min with 35min for the alcohol clamp – details below and in appendix flow sheets). The Pharmaceutical Development Service (PDS) of the NIH CRC will prepare the ghrelin bags ready for IV infusion. Vials of ghrelin and saline will be indistinguishable visually.

Dose.

A bolus of IV ghrelin followed by an infusion of IV ghrelin will be employed, as detailed next.

- An IV administration of 3 mcg/kg bolus of ghrelin. The injection of the bolus will start 10 minutes before the start of the other lab procedures (PR for Visits #2 and #3; "fMRI/alcohol clamp" for Visits #4 and #5) and will last 10 minutes;
- An infusion of 5 pmol/Kg/min (that is 16.9 ng/kg/min) of ghrelin for 120 minutes (Visits #2 and #3) or 65 minutes (Visits #4 and #5). In fact, the infusion will last for the duration of the PR (120 minutes at Visits #2 and #3) and for a portion of the 95 min "fMRI/alcohol clamp" (65 minutes at at Visits #4 and #5).

Dose justification.

1. Ghrelin dose is adjusted per weight. Although some studies used a fixed dose[47], the majority of the studies used a dose of ghrelin adjusted per weight (see Tables 1-2). Consistent with the majority of the literature, we will use a dose of ghrelin based on body weight. To now, there are no published data showing that ghrelin dose should also be based on other parameters, such as body water and/or gender. However, it is worth noting that

two studies showed that IV ghrelin effects are independent of gender[42,47]. We will exclude subjects affected by obesity (BMI ≥ 30 Kg/m^2) or subjects with weight ≥ 120 Kg, for at least three reasons: 1) to keep doses of ghrelin administered at a low-medium level, given we will adjust the dose per weight; 2) to avoid enrolling subjects with a possible 'ghrelin resistance' which may mask the effects of IV ghrelin administration; and 3) to avoid enrolling subjects with other psychiatric disorders (e.g. binge eating), as we want to focus specifically on the effects of ghrelin on alcohol reward.

2. Ghrelin is administered as a bolus followed by an infusion. In the previous study at Brown University, only a bolus of 1 mcg/kg or 3 mcg/kg was used because a longer exposure to ghrelin was not needed given the short duration of the human lab session (e.g. the cue-reactivity experiment last ~30 minutes). By contrast, in this protocol, it is important that ghrelin's effects last longer in order to fully investigate the possible effects of ghrelin on alcohol reward, alcohol craving, alcohol sensitivity and fMRI outcomes during the entire duration of the human lab sessions performed at Visits #2-5. We will still employ an initial bolus, as the preliminary data from Brown University (see Figure 2) suggest that a bolus might acutely and temporarily increase alcohol craving. Specifically, we will use a bolus of 3 mcg/kg because the preliminary data (see Figure 1 above) might suggest a possible (albeit not significant - interim analysis) dose-response effect on alcohol craving, thus the higher dose should be used to maximize the probability of finding a drug effect. After the 10-minute ghrelin bolus, an infusion of 5 pmol/Kg/min of ghrelin for 120 minutes (Visits #2 and #3) or 65 minutes (Visits #4 and #5) will take place. The dose of 5 pmol/Kg/min (= 16.9 ng/kg/min) is that most frequently used in previous studies with healthy controls or subjects with other chronic conditions, as detailed in the Tables 1 and 2. For example, Stephen Bloom's lab at the Imperial College of London has demonstrated that 5 pmol/Kg/min (as compared not only to placebo, but also to 1 pmol/Kg/min) is able to reproduce the physiological effects of ghrelin. Although our dose schedule includes a bolus of 3 microg/Kg before the infusion of 5 pmol/Kg/min, it is important to note that other studies have used even higher doses of ghrelin (e.g. higher total daily exposure) and still with a very safe profile. For example, Lavin et al.[49] used an IV ghrelin dose of 10 pmol/Kg/min for 180 minutes, and participants did not report any side-effect. Anna Cappola's lab at UPenn has recently performed a 3-hr IV ghrelin study[69] where ghrelin was infused at a rate of 2.5pmol/kg/min for the first 60 minutes, 5 pmol/Kg/min for the second 60 minutes, and 10 pmol/kg/min for the last 60 minutes. The only transitory side-effect was a feeling of warmth. Notably, Cappola's study was conducted in individuals who were generally more prone to develop side-effects, e.g. individuals > 70 years old with geriatric frailty (while here we will exclude anyone > 60-yr-old). In this study, we propose a dose whose total daily exposure is significantly lower compared to the studies conducted by Lavin's[49] and Cappola's lab[69].

PR sessions (visits #2 & #3)

Breath ethanol levels will be measured, a urine drug screen and, in women, a urine pregnancy test will be performed at the start of these sessions. Information regarding alcohol drinking, cigarette smoking, mood and anxiety states and symptoms will be collected. Food craving and urge to drink alcohol will also be collected.

Prior to the start of the session, two indwelling IV catheters are inserted using sterile technique. One of the IV catheters is used for the ethanol IV infusion, the other for the ghrelin IV infusion.

One of the IV catheters includes a bidirectional valve that allows for both infusion and blood sampling during the experiment. The subject is seated in a comfortable chair, out of sight of the infusion pumps, and instructed in the procedures. The experimenter will be available to answer any questions raised by the participants and will occasionally inquire about the well-being of the participant.

Progressive Ratio Schedule Self-administration (Visits #2 and #3).
The PR schedule self-administration will be conducted using the Computer-Assisted Self-infusion of Ethanol (CASE) system (currently being used in two NIH protocols 08-AA-0137 and 08-AA-0178). Just prior to the start of the PR schedule session, the subject will be reminded of the procedure for obtaining alcohol during the session. Following a 15-min priming interval (that will not applied here, as our 'priming' is the IV bolus of ghrelin), the PR period lasting 120 minutes begins. Each standardized exposure will follow a slopelet pattern (linear increase of 7.5 mg% over 2.5 min followed by a linear decrease of 0.5 mg%/min until the next exposure). The PR schedule will escalate geometrically from using a progressive ratio that will be empirically established. An initial PR schedule will be established based on simulations using the model published by Richardson and Roberts.[147] For practical considerations, we will select a schedule that requires 2 button presses for the first exposure and escalates exponentially to requiring ~15,000 button presses for the 20th exposure. This initial schedule will be tested in the first 6 subjects, and the range of break-points obtained will be evaluated. If there is insufficient variability in the break-points across the first 6 subjects, the schedule may be adjusted before proceeding with the enrollment of other subjects. Within 0.5 seconds of pushing the drink button, the CASE system software will begin infusing the alcohol necessary to produce the desired increase in BrAC exposure from the BrAC level at the time the button was pushed. Also, if the CASE system predicts that an additional drink selected by the participant would result in a peak BrAC 0.12 (which is the upper limit for BrACs achieved in this study), then the push button will be inactivated, with the subject's knowledge, until the time when the predicted peak BrAC is below the upper limit.

In an attempt to control the ambient environment during the self-administration session, participants will be offered one of five choices of activity: 1) listening to music; 2) watching videos of television comedy shows; 3) watching videos of nature documentaries; 4) watching sports videos; or 5) browsing the internet on a laptop or tablet. The participant's choice will be documented and any effect of the participant's activity on self-administration measures will be explored. The experimenter will be available to answer any questions raised by the participants and will occasionally enquire about the well-being of the participant. At 15-minute intervals, the experimenter will obtain a BrAC reading. Participants will be asked to complete a Drug Effects Questionnaire (DEQ) which assesses feelings of "high" and "intoxication" and how much they "like" the feelings they perceive using visual analog scales,[148] and the Biphasic Alcohol Effects Scale (BAES)[149] to assess the stimulant and sedative effect of alcohol. Participants will also complete the Alcohol Visual Analogue Scale (A-VAS), the Alcohol Urge Questionnaire (AUQ) to assess the urge to consume alcohol,[150] a mood questionnaire (Profile of Mood States, POMS[151]), and the CASE Experience Questionnaire (CEQ) to determine craving and if participant's can gauge the amount of ethanol infused. At the end of the session, the infusion pump will be disconnected and the IV catheters will be removed from the participant's arm. Lunch will be provided and serial breathalyzer tests will be done to track the BrAC level until it

reaches 0.00%. Then, participant will be asked to stay in the Clinical Center overnight and the morning after participant will have breakfast, receive debriefing and then released.

Infusion profile computation using the CASE system: The infusion rate profile for each participant is calculated using CASE system software, which exercises a modification of the physiologically-based pharmacokinetic model of ethanol distribution and elimination being used in completed and ongoing LCTS alcohol infusion studies (03-AA-0283, 04-AA-0060, 07-AA-0026, 08-AA-0125, 08-AA-0137, 08-AA-0178, 11-AA-0180). Currently, physiologically-based pharmacokinetic model parameters are computed for each individual participant by transformation of the subject's age, height weight and gender into the physiologic parameters of the model. Using a high gain on the simulated error between model and desired BrAC as the infused alcohol, the model is forced to follow the desired time course of BrAC, thereby computing the prescribed infusion rate profile as a continuous variable. A pump driver software provides the interface between the buffer and the infusion pumps, using the computer's real-time system clock to administer the profile during the experimental session. Since the modeling solutions are calculated at a thousand-times real-time, the delay between the subject electing another drink and the consequent adjustment to the infusion profile is negligible. The software will manage adherence to the limits on availability of another drink by computing the predicted peak BrAC for an additional drink and making the push button unavailable until the predicted peak BrAC is below the pre-set upper limit for BrAC exposure.

<u>Additional baseline assessments performed at Visit# 2 for exploratory analyses.</u>
Sweet Preference Test. In this study, we will explore if responses to ghrelin administration differ between sweet-liking and sweet-disliking individuals. A growing body of evidence in fact indicates that the hedonic response to sweet taste is associated with genetic risk of alcohol dependence (AD) in humans. Given that we are investigating a peptide that physiologically is involved with food reward and appetitive behaviors, it will be scientifically interesting to explore possible differences between sweet-liking and sweet-disliking individuals in this study. Sweet preference testing will be conducted as previously described (e.g.[152.153]). Each subject will be instructed to sip, swish around his/her mouth and then spit out five different concentrations of a sweet solution (0.05, 0.10, 0.21, 0.42 and 0.83 M) each of which will be presented five times in a pseudorandom order for a total of 25 tastings. After each tasting, participants will rinse their mouths with distilled water before proceeding to the next solution. The participants then will rate the intensity and pleasurableness of each tasting using a 200 mm analog scale. Subjects will be assigned to one of two categories— sweet-liking or sweet-disliking —based upon their hedonic response to the various sucrose concentrations. To be categorized as a sweet liker, a subject must rate the highest concentration of sucrose (0.83 M) as the most pleasurable. Sweet-dislikers could prefer any of the other four concentrations. Such categorization has been shown to be stable over time.

Brief Addictive Behavior Social Density Assessment (BASDA). We will administer the alcohol component of the abbreviated version of the BASDA, as recently developed at the University of Georgia.[154] The BASDA measures the perception of alcohol use for each participant's 1st, 2nd, 3rd, & 4th closest non-biological associates, mother, father, and two closest siblings in age, as self-reported by the study participant. The BASDA alcohol use subscale consists of the first three questions of the Alcohol Use Disorders Identification Test (AUDIT). The goal of this assessment

is to explore the social milieu that surrounds the participant and if this information might help to predict different responses to drinking behavior in our human laboratory setting. The BASDA is a self-reported assessment where participants self-report on their closest associates' drinking behaviors. Participants are not asked to provide the names of their participants' closest biological and non-biological associates.

Alcohol effects questionnaire (AEFQ) version s. The alcohol effects questionnaire is a revision of the alcohol expectancy questionnaire and is used to assess the positive and negative effects people expect to experience from alcohol. The brief questionnaire consists of 40 true/false items with 8 subscales that aims to assess personal beliefs regarding the both the reinforcing and undesirable effects of alcohol. The 8 subscales are: global positive, social and physical pleasure, sexual enhancement, power and aggression, social expressiveness, relaxation and tension reduction, cognitive and physical impairment, and careless unconcern. In research studies, AEFQ expectancy scores have correlated with drinking problems, alcohol cue reactivity, and number of lever presses during IV alcohol self-administration (unpublished data from Dr. Ramchandani's lab).

Behavioral approach system (BAS)/behavioral inhibition system (BIS) scale. The BIS/BAS scale was developed to measure individual differences in the sensitivity of the BIS and BAS.[155] The scale is divided into 4 subscales: BAS Drive, BAS Fun Seeking, BAS Reward Responsiveness, and BIS. Much research suggests these systems play an important role in vulnerability to addiction. In fact, high BAS Fun Seeking scores have been shown to be associated with lifetime drug abuse/dependence,[156] and high BAS Drive scores have been shown to predict alcohol craving during exposure to alcohol cues.[157] The individual differences in reward sensitivity measured by these scales are especially relevant to addiction when examining the role of the DA reward system. The goal of this assessment in the current study is to further explore how BAS and BIS personality traits relate to alcohol craving and the activation of reward circuitry.

Finally, although this protocol will not assess formally smoking-related outcomes, we are also aware of the high comorbidity between alcoholism and smoking. Therefore, at Visit 2 (as well as at Visits 3, 4 and 5), we will assess breath carbon monoxide (BrCO) for possible between-group differences and possible post-hoc analyses.

fMRI/alcohol clamp sessions (visits #4 & #5)
Breath ethanol levels will be measured, a urine drug screen and, in women, a urine pregnancy test will be performed at the start of these sessions. The two "fMRI/alcohol clamp" sessions will be identical (but the double-blind drug condition will be ghrelin or placebo) and they will be scheduled at approximately the same time of the day with at least 3 days washout window between them. A washout period of at least 3 days will also be applied between visits #3 and #4. fMRI scanning in this protocol is an investigational procedure. The administration of alcohol is performed following the standardized alcohol clamp paradigm for which our research team at LCTS has considerable experience (e.g. 03-AA-0283, 04-AA-0060, 07-AA-0026, 08-AA-0125 and 11-AA-0180). The fMRI procedures will be similar to those used in protocols 98-AA-0056 and 04-AA-0060. Also, the combined "fMRI/alcohol clamp" procedures will be identical to those used in the current varenicline protocol (08-AA-0137), therefore the research team has already experience in this combined paradigm. BrAC testing will be done immediately prior to

the scan and subjects whose BrAC exceeds 0.000% will not be scanned. An IV catheter will be inserted in each forearm; one of the IV catheters is used for the ethanol IV infusion, the other for the ghrelin IV infusion. The IV catheter includes a bidirectional valve that allows for both infusion (e.g. ethanol) and blood sampling during the experiment.

Imaging will take place in the NIH NMR center using one of the center's 3T scanners. An initial structural scan will be collected on each subject for later co-registration of functional images. The structural scan will last less than 10 minutes. Functional scans will be acquired using a T2*-EPIRT sequence that measure changes in blood oxygen level dependent (BOLD) contrast. Visual stimuli will be projected onto an opaque screen at the foot of the imaging machine via a liquid crystal diode projector. Subjects will be able to view the images via a mirror placed above their head in the scanner. Subjects will have an opportunity for a break between structural and functional imaging, and will remain in contact with the experimenters at all times via an intercom system built into the scanner. The functional scans, (see appendix flow sheet for scan times), will be acquired while the subjects are at rest and while they participate in a modified version of the MID task in which they will respond to cues that indicate the opportunity to press a button to gain a reward. On some trials the reward will be points that can be exchanged for snack foods (subjects can choose items from a hamper containing candy bars, bags of chips and crackers provided by the Nutrition Department of the NIH Clinical Center) while on other trials the reward will be points that will determine how much intravenous alcohol a subject will be given during the "alcohol clamp" procedure which will immediately follow the modified MID task. In the previous IV ghrelin alcohol study at Brown, we included a palatable nonalcoholic beverage to investigate whether the effects of IV ghrelin occur across caloric appetitive beverage cues. For the same reasons, and consistent with the role of ghrelin in controlling food intake, in this protocol, participants will be exposed to both alcoholic and food-related cues in order to tease a part possible non-specific effects of ghrelin on appetitive behaviors vs. specific effects on alcohol-related behaviors.

After completion of the baseline modified MID task, subjects will receive the ghrelin/placebo bolus and followed by a continuous infusion as described above. During ghrelin/placebo infusion subjects will repeat the MID task and resting scans. Next, alcohol infusion begins, which will be given as described in protocol 08-AA-0137. Ethanol will be infused as a 6% v/v solution in saline. The infusion rates will be based on a physiologically-based pharmacokinetic model for ethanol – the same model used to compute the infusion rates for the alcohol self-administration sessions described above. Blood samples will be obtained during the clamp. The alcohol clamp method has been used successfully in several studies of the pharmacokinetics and pharmacological effects of ethanol in humans,[158-165] and is has been used, for example, in NIAAA protocols listed above.

During the alcohol clamp, subjects will have resting scans and repeat the MID task. (See fMRI appendix flow sheet for details). At the end of the infusion, the catheters will be removed, lunch will be served and repeated BrACs will be performed until BrAC returns to 0.00 g%.

Diet (visits #2-5)

The night before each session, participants are admitted to the Clinical Center and will receive a standardized dinner. After overnight stay, participants will receive a fixed breakfast in the morning. These two courses will be standardized in agreement with the NIH CC Nutrition Service. Then, before starting the session, a fixed light snack is served (≈167 Kcal; approximately 62% carbohydrate, 13% protein, 25% fat) to standardize food intake before IV

ghrelin. The use of a standardized caloric food intake before the experiment: i) is a procedure used in human studies testing ghrelin i.v.[35,37,75,]; ii) is consistent with studies testing the role of ghrelin on alcohol reward in animals without food deprivation effect[98,122]; iii) avoids the presence of elevated baseline ghrelin levels (present in food deprivation conditions[34,74,81,91]), which may be a confounding factor for our controlled experiment (exogenous ghrelin vs. saline solution); iv) does not represent a confounding factor for the ethanol administration, as it is infused IV and not orally; and v) it is consistent with our similar procedure used in the IV ghrelin alcohol study at Brown University. Lunch will be provided after the end of each session. Water will be permitted *ad libitum*, while other beverages (e.g. soda) will not be permitted.

In order to standardize the appetitive/metabolic conditions of the study participants, we also plan the following:

- Nutrition Department staff at the NIH Clinical Center will meet with the participants to perform a 24-hour recall of everything the participant consumed prior to admission. Participants will also be instructed to eat as similarly as possible during the 24 hours prior to each of their visits #2-5; capturing the 24-hr recall will allow for checking compliance with this. (This is not meant to say that all participants have to eat the same foods, just that participant '001' should eat the same things each time and participant '002' should eat the same things each time, even though '001' and '002' will be eating different things.). Information on food intake and related calorie intake during the entire stay in our Unit at the Clinical Center will be also recorded.
- Participants will be given a choice of a few lunch and breakfast options, but all options will be the same in kcal and macronutrient content. Tentatively, each participant will receive the same meals for all the visits (e.g., if participant '001' picks lunch A for the first visit, s/he will get lunch A for all her/his visits.), although participants' requests to change their choice among the given options may be satisfied.

Blood Sampling:

Furthermore, at admission at each visit (Visit #2-5), we will also collect blood samples in order to control appetitive/metabolic indicators prior to the initiation of the dietary regimen. Specifically, during the PR sessions and the fMRI/alcohol clamp sessions, at baseline we will collect:

*Blood Test Panel I: blood glucose, cholesterol, triglycerides, GH, insulin, IGF-1, cortisol, ACTH, PRL, FSH, LH, testosterone total and free, T3-free, T4-free, TSH;

**Blood Test Panel II: GH, insulin, cortisol, PRL and other feeding-related (or stress-related hormones). During ghrelin/placebo/ETOH infusions, Blood Test Panel II will be repeated every 30 minutes during the PR sessions and every 15 minutes during the fMRI/alcohol clamp sessions. In addition, BAC will be measured twice while participants are in the scanner.

The timepoints for blood collection in the flowsheets of this protocol represent the maximum number of blood sampling for this protocol. While our ideal goal is to collect all of them, we appreciate that occasionally blood collection may be missed due to expected technical problems during the study procedures. Furthermore, if there are blood draw issues with both IV lines, we might also attempt to draw blood using regular or butterfly needles.

Samples will be stored for subsequent analyses of ghrelin levels and other appetitive hormones (and stress hormones), including (but not limited to) insulin, leptin and GLP-1. Some of these stored samples will be shipped in a fully de-identified form to AI Fatemeh Akhlaghi's laboratory to be processed for a metabolic panel that includes appetitive and stress peptides like ghrelin, insulin, leptin, GLP-1 and others. No samples will be shipped until MTA is fully executed between NIAAA and URI. Furthermore, additional metabolic indicators (e.g. glucose, cholesterol, triglycerides, thyroid hormones) will also be assessed prior to the initiation of the dietary regimen.

Overnight stays (visits #2-5)
This study will enroll non-treatment seeking heavy drinking outpatient individuals. Nonetheless, two overnight stays will take place in the Clinical Center (e.g. Metabolic Unit or NIAAA-LCTS inpatient unit, pending on availability) per each session:

- The night before each session, i.e. Visits #2-5. This allows for controlling for two important factors in this study:
    i) Alcohol consumption before each session, which includes the IV administration of alcohol and ghrelin. In fact, while the baseline BrAC allows for controlling a recent consumption of alcohol, the overnight staying allows for a much longer and thus more rigorous control of alcohol consumption, thus standardizing alcohol consumption across all participants. Additionally, the overnight stay before Visits #2-5 increases the feasibility of the study as it does assure that baseline BrAC will be 0.00 before each session. In fact, if participants came to the clinic on the same day of the session, there is a risk that their BrAC could be positive, which, in turn, would require delaying the session or indeed withdraw the subject if BrAC was > 0.08;
    ii) The night before each session, each participant will receive a standard pre-fixed dinner. Given the key role of ghrelin in food intake and metabolism, the overnight stay allows for controlling carefully caloric intake during the night before the experiment, thus standardizing food intake across all participants.
- The night following each session. This overnight will permit additional monitoring for safety reasons. Specifically, after the session is complete and subjects have lunch, vital signs and general well-being will be assessed approximately every 30 minutes until BrAC will zero out. A non-standardized dinner will served in the evening. Finally, participants will be debriefed on the following morning before being sent home.

e. End of Participation

Follow-up (visit #6) (lasting 30 minutes). One week after the last fMRI/alcohol clamp session (Visit #5), subjects will return to the NIH CRC for clinical evaluation, including assessment for compliance with the previous recommendations to reduce alcohol consumption.
At this visit they will be informed about the results of their clinical evaluation for alcohol use disorders. We will follow the clinical guidelines for treatment of alcohol use disorders as described in the NIAAA publication "*Helping patients who drink too much: a clinician's guide*". These guidelines prescribe a "Brief Intervention" during which the patient is informed of the diagnosis and a clear recommendation to stop drinking alcohol is made. The possible use of medication to reduce alcohol use as well as possible participation in a self-help group will be discussed, and a follow-up appointment in the NIAAA outpatient clinic will be scheduled if desired. After the follow-up visit, subjects will be considered to have completed the protocol.

Only information that a subject specifically requests in writing will be shared with other healthcare providers or third parties.

f. Future Contact

While all steps are always taken to ensure quality data from all participants, we may ask participants who generate unusable data (or if there is an equipment failure) on one of their experimental sessions to repeat that session. This will have the effect of maximizing the risk/benefit ratio as it will prevent having to discard usable data from one session and will minimize the number of participants recruited overall to obtain sufficient data to meet power requirements. We may ask the participant to return at a later date or to stay an additional night on the clinic unit. Subjects will receive additional compensation if experimental sessions are repeated; compensation for this *as needed* research procedures may vary based on the inconvenience units and will be based on the NIDA/NIAAA recommendations.

5. Storage of data and samples

Data will be stored on an NIH data server, under the management of the ORIT, NIAAA. The existence and types of information contained in the data management system have been publicly reported as required by the FOIA.

It should be mentioned that the timepoints for the data collection outlined in the flowsheets of this protocol represent the maximum number that will be collected. While our ideal goal is to collect all of them, we appreciate that occasionally questionnaires and other data collection may be missed (missing data) as in any clinical study, due to time constraints resulting from possible technical problems during the study procedures (e.g., a problem with the IV line or with the computer used to administer questionnaires, etc.).

Plasma and serum for blood levels of ghrelin, GH and other feeding-related and stress-related hormones will be collected from each subject on repeated occasions, as detailed in the study flow sheets. Blood not processed via the Department of Laboratory Medicine at the Clinical Center or will be in -80 freezers. All biological specimens obtained under this protocol will be stored in coded form (protocol plus subject number) in freezers located in the access-controlled laboratory area of LCTS.

Additional blood samples will be collected for DNA extraction and future genetic analysis under the NIAAA Screening Protocol (PI: Reza Momenan, Ph.D.Genetic analyses include loci encoding pro-ghrelin (GHRL) and its receptor (GHSR).

6. Additional Considerations

The present protocol will be submitted for review to the FDA. Dr. Kenna holds an IND for the use of IV ghrelin in alcohol-dependent heavy drinkers for the trial performed at Brown University. The FDA submission for this protocol will cross-reference the previous IND (notably, we will use ghrelin from the same lot#). Furthermore, Dr. Ramchandani holds an IND for the use of IV ethanol, which will be cross-referenced as well.

There is a possibility that subjects might identify if they received IV ghrelin or placebo. Therefore, at the end of each session, we will ask subjects if they believe they received ghrelin or placebo and this information will be recorded.

7. Risks and discomforts

IV Ghrelin administration

There is a potential risk of side-effects from infusion of ghrelin, although side-effects have not been frequent, and when side-effects were reported, they were all in the mild range (see Tables 1 and 2 above). No clinically significant, serious or unexpected side-effects have ever been reported in the published literature to data. It should also be noted that the previous study conducted at Brown by Dr. Leggio shows a very safe profile of IV ghrelin when administered to non-treatment seeking heavy drinking alcohol-dependent volunteers.

Risks will be minimized through careful and detailed screening of participants to minimize recruitment of those who might be at higher risk for adverse events, as well as careful and detailed monitoring of adverse events and participant well-being during the laboratory sessions.

1. Consistent with the well-known effect of ghrelin on food intake, hunger and craving for food may occur after IV ghrelin administration.
2. IV Ghrelin has been associated with flushing, facial warmth and sweating. This side-effect, however, was never reported in the IV ghrelin study with alcoholic individuals at Brown University, and in that study history of flushing (e.g. alcohol-induced flushing reactions) was not an exclusion criterion. Nonetheless, in this study involving the IV administrations of ghrelin and alcohol, potential individuals with current or prior history of alcohol-induced flushing reactions will be excluded.
3. IV Ghrelin has been associated with decreased arterial pressure and secondary increased heart rate can also occur. In this study, we will exclude individuals with history of hypotension clinically significant (e.g.: history of fainting and/or syncopal attacks) as well as those with resting systolic BP < 100mg.
4. Rarely, elevated mood, abdominal discomfort, somnolence, fever, diarrhoea, mild glucosuria, vertigo and emesis have been reported in individuals receiving IV ghrelin. Patients with chronic intestinal diseases and/or medical history of diarrhoea and metabolic disorders, including diabetes and obesity will be excluded. Furthermore, in order to maximize safety, blood panels will include assessments of several metabolic hormones in order to monitor ghrelin's effects on their levels.
5. There are no enough data on the exposure of elder individuals to IV ghrelin. Specifically, among all of the studies testing IV ghrelin in humans (Tables 1-2), only one study[69] included individuals > 70 years. Therefore, consistent with the majority of the published literature, participants > 70 years old will be excluded in the present study (i.e. we will not enroll individuals who are > 60).
6. Beyond the risks already described when ghrelin was either infused or injected, we are aware that consistent with the main hypothesis of this study, IV ghrelin may, at least temporarily, increase alcohol craving (a symptom provocation study). Therefore, this study includes a post-session debriefing after each of the four sessions. The Investigators have all experience in the management of this population, including non-treatment seekers taking part in alcohol laboratory studies. In particular, all NIAAA LCTS Investigators of this protocol have experience with challenge experiments (for example, alcohol, meta-

chlorophenylpiperazine, yohimbine, lipopolysaccharide) that have been used previously or currently in patients with addictive disorders within the NIAAA intramural program, and no serious adverse events have been encountered in those studies. Notably, unlike other compounds used for challenge studies, ghrelin is a naturally occurring hormone. Importantly, since the brief half-life of IV ghrelin, we do not expect that the effects of the peptide on craving will be too long once the IV administration is over. Safety is maximized by the fact that participants will be kept overnight at the NIH Clinical Center after each session. This will allow for continued monitoring as needed, and the morning after the session, subjects will be debriefed on their drinking and receive counseling, using motivational enhancement techniques, aimed at enhancing their readiness for behavioral change and seeking treatment if needed, as recommended by the NIAAA guidelines.[166] Prior to discharge, subjects will be asked about the severity of their urges to drink, as well as any untoward symptoms they might be experiencing. Recommendations to reduce or stop drinking again will be given to them prior to discharge. We also note that subjects will receive a meal right after each session, and it is demonstrated that food suppresses plasma ghrelin levels.

It should be also noted that we are applying a conservative approach, e.g. we will keep participants overnight at the NIH Clinical Center after each alcohol/ghrelin infusion. Dr. Leggio's study at Brown was an outpatient study and, there was no need to hospitalize any of the participants (although included in the protocol as a possible additional safety action). No serious adverse events (SAEs) occurred during the ghrelin study at Brown University. All debriefing sessions were conducted successfully, e.g. all participants were released after A-VAS craving score was equal or lower to the baseline (pre-infusion IV ghrelin) A-VAS craving. There were no cases of persistent high craving at the end of the session.

It is unknown if administration of ghrelin to pregnant women might cause harm to the fetus. No fetus will be exposed to exogenous ghrelin. A urine pregnancy test will be administered during each study visit block to all women. A positive test for beta-hCG would exclude participation in the study. Should a positive test occur, the investigators will be informed, and the PI or Medical Investigator will be involved in gently informing the participant of the fact, and will offer referral to an obstetrician.

IV alcohol administration

There is a potential risk of side-effects from infusion of alcohol. Ethanol is primarily a depressant of the Central Nervous System. Its effects, in general, are proportional to the BrAC reached after alcohol administration. Alcohol may produce motor in-coordination, deficits in concentration, memory impairment and a feeling of expansiveness or euphoria, possibly followed by sleepiness. Nausea and vomiting could rarely occur.

1. The maximum BrACs that may be achieved during the IV alcohol PR sessions will be 0.12, while during the fMRI/alcohol clamp sessions the max will be 0.08. This research team has reached these levels in previous/ongoing studies, and no subject has reported nausea, experienced vomiting or other clinically important side-effects.
2. The alcohol infusion procedure is considered to be relatively safe: the concentration of alcohol in the infusate is never greater than 6% v/v and the limit on the amount of alcohol that can be infused is determined by hanging only two 1000 ml capacity bags of the infusate (6% v/v alcohol ~ 4 oz. ethanol at most).

3. During all alcohol exposure sessions, participants will be monitored by the nursing and research staff for any untoward effect and the session will be terminated if any major or unexpected adverse events occur. The physician will be on-call to respond immediately to any problems.
4. Participants will be kept overnight at the hospital after each session. The Investigators have several years of experience in studies administering intravenous alcohol to human participants without any major adverse events, and the nursing staff is well experienced in managing intoxicated participants.
5. The procedures are consistent with the "*Recommended Guidelines on Ethyl Alcohol Administration in Human Experimentation*" prepared by the National Advisory Council on Alcohol Abuse and Alcoholism, Dept. of Health and Human Services.

Administration of alcohol to pregnant women might cause harm to the fetus. No fetus will be exposed to ethanol. A urine pregnancy test will be administered during each study visit block to all women. A positive test for beta-hCG would exclude participation in the study. Should a positive test occur, the investigators will be informed, and the PI or Medical Investigator will be involved in gently informing the participant of the fact, and will offer referral to an obstetrician.

IV Ghrelin and IV alcohol co-administration

There is a potential risk for additive effects of ghrelin and alcohol co-administered, such as:

- Flushing and facial warmth; thus, we will exclude individuals with current or prior history of alcohol-induced flushing reactions.
- Sedation; thus, we will exclude subjects who take medications that may amplify alcohol's sedative effects and we will only enroll individuals who are not older than 60.
- Decreased arterial pressure; thus, we will exclude individuals with history of hypotension clinically significant, and blood pressure and continuous heart rate will be monitored during the lab and fMRIsessions. Furthermore, subjects whose screening will show resting systolic BP < 100 mmHg are not enrolled in this study.

  As shown in Tables 1 and 2 above, the IV administration of ghrelin has a very safe profile, and we anticipate that its administration together with alcohol administration will be safe and well-tolerated. Also, subjects will be continuously monitored during the lab sessions and alcohol and ghrelin administrations will be stopped (and the IRB notified) if a subject should develop any significant symptoms requiring to do so. Not only the sessions are performed in a well-controlled environment in the NIH Clinical Center, but subjects will be kept overnight at the hospital and only released the morning after in order to maximize their safety and monitoring.

Risk of fMRI

1. *Anxiety about enclosed spaces*- Some individuals experience claustrophobia when placed inside the magnet bore of the scanner. Participants will be warned about this in advance and advised not to participate if they are prone to experiencing anxiety in enclosed places.
2. *Nerve stimulation*- The use of a 3T field strength magnet sometimes induces "peripheral nerve stimulation," in which peripheral muscles twitch in association with scanning. Subjects will be notified of this possibility in the consent form. The twitching does not occur in everyone, is not painful, and usually lasts for a short time. Some individuals may find this mildly annoying and we will terminate the scanning if they feel uncomfortable. Finally,

fMRI is a noisy process, so we require participants to wear foam earplugs while inside the scanner.

Insertion of IV lines
The total amount of blood to be drawn for this protocol will be approximately 428cc (= 87 US teaspoons). There is a slight risk of bruising or, rarely, infection from insertion of indwelling peripheral venous catheters for infusion and blood sampling. Risks from IV catheterization are minimized by experienced medical personnel who will perform catheter placement using sterile technique and following universal precautions. Participants will be seated or supine when catheters are placed.

8. Subject Safety monitoring

Subjects will be monitored regularly and frequently during their participation in the study. They will undergo standard assessment battery (described below) at each study visit.

Parameters to be monitored (see Study flow sheet for times of monitoring individual items):

1. Weight
2. Vital signs
3. Continuous heart rate monitoring will be recorded during IV Ghrelin and IV alcohol co-administration.
4. BrAC, breath carbon monoxide (BrCO); Blood Alcohol Content (BAC) when participants are in the fMRI scanner.
5. Drug use: Urine Drug Test (UDT)
6. Blood Test Panel I: blood glucose, cholesterol, triglycerides, GH, insulin, IGF-1, cortisol, ACTH, PRL, FSH, LH, testosterone total and free, T3-free, T4-free, TSH.
   Blood Test Panel II: GH, insulin, cortisol, PRL (and other feeding and stress related hormones).
7. Urinalysis
8. Pregnancy: urine pregnancy testing
9. Mood and anxiety level and suicidality (Comprehensive Psychopathology Rating Scale – CPRS), Columbia Suicide Severity Rating Scale (C-SSRS).
10. Alcohol consumption- TLFB and alcohol craving (A-VAS and AUQ)
11. NMR Safety Questionnaire- questionnaire developed by NMR Center to verify absence of any exclusions to MRI scanning (to be completed at screening and visits 4 and 5).

Toxicity tables/criteria to be used
NCI Common Terminology Criteria for Adverse Events v. 3.0 will be used for grading and reporting adverse events.[167]

Criteria for individual subject withdrawal

- A Serious Adverse Event (SAE) that is judged by the Investigators as being related to the study drug;
- Pregnancy;
- At the discretion of the Principal Investigator (PI) based on adverse event (AE) severity;
- Non-compliance with protocol procedures or investigator request(s);
- Patient request.

Enrolled subjects who show up with positive UDT or BrAC ≥ 0.08% at one of the four experimental sessions (excluding the night before visits 2-5) will not perform the session. In this case, the experimental session may be re-scheduled at the PI discretion. Additionally, a session may be rescheduled for other medical reasons for which the PI and/or MAI think it is in the best interest of the participant to re-schedule the session. However, if the subject will repeatedly show up with positive UDT or BrAC ≥ 0.08% at repeated visits, the PI may withdraw him/her from the study.

9. Outcome measures

a. Primary outcome measures

The primary measure of this study will be the breakpoint, which is the schedule (number of button presses) at which the individual stops to work for more alcohol during the PR sessions.

b. Secondary outcome measures

Alcohol exposure will be measured via BrAC exposure measures, i.e. highest BrAC, average BrAC and the area under the BrAC-time curve and by BAC when subjects are in the fMRI scanner. Alcohol craving will be measured using the Alcohol Visual Analogue Scale (A-VAS) and the Alcohol Urge Questionnaire (AUQ). Sensitivity to alcohol will be measured using the Drug Effects Questionnaire (DEQ), Biphasic Alcohol Effects Scale (BAES), and the Profile of Mood States (POMS), repeatedly during the PR session and the CASE Experience Questionnaire (CEQ)
at the end of all sessions. fMRI BOLD signal in brain areas associated with incentive salience and areas associated with reward circuitry (including the ventral striatum) will be measured during the "fMRI/alcohol clamp" session.

10. Statistical Analysis

1. Analysis of data/ study outcomes

Analysis of data/study outcomes

Outcome data will be examined for homogeneity of variance, and if necessary transformed to meet this criterion. Most of the analyses will consist of repeated measures ANCOVAs to accommodate dependent measures assessed at multiple time points. When the dependent measure is just assessed once then a regular ANCOVA will be used. We will examine medication main effects across several different dependent measures.

Criteria for significance

A probability level of 0.05 or lower will be considered significant.

2. Power analysis

Power analysis

A generic calculation of power for a two-means comparison to detect an effect size of Cohen's f ≥ 0.4 (large effect size) with a sample size of 50 yields a power estimate ≥ 0.80 at alpha=0.05. The relative effect size chosen ("large" effect size) for the power calculation is based on the observation that laboratory studies typically yield larger effect sizes than clinical trials, including, for example, our recent (unpublished; PI: Ramchandani) human lab studies using the

IV Alcohol PR procedure, described above and representing the study our primary aim (number of button presses) is based upon.
Furthermore, the sample proposed here provides sufficient power (power estimate ≥ 0.80 at alpha=0.05) to detect ghrelin's effects on fMRI BOLD activation in the VS, consistent with the unpublished data (PI: Ramchandani; data already mentioned above) with varenicline (n = 8) vs. placebo (n = 7) indicating significantly higher BOLD activation in the varenicline group.

Accrual number request, taking into account screening/ dropouts
In the Brown University study with IV ghrelin, inclusionary/exclusionary criteria were similar and about 50% of the subjects screened were eligible and randomized. Consistent with other human laboratory studies with a similar study length, we also expect that a number up to 20% will not complete the study. Therefore, we anticipate a number-needed-to-screen of 124 subjects, with 62 being randomized and 50 completers.

11. Human Subjects Protection

a. Subject selection

Adults who fulfill the qualification criteria will be included, regardless of race, ethnicity, sex, or religious affiliation.

b. Justification for inclusion of children

Children will not be included because the study proposed involves the administration of alcohol. It is illegal to give alcohol to individuals below 21 years of age.

c. Justification for inclusion or exclusion of other vulnerable subjects

Vulnerable participants will not be enrolled. Females who show a positive urine pregnancy test will be withdrawn from the study. Women of child-bearing potential (i.e. who are pregnant or who are trying to become pregnant) will not be included, because it is inappropriate and unethical to give alcohol to women who are pregnant because of the risk of fetal alcohol syndrome. Females of childbearing potential who are sexually active and have not been surgically sterilized must agree to use a reliable method of birth control during the study **(details above in section 3b, Inclusion criteria, #4).** Furthermore, a urine pregnancy test will be performed at each session.

d. Justification of sensitive procedures

This study employs the use of placebo to evaluate the effects of ghrelin. This is the required standard set by the FDA for evaluation of drugs. The previous outpatient human laboratory study conducted at Brown University was a placebo-controlled study and FDA reviewed its protocol.

The hypothesis of this protocol is that IV ghrelin will temporarily increase the likehood that participants will self-administer alcohol during the PR session and will increase craving. The possible temporary increase in alcohol administration and craving is a necessary challenge to evaluate the ghrelin system as a novel neuropharmacological target for alcoholism. It should be noted that at this stage ghrelin administration cannot be considered a standard provocative test. Although very preliminary data from the Brown study suggest so, this is a very early stage research. The possibility that IV ghrelin increases temporarily alcohol administration and craving is in fact the main objective measure of this project. The study at Brown was an outpatient study

and its safety profile was excellent. This protocol will enroll outpatients who, however, will stay overnight at the NIH Clinical Center before and after each session (two nights per session).

e. Safeguards for vulnerable populations

Vulnerable populations will not be studied. Urine pregnancy tests will ensure that alcohol is not administered to women who may be pregnant.

f. Qualifications of investigators

The NIAAA medications development program at the Laboratory of Clinical and Translational Studies is among the leading sites worldwide for this type of translational medicine studies. The program has expertise in addiction medicine, general psychiatry and internal medicine, pharmacology and is supported by a nursing staff with combined expertise in medicine and behavioral health. The investigators have also several years of experience in conducting alcohol infusion studies and neuro-imaging studies.

Lorenzo Leggio, M.D., Ph.D., M.Sc., is the Chief of the Section of Psychoneuroendocrinology and Neuropsychopharmacology, LCTS. He has several years of clinical and academic experience in the field of addiction medicine. He has extensive experience in direct patient care, and direction and execution of clinical trials. Before joining NIH, he was Core Faculty of the Brown University Center for Alcohol and Addiction Studies and Assistant Professor in the Brown University Medical School. In this capacity, he served as PI for several clinical trials funded by NIAAA or Foundations. Dr. Leggio has considerable experience with pharmacotherapy human laboratory studies, including a NIAAA-funded study testing IV ghrelin in alcoholic individuals and conducted at Brown University. Dr. Leggio is the PI of this study and will be responsible for the conduct, analysis and publication of the results of the study. He will obtain consent for this study.

Vijay A. Ramchandani, Ph.D., is the Chief of the Section of Human Psychopharmacology, Laboratory of Clinical and Translational Studies. He is a clinical pharmacologist with several years of experience conducting alcohol administration studies, particularly using the alcohol infusion method, which he co-developed while at Indiana University, prior to joining NIH. He will obtain consent for this study.

Mary R. Lee M.D. is a Staff Clinician in the Section on Clinical Psychoneuroendocrinology and Neuropsychopharmacology (SCPN), LCTS, NIAAA. She completed medical school and internal medicine residency at Columbia Presbyterian Medical Center in New York and psychiatry residency at George Washington University Medical Center. She is board-certified in internal medicine and addiction medicine. She has over 15 years experience in psychiatric and addiction research. Dr. Lee will assist the PI in conducting experimental procedures and publishing findings from the study. She will also serve as Medical Advisory Investigator (MAI) and will obtain consent.

David T. George, M.D. is a Board Certified Psychiatrist and Chief of the Section of Clinical Assessment and Treatment Evaluation, Laboratory of Clinical and Translational Studies. He has more than 20 years experience in alcoholism research and treatment. Dr. George will support the

MAI in the conduct of the study and will be involved in aspects of clinical care. Dr. George will obtain consent for this study.

Melanie Schwandt, Ph.D., is a Staff Scientist in the Laboratory of Clinical and Translational Studies. She has more than 10 years experience constructing, managing and analyzing large datasets that combine behavioral and physiological outcomes, including over 5 years experience in pre-clinical research involving alcohol-related phenotypes and more than 2 years experience analyzing data from clinical experimental medicine studies. She will assist the PI in the analysis of the data. She will not obtain consent for the study.

Reza Momenan, Ph.D., is a Staff Scientist in the Section of Brain Electrophysiology and Imaging, Laboratory of Clinical and Translational Studies. He has extensive experience in human brain imaging including PET, MRI and fMRI and will guide the image processing and image analysis methods. He will obtain consent for this study.

LaToya S. Sewell, MSN, CRNP is a Family Nurse Practitioner in the Laboratory of Clinical and Translational Studies. She completed her graduate nursing education at the University of Maryland, Baltimore and has extensive training at the NIH in the areas of research and Neuroscience. Her background includes clinical management of patients with autonomic function disorders, as well as expertise in neuroimaging analysis. Within this lab Nurse Practitioner Sewell serves on the research team as a clinical provider in support of many NIAAA studies. Ms. Sewell will obtain informed consent.

Mehdi Farokhnia, M.D is a Post-Doctoral fellow in the Section on Clinical Psychoneuroendocrinology and Neuropsychopharmacology (CPN), LCTS, NIAAA. He completed medical school at Tehran University of Medical Sciences and has over 5 years experience in psychiatric research. During medical school and after that, he has been involved in conducting a variety of research projects while working as a research associate at the Psychiatric Research Center, Roozbeh Psychiatric Hospital. Dr. Farokhnia will assist the PI serving in the capacity of Lead Associate Investigator in conducting experimental procedures, analyzing the data, and publishing findings from the study. He will also obtain informed consent.

Fatemeh Akhlaghi, Pharm. D., Ph.D., is a tenured full professor in the College of Pharmacy, University of Rhode Island (URI) and adjunct professor of Medicine at Brown University Medical School. Her area of specialty is clinical pharmacology in diabetes and transplantation; she also conducts clinical pharmacokinetics research at Rhode Island Hospital, Providence, RI. Dr. Akhlaghi is also a member of the IRB at URI. Dr. Akhlaghi is the Co-PI, together with Dr. Leggio, of the multiple-PI UH2/UH3 grant funded by NCATS. Her laboratory at URI will receive samples that are de-identified from this study. She or her lab personnel will not have any means of linking the study codes to subjects' personal information. She will not obtain consent for this study.

12. Anticipated Benefit

This study does not offer direct benefit to participants but is likely to yield generalizable knowledge about the possible role of ghrelin in alcohol-related behaviors (e.g. drinking, craving,

sensitivity). This will markedly facilitate the identification of a novel neuropharmacological target, thus facilitating the development of novel alcoholism treatments.

13. Classification of risk

Overall risk and discomfort in this study is "more than minimal". The increase over minimal risk, discomfort is minor, and every precaution and protection to minimize any risks will be taken as described in a previous section. This level is justified, because it will markedly facilitate medication development for alcoholism, and thus contribute to addressing major unmet medical needs. As such, the benefit:risk ratio of the proposed study is favorable.

14. Consent documents and process

a. Designation of those obtaining consent

Study investigators designated as able to obtain consent in section '11f' above, will obtain informed consent for this protocol.

b. Consent procedures

At Visit 1 (Screening), subjects are screened throughout the NIAAA Screening protocol 98-AA-0009 (PI: Reza Momenan, Ph.D.) or 14-AA-0181 after consenting participants with an already IRB-approved consent form, specific for the 98-AA-0009 or 14-AA-0181 protocol.
Eligible subjects are scheduled for Visit 2; the study-specific consent form for ghrelin is obtained at Visit 2 before starting the other procedures outlined for Visit 2. Nonetheless, at Visit 1, if all information necessary to determine eligibility is obtained and the participant is still in our Clinic, then the ghrelin-specific consent form can be obtained at the end of Visit 1 (although we predict this will be not a frequent scenario). In summary, the ghrelin-specific consent form will be typically obtained at Visit 2, and occasionally at the end of the Visit 1. Participants who might be eligible at the end of Visit 1 (but we will make clear that their eligibility will have to be confirmed) will be allowed to take the consent document home before Visit 2 is scheduled. As such, potential participants have the option to read the consent home, call us with questions, and consult (if they want) family members and/or their private health care professional. The ghrelin-specific consent form will be obtained before starting the procedures scheduled for Visit 2, and all procedures of Visit 2 will always take place on a different subsequent day after Visit 1.
All participants will receive a verbal explanation in terms suited to their comprehension of the purposes, procedures and potential risks of the study and of their rights as research participants. Participants will have the opportunity to carefully review the written consent form and ask questions regarding this study prior to signing. Participants will be asked to sign and date the informed consent. A copy of the signed and dated consent will be provided to each participant.
In regards to consenting participants who might be impaired, our standard procedure is for the nurses to do a breathalyzer test upon arrival (covered generally under the standard hospital consent that all subjects admitted to the NIH Clinical Center sign when they check in at admissions). If the results is >0.03, then the subject is informed that we cannot proceed, and they are given the option of waiting until their level falls below 0.02 and/or sending them back in a cab. If the reading is >0.08, we strongly encourage them to stay at least until they drop below the legal limit (and if participants came by car and don't want to wait until < 0.08, then the NIH police is notified, as already specified in the consent). If the reading is ≤0.03, we let the subject know that we will need to wait 1-2 hours to ensure their readings are back to zero before they can sign the consent form and proceed.

After negative breathalyzer is obtained, the consent process will take place and then study procedures will start. The NIH CC nursing staff in charge of the participant will be required to first complete and sign a 2-item checklist [*i.e.: a) ask to the participant whether or not they have been consented; and b) verify consent was signed*] before study procedures take place at the time of the admission (Visit 1).As part of the consent procedure, participants' ability to understand the consent will be evaluated using the '*NIDA/NIAAA Evaluation of Potential Research Participants' Ability to Consent*'.

c. Consent documents

The consent form attached in the Appendix contains all required elements. The original consent is filed with their NIH medical record and a digital copy is uploaded into the CRIS system. A copy of the signed consent is also made for the subject's CRF binder for regulatory purposes.

Data and Safety monitoring

d. Data and Safety monitor

Data and safety monitoring commensurate with the degree of risk involved in participation in a small, single-site trial like the present one and will be performed by an independent safety monitor (ISM).

e. Data and safety monitoring plan

The ISM is a physician with relevant expertise, whose primary responsibility is to provide independent safety monitoring in a timely fashion. To ensure expertise in clinical addiction research as well as independence, the ISM for the current study will be a physician from a different Section of NIAAA. The ISM responsibilities will be assumed by Nancy Diazgranados, M.D., NIAAA or Falk Lohoff, M.D. NIAAA.

The ISM evaluation includes the progress of the research study as documented in the "ISM Monitoring Report and Log", data quality from review of adverse events, lab data and relevant clinical data collected immediately after they occur or are reported, with follow-up through resolution, and participant accrual in the Screening and Enrollment Log. The ISM reviews on an ongoing basis individual and cumulative participant data, and makes recommendations regarding the safe continuation of the study, or its termination. The ISM may break the study blind if needed. Weekly safety data monitoring reports are generated by NIAAA Staff Scientist and sent via encrypted email to the MAI, ISM and PI for review and consideration. Appropriate action is then conveyed to the research team.

The ISM will submit their review findings on the "Independent Safety Monitoring Report and Log" (stored in regulatory binder) for the PI and MAI to review. The PI will submit all findings from this review at the time of continuing review and/or in the case of a PR report the timing of which is in compliance with OHSRP guidelines.

Adverse Event reporting and/or Problem Reporting will be performed consistent with current OHSRP and NIH SOP Guidelines including; report submission in PTMS for OCD and IRB review, email notification to the study sponsor if this is a separate individual from the PI, hard copy notification in triplicate via Fed Ex/U.S. Mail to the FDA.

Criteria for stopping the study or suspending enrollment or procedures
The study will be terminated if more than five individuals experience serious adverse events (SAEs) related to the study drug [> 5 SAEs].
There is no plan at this stage to conduct an interim analysis.

f. Criteria for suspending study procedures on an individual basis: Administration of study medication or alcohol will be suspended on a case- by-case basis as per the PI and MAI request in accordance with the following criteria

Checklist:

- Clinically significant findings including changes in mood or behavior such as aggression, agitation, depression or suicidal ideation or suicidal behavior.
- Changes in concomitant medications that may interact with study medication or alcohol.
- At the discretion of the Principal Investigator (PI) and Medical Investigator, based on adverse event (AE) severity
- Non-compliance with protocol procedures or investigator request(s)
- Patient request
- Equipment failure

Please see also "Criteria for individual subject withdrawal", Section 8. **Subject Safety monitoring**

15. Clinical Monitoring/Quality Assurance

Quality assurance will be monitored by the PI and research team and the NIMH/NIAAA/NIDA Combined Monitoring Plan, Intramural Research Program Auditing Committee (IRPAC) (Please see Appendix (E) – Standard Operating Procedures for the Intramural NIMH, NIAAA and NIDA Combined Monitoring Plan) coordinated by the Offices of the Clinical Director at NIMH,NIAAA and NIDA.
The NIMH/NIAAA/NIDA Combined Monitoring Plan, Intramural Research Program Auditing Committee (IRPAC) monitors intramural research studies to ensure compliance with GCP, organizational policies and applicable federal, state and local laws and the reliability of study data.

a. Quality assurance monitor

- Quality assurance (QA) will be performed by the PI, as well as by an independent QA monitor, which according to established practice is considered sufficient for small, single-site trials, like the present project. The QA monitor for this study will have considerable expertise in clinical trials in alcohol dependence, and will have completed training in Clinical Research Monitoring and GCP Compliance.

b. Quality assurance plan

- All participants' data and binders will be reviewed on a monthly basis.

16. Adverse event and unanticipated problem reporting

Adverse events, protocol deviations, unanticipated problems (UP), Unanticipated Adverse Device Effects (UADEs), serious adverse events are defined as described in NIH HRPP SOP 16 ("Reporting Requirements for Unanticipated Problems, Adverse Events and Protocol Deviations."). The Principal Investigator is responsible for detecting, documenting, and reporting unanticipated problems, adverse events (AEs), including serious adverse events (SAEs), and deviations in accordance with NIH policy, IRB requirements, and federal regulations. Relatedness to the research of all serious adverse events will be determined by the PI in consultation with the CD.

Serious unanticipated problems and serious protocol deviations will be reported to the IRB and CD as soon as possible and in writing on the Problem Report Form not more than 7 days after the PI first learns of the event. Not serious unanticipated problems and not serious deviations will be reported to the IRB and CD as soon as possible and in writing on the Problem Report Form not more than 14 days after the PI first learns of the event. Deaths will be reported to the Clinical Director within 7 days after the PI first learns of the event. All adverse events, deviations, and unanticipated problems will be summarized and reported at the time of Continuing Review.

17. **Alternatives to participation** or **Alternative therapies**

To be eligible for this study, subjects must not be seeking help for drinking problems. Those who are seeking help for a drinking problem will be referred to standard therapy for heavy drinking and will not be allowed to participate in this study.

18. Confidentiality

This protocol is covered by a Certificate of Confidentiality (CoC) issued by the US Department of Health and Human Services. Strict subject confidentiality will be maintained throughout the study. Confidentiality and information technology standards are in place at the NIAAA/LCTS intramural program to protect electronic repositories of patient data as well as other clinical patient related material. It is reasonably expected that these safeguards will protect participants' medical and personal health information, ensuring their privacy.

Information obtained in the course of being screened for or participating in this protocol will become part of the patient's NIH medical record. This includes potentially sensitive information, such as urine tests positive for illicit drugs. Access to this information may be requested by third parties. Such access will not be granted without the explicit, written consent of the subjects.

Samples and data will be stored using codes that we assign. Data will be kept in password protected computers. Samples will be kept in locked storage. Only study personnel will have access to the samples and data.

20. Conflict of Interest

a. Distribution of NIH Guidelines

NIH guidelines on conflict of interest have been distributed to all investigators.

b. Conflict of interest

There are no conflicts-of-interest to report.

c. Role of a commercial company or sponsor

There is no drug company involved.

21. Technology Transfer

No technology will be subject to transfer to agencies outside the US Government.

22. Compensation

Volunteers will be compensated for time and research-related inconveniences, to the extent to which they complete them, as follows:

Visit #	Compensation
Visit 2 (alcohol PR)	$250
Visit 3 (alcohol PR)	$250
Visit 4 (fMRI/alcohol clamp)	$250
Visit 5 (fMRI/alcohol clamp)	$250
Visit 6 (Follow-up: $25 + bonus for completing all parts of the study: $100)	$125
TOTAL	**$1,125**

Compensation will be prorated for parts completed if subjects do not complete the study. If needed, subjects will be provided with a taxi paid for by NIH.

Visit 1 (Screening) is done under a separate NIAAA protocol, i.e. the NIAAA Screening Protocol 98-AA-0009, (PI: Reza Momenan, Ph.D.) or 14-AA-0181. The 98-AA-0009 protocol has been routinely used in the NIAAA Clinical Program for several years to screen non-treatment seeking individuals who are then enrolled - pending on their eligibility - in more specific studies, such as this proposed study with ghrelin. Therefore, all human lab studies with non-treatment populations performed in the NIAAA Clinical Program enroll participants who were first screened via the 98-AA-0009 protocol or the 14-AA-0181.

22. References

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402: 656-60, 1999. PMID: 10604470.
2. Cummings DE, Naleid AM, Figlewicz Lattemann DP. Ghrelin: a link between energy homeostasis and drug abuse? Addict Biol 12: 1-5, 2007. PMID: 17407491.
3. Jerlhag E, Egecioglu E, Dickson SL, Andersson M, Svensson L, Engel JA. Ghrelin Stimulates Locomotor Activity and Accumbal Dopamine-Overflow via Central Cholinergic Systems in Mice: Implications for its Involvement in Brain Reward. Addict Biol 11: 45-54, 2006. PMID: 16759336

4. Jerlhag E, Egecioglu E, Dickson SL, Douhan A, Svensson L, Engel JA. Ghrelin administration into tegmental areas stimulates locomotor activity and increases extracellular concentration of dopamine in the nucleus accumbens. Addict Biol 12: 6-16, 2007. PMID: 17407492.
5. Addolorato G, Capristo E, Leggio L, Ferrulli A, Abenavoli L, Malandrino N, Farnetti S, Domenicali M, D'Angelo C, Vonghia L, Mirijello A, Cardone S, Gasbarrini G. Relationship between ghrelin levels, alcohol craving, and nutritional status in current alcoholic patients. Alcohol Clin Exp Res 30: 1933-7, 2006. PMID: 17067359.
6. Hillemacher T, Kraus T, Rauh J, Weiss J, Schanze A, Frieling H, Wilhelm J, Heberlein A, Gröschl M, Sperling W, Kornhuber J, Bleich S. Role of appetite-regulating peptides in alcohol craving: an analysis in respect to subtypes and different consumption patterns in alcoholism. Alcohol Clin Exp Res 31: 950-4, 2007. PMID: 17433008.
7. Wurst FM, Graf I, Ehrenthal HD, Klein S, Backhaus J, Blank S, Graf M, Pridzun L, Wiesbeck GA, Junghanns K. Gender differences for ghrelin levels in alcohol-dependent patients and differences between alcoholics and healthy controls. Alcohol Clin Exp Res 31:2006-11, 2007. PMID: 17949465.
8. Anton RF. What is craving? Models and implications for treatment. Alcohol Res Health 23:165-73, 1999. PMID: 10890811.
9. Monti PM, Rohsenow DJ, Hutchison KE. Toward bridging the gap between biological, psychobiological and psychosocial models of alcohol craving. Addiction 95(Suppl 2):229S-236S, 2000. PMID: 11002917.
10. Hutchison KE, Wooden AL, Swift RM Smolen A, McGeary JE, Adler L, Paris L. Olanzapine reduces craving for alcohol: a DRD4 VNTR polymorphism by pharmacotherapy interaction. Neuropsychopharmacology 28: 1882-8, 2003. PMID: 12888781.
11. National Institute on Alcohol Abuse and Alcoholism (2000). Tenth Special Report to the U.S. Congress on Alcohol and Health, Washington, DC: U.S. Department of Health and Human Services.
12. Swift RM. Drug therapy for alcohol dependence. New Engl J Med 340:1482-90, 1999. PMID: 10320388.
13. Addolorato G, Abenavoli L, Leggio L, Gasbarrini G. How many cravings? Pharmacological aspects of craving treatment in alcohol addiction: a review. Neuropsychobiology 51: 59-66, 2005. PMID: 15741745.
14. Miller WR, Walters ST, Bennett ME. How effective is alcoholism treatment in the United States? J Stud Alcohol 62: 211-20, 2001. PMID: 11327187.
15. Garbutt JC, West SL, Carey TS, Lohr KN, Crews FT. Pharmacological treatment of alcohol dependence: A Review of the Evidence, JAMA 281:1318-25, 1999. PMID: 10208148.
16. Kranzler H, Van Kirk J. Efficacy of naltrexone and Acamprosate for alcoholism treatment: A meta analysis. Alcohol Clin Exp Res 25:1335-41, 2001. PMID: 11584154.
17. Litten RZ, Allen JP. Reducing the desire to drink. Pharmacology and neurobiology. Recent Dev Alcohol 11:325-44, 1993. PMID: 8234930.
18. Heilig M, Egli M. Pharmacological treatment of alcohol dependence: target symptoms and target mechanisms. Pharmacol Ther 111:855-76, 2006. PMID: 16545872.
19. Arora S, Anubhuti. Role of neuropeptides in appetite regulation and obesity--a review. Neuropeptides 40:375-401, 2006. PMID: 16935329.
20. Addolorato G, Leggio L, Abenavoli L, Gasbarrini G. Neurobiochemical and clinical aspects of craving in alcohol addiction: a review. Addict Behav 30:1209–24, 2005. PMID: 15925129.

21. Blum K, Braverman ER, Holder JM, Lubar JF, Monastra VJ, Miller D, Lubar JO, Chen TJ, Comings DE. Reward deficiency syndrome: a biogenetic model for the diagnosis and treatment of impulsive, addictive, and compulsive behaviors. J Psychoactive Drugs 32(Suppl. i-iv):1–112, 2000. PMID: 11280926.
22. Ramoz N, Versini A, Gorwood P. Eating disorders: an overview of treatment responses and the potential impact of vulnerability genes and endophenotypes. Expert Opin Pharmacother; 8: 2029-44, 2007. PMID: 17714058.
23. Aydin S. Discovery of ghrelin hormone, research and clinical applications. Turkish J Biochem 32:76-89, 2007.
24. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. A role for ghrelin in the central regulation of feeding. Nature 409:194–8, 2001. PMID: 11196643.
25. Toshinai K, Date Y, Murakami N, Shimada M, Mondal MS, Shimbara T, Guan JL, Wang QP, Funahashi H, Sakurai T, Shioda S, Matsukura S, Kangawa K, Nakazato M. Ghrelin-induced food intake is mediated via the orexin pathway. Endocrinology 144:1506–12, 2003. PMID: 12639935.
26. Solomon A, De Fanti BA, Martinez JA. Peripheral ghrelin participates in glucostatic feeding mechanisms and in the anorexigenic signalling mediated by CART and CRF neurons. Nutr Neurosci 8:287–95, 2005. PMID: 16669599.
27. Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 37:649–61, 2003. PMID: 12597862.
28. Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature 407:908–13, 2000. PMID: 11057670.
29. Furuse M, Tachibana T, Ohgushi A, Ando R, Yoshimatsu T, Denbow DM. Intracerebroventricular injection of ghrelin and growth hormone releasing factor inhibits food intake in neonatal chicks. Neurosci Lett 301:123–6, 2001. PMID: 11248438.
30. Unniappan S, Lin X, Cervini L, Rivier J, Kaiya H, Kangawa K, Peter RE. Goldfish ghrelin: molecular characterization of the complementary deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake. Endocrinology 143:4143–6, 2002. PMID: 12239128.
31. Tritos NA, Kokkotou EG. The physiology and potential clinical applications of ghrelin, a novel peptide hormone. Mayo Clin Proc 81(5):653-60, 2006. PMID: 16706263.
32. Nagaya N, Kojima M, Uematsu M, Yamagishi M, Hosoda H, Oya H, Hayashi Y, Kangawa K. Hemodynamic and hormonal effects of human ghrelin in healthy volunteers. Am J Physiol Regul Integr Comp Physiol 280: R1483–R1487, 2001. PMID: 11294772.
33. Akamizu T, Takaya K, Irako T, Hosoda H, Teramukai S, Matsuyama A, Tada H, Miura K, Shimizu A, Fukushima M, Yokode M, Tanaka K, Kangawa K. Pharmacokinetics, safety, and endocrine and appetite effects of ghrelin administration in young healthy subjects. Eur J Endocrinol 150: 447–55, 2004. PMID: 15080773.
34. Vestergaard ET, Hansen TK, Gormsen LC, Jakobsen P, Moller N, Christiansen JS, Jorgensen JO. Constant intravenous ghrelin infusion in healthy young men: clinical pharmacokinetics and metabolic effects. Am J Physiol Endocrinol Metab 292:E1829-36, 2007. PMID: 17311892.

35. Paulo RC, Brundage R, Cosma M, Mielke KL, Bowers CY, Veldhuis JD. Estrogen Elevates the Peak Overnight Production Rate of Acylated Ghrelin. J Clin Endocrinol Metab 93: 4440-7, 2008. PMID: 18697865.
36. Arvat E, Di Vito L, Broglio F, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F, Ghigo E. Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans. J Endocrinol Invest 23: 493-5, 2000. PMID: 11021763.
37. Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA, Bloom SR. Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab 86: 5992, 2001. PMID: 11739476.
38. Arvat E, Maccario M, Di Vito L, Broglio F, Benso A, Gottero C, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F, Ghigo E. Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone. J Clin Endocrinol Metab 86: 1169-74, 2001. PMID: 11238504.
39. Broglio F, Arvat E, Benso A, Gottero C, Muccioli G, Papotti M, van der Lely AJ, Deghenghi R, Ghigo E. Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. J Clin Endocrinol Metab 86:5083-6, 2001. PMID: 11600590.
40. Weikel JC, Wichniak A, Ising M, Brunner H, Friess E, Held K, Mathias S, Schmid DA, Uhr M, Steiger A. Ghrelin promotes slow-wave sleep in humans. Am J Physiol Endocrinol Metab 284:E407-15, 2003. PMID: 12388174.
41. Broglio F, Gottero C, Benso A, Prodam F, Casanueva FF, Dieguez C, van der Lely AJ, Deghenghi R, Arvat E, Ghigo E. Acetylcholine does not play a major role in mediating the endocrine responses to ghrelin, a natural ligand of the GH secretagogue receptor, in humans. Clin Endocrinol (Oxf) 58: 92-8, 2003. PMID: 12519418.
42. Broglio F, Benso A, Castiglioni C, Gottero C, Prodam F, Destefanis S, Gauna C, van der Lely AJ, Deghenghi R, Bo M, Arvat E, Ghigo E. The endocrine response to ghrelin as a function of gender in humans in young and elderly subjects. J Clin Endocrinol Metab 88: 1537-42, 2003. PMID: 12679436.
43. Broglio F, Gottero C, Benso A, Prodam F, Destefanis S, Gauna C, Maccario M, Deghenghi R, van der Lely AJ, Ghigo E. Effects of ghrelin on the insulin and glycemic responses to glucose, arginine, or free fatty acids load in humans. J Clin Endocrinol Metab 88: 4268-72, 2003. PMID: 12970297.
44. Tassone F, Broglio F, Destefanis S, Rovere S, Benso A, Gottero C, Prodam F, Rossetto R, Gauna C, van der Lely AJ, Ghigo E, Maccario M. Neuroendocrine and metabolic effects of acute ghrelin administration in human obesity. J Clin Endocrinol Metab 88: 5478-83, 2003. PMID: 14602793.
45. Alvarez-Castro P, Isidro ML, Garcia-Buela J, Leal-Cerro A, Broglio F, Tassone F, Ghigo E, Dieguez C, Casanueva FF, Cordido F. Marked GH secretion after ghrelin alone or combined with GH-releasing hormone GHRH) in obese patients. Clin Endocrinol (Oxf) 61: 250-5, 2004. PMID: 15272922.
46. Druce MR, Wren AM, Park AJ, Milton JE, Patterson M, Frost G, Ghatei MA, Small C, Bloom SR. Ghrelin increases food intake in obese as well as lean subjects. Int J Obes (Lond) 29(9):1130-6, 2005. PMID: 15917842.

47. Schmid DA, Held K, Ising M, Uhr M, Weikel JC, Steiger A. Ghrelin stimulates appetite, imagination of food, GH, ACTH, and cortisol, but does not affect leptin in normal controls. Neuropsychopharmacology 30: 1187-92, 2005. PMID: 15688086.
48. Fassino S, Daga GA, Mondelli V, Pierò A, Broglio F, Picu A, Giordano R, Baldi M, Arvat E, Ghigo E, Gianotti L. Hormonal and metabolic responses to acute ghrelin administration in patients with bulimia nervosa. Psychoneuroendocrinology 30: 534-40, 2005. PMID: 15808922.
49. Levin F, Edholm T, Schmidt PT, Grybäck P, Jacobsson H, Degerblad M, Höybye C, Holst JJ, Rehfeld JF, Hellström PM, Näslund E. Ghrelin stimulates gastric emptying and hunger in normal-weight humans. J Clin Endocrinol Metab 91: 3296-302, 2006. PMID: 16772353.
50. Tack J, Depoortere I, Bisschops R, Delporte C, Coulie B, Meulemans A, Janssens J, Peeters T. Influence of ghrelin on interdigestive gastrointestinal motility in humans. Gut 55: 327-33, 2006. PMID: 16216827.
51. Miljic D, Djurovic M, Pekic S, Doknic M, Stojanovic M, Milic N, Casanueva FF, Ghatei M, Popovic V. Glucose metabolism during ghrelin infusion in patients with anorexia nervosa. J Endocrinol Invest 30:771-5, 2007. PMID: 17993770.
52. Miljic D, Pekic S, Djurovic M, Doknic M, Milic N, Casanueva FF, Ghatei M, Popovic V. Ghrelin has partial or no effect on appetite, growth hormone, prolactin, and cortisol release in patients with anorexia nervosa. J Clin Endocrinol Metab 91: 1491-5, 2006. PMID: 16449333.
53. Veldhuis JD, Keenan DM, Iranmanesh A, Mielke K, Miles JM, Bowers CY. Estradiol potentiates ghrelin-stimulated pulsatile growth hormone secretion in postmenopausal women. J Clin Endocrinol Metab 91: 3559-65, 2006. PMID: 16804038.
54. Veldhuis JD, Iranmanesh A, Mielke K, Miles JM, Carpenter PC, Bowers CY. Ghrelin potentiates growth hormone secretion driven by putative somatostatin withdrawal and resists inhibition by human corticotropin-releasing hormone. J Clin Endocrinol Metab 91: 2441-6, 2006. PMID: 16537682.
55. Vestergaard ET, Andersen NH, Hansen TK, Rasmussen LM, Moller N, Sorensen KE, Sloth E, Jorgensen JO. Cardiovascular effects of intravenous ghrelin infusion in healthy young men. Am J Physiol Heart Circ Physiol 293: H3020-6, 2007. PMID: 17873022.
56. Kluge M, Schüssler P, Zuber V, Kleyer S, Yassouridis A, Dresler M, Uhr M, Steiger A. Ghrelin enhances the nocturnal secretion of cortisol and growth hormone in young females without influencing sleep. Psychoneuroendocrinology 32: 1079-85, 2007. PMID: 17945435.
57. Kluge M, Schüssler P, Bleninger P, Kleyer S, Uhr M, Weikel JC, Yassouridis A, Zuber V, Steiger A. Ghrelin alone or co-administered with GHRH or CRH increases non-REM sleep and decreases REM sleep in young males. Psychoneuroendocrinology 33: 497-506, 2008. PMID: 18329818.
58. Vestergaard ET, Djurhuus CB, Gjedsted J, Nielsen S, Møller N, Holst JJ, Jørgensen JO, Schmitz O. Acute effects of ghrelin administration on glucose and lipid metabolism. J Clin Endocrinol Metab 93: 438-44, 2008. PMID: 18042651.
59. Lanfranco F, Bonelli L, Baldi M, Me E, Broglio F, Ghigo E. Acylated ghrelin inhibits spontaneous LH pulsatility and responsiveness to naloxone but not that to GnRH in young men: evidence for a central inhibitory action of ghrelin on the gonadal axis J Clin Endocrinol Metab 93: 3633-9, 2008 PMID: 18559917.
60. Coiro V, Saccani-Jotti G, Rubino P, Manfredi G, Vacca P, Volta E, Chiodera P. Oxytocin inhibits the stimulatory effect of ghrelin on circulating neuropeptide Y levels in humans. J Neural Transm 115:1265-7, 2008. PMID: 18461271.

61. Veldhuis JD, Reynolds GA, Iranmanesh A, Bowers CY. Twenty-Four Hour Continuous Ghrelin Infusion Augments Physiologically Pulsatile, Nycthemeral and Entropic (Feedback-Regulated) Modes of GH Secretion. J Clin Endocrinol Metab 93: 3597–603, 2008. PMID: 18593763.
62. Kok P, Paulo RC, Cosma M, Mielke KL, Miles JM, Bowers CY, Veldhuis JD. Estrogen Supplementation Selectively Enhances Hypothalamo-Pituitary Sensitivity to Ghrelin in Postmenopausal Women. J Clin Endocrinol Metab 93:4020-6, 2008. PMID: 18611978.
63. Broglio F, Prodam F, Riganti F, Gottero C, Destefanis S, Granata R, Muccioli G, Abribat T, van der Lely AJ, Ghigo E. The continuous infusion of acylated ghrelin enhances growth hormone secretion and worsens glucose metabolism in humans. J Endocrinol Invest 31: 788-94 2008. PMID: 18997491.
64. Benso A, Gramaglia E, Prodam F, Riganti F, Gigliardi VR, Lucatello B, Olivetti I, St Pierre D, Ghigo E, Broglio F. Beta-adrenergic agonism does not impair the GH response to acylated ghrelin in humans. Clin Endocrinol (Oxf) 71: 234-6, 2009. PMID: 19067721.
65. Huda MS, Dovey T, Wong SP, English PJ, Halford J, McCulloch P, Cleator J, Martin B, Cashen J, Hayden K, Wilding JP, Pinkney J. Ghrelin restores 'lean-type' hunger and energy expenditure profiles in morbidly obese subjects but has no effect on postgastrectomy subjects. Int J Obes 33: 317-25. PMID: 19188925.
66. Kluge M, Gazea M, Schüssler P, Genzel L, Dresler M, Kleyer S, Uhr M, Yassouridis A, Steiger A. Ghrelin increases slow wave sleep and stage 2 sleep and decreases stage 1 sleep and REM sleep in elderly men but does not affect sleep in elderly women. Psychoneuroendocrinology Jul 31, 2009. PMID: 19647945.
67. Messini CI, Dafopoulos K, Chalvatzas N, Georgoulias P, Messinis IE. Effect of ghrelin on gonadotrophin secretion in women during the menstrual cycle. Hum Reprod 24: 976-81, 2009. PMID: 19095668.
68. Ang D, Nicolai H, Vos R, Mimidis K, Akyuz F, Kindt S, Vanden Berghe P, Sifrim D, Depoortere I, Peeters T, Tack J. Influence of ghrelin on the gastric accommodation reflex and on meal-induced satiety in man. Neurogastroenterol Motil 21: 528-33, e8-9. 2009 PMID: 19210631.
69. Burns CM, Ratcliffe SJ, Gooneratne N, Scattergood T, Waring AC, Cappola AR. Effects of ghrelin administration in frail and healthy older women. The Endocrine Society's Annual Meeting, Washington, D.C., Abstract#P3-495; 12 June 2009.
70. Nagaya N, Miyatake K, Uematsu M, Oya H, Shimizu W, Hosoda H, Kojima M, Nakanishi N, Mori H, Kangawa K. Hemodynamic, renal, and hormonal effects of ghrelin infusion in patients with chronic heart failure. J Clin Endocrinol Metab 86: 5854-9, 2001. PMID: 11739451.
71. Aimaretti G, Baffoni C, Broglio F, Janssen JA, Corneli G, Deghenghi R, van der Lely AJ, Ghigo E, Arvat E. Endocrine responses to ghrelin in adult patients with isolated childhood-onset growth hormone deficiency. Clin Endocrinol (Oxf) 56: 765-71, 2002. PMID: 12072046.
72. Janssen JA, Poldermans D, Hofland LJ, Vourvouri EC, Muller AF, Bax JJ, Deghenghi R, Broglio F, Ghigo E, van der Lely AJ. There are no acute cardiac effects of a single IV dose of human ghrelin in severe growth hormone deficient patients. J Endocrinol Invest 27: 659-64, 2004. PMID: 15505990.
73. Gauna C, Meyler FM, Janssen JA, Delhanty PJ, Abribat T, van Koetsveld P, Hofland LJ, Broglio F, Ghigo E, van der Lely AJ. Administration of acylated ghrelin reduces insulin

sensitivity, whereas the combination of acylated plus unacylated ghrelin strongly improves insulin sensitivity. J Clin Endocrinol Metab 89: 5035-42, 2004. PMID: 15472202.
74. Broglio F, Gianotti L, Destefanis S, Fassino S, Abbate Daga G, Mondelli V, Lanfranco F, Gottero C, Gauna C, Hofland L, Van der Lely AJ, Ghigo E. The endocrine response to acute ghrelin administration is blunted in patients with anorexia nervosa, a ghrelin hypersecretory state. Clin Endocrinol (Oxf) 60: 592-9, 2004. PMID: 15104562.
75. Neary NM, Small CJ, Wren AM, Lee JL, Druce MR, Palmieri C, Frost GS, Ghatei MA, Coombes RC, Bloom SR. Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial. J Clin Endocrinol Metab 89: 2832-6, 2004. PMID: 15181065.
76. Tack J, Depoortere I, Bisschops R, Verbeke K, Janssens J, Peeters T. Influence of ghrelin on gastric emptying and meal-related symptoms in idiopathic gastroparesis. Aliment Pharmacol Ther 22:847-53, 2005. PMID: 16225494.
77. Tesauro M, Schinzari F, Iantorno M, Rizza S, Melina D, Lauro D, Cardillo C. Ghrelin improves endothelial function in patients with metabolic syndrome. Circulation 112: 2986-92, 2005. PMID: 16260640.
78. Murray CD, Martin NM, Patterson M, Taylor SA, Ghatei MA, Kamm MA, Johnston C, Bloom SR, Emmanuel AV. Ghrelin enhances gastric emptying in diabetic gastroparesis: a double blind, placebo controlled, crossover study. Gut 54: 1693-8, 2005. PMID: 16085693.
79. Wynne K, Giannitsopoulou K, Small CJ, Patterson M, Frost G, Ghatei MA, Brown EA, Bloom SR, Choi P. Subcutaneous ghrelin enhances acute food intake in malnourished patients who receive maintenance peritoneal dialysis: a randomized, placebo-controlled trial. J Am Soc Nephrol 16:2111-8, 2005. PMID: 15888560.
80. Damjanovic SS, Lalic NM, Pesko PM, Petakov MS, Jotic A, Miljic D, Lalic KS, Lukic L, Djurovic M, Djukic VB. Acute effects of ghrelin on insulin secretion and glucose disposal rate in gastrectomized patients. J Clin Endocrinol Metab 91:2574-81, 2006. PMID: 16621911.
81. Cremonini F, Camilleri M, Vazquez Roque M, McKinzie S, Burton D, Baxter K, Zinsmeister AR. Obesity does not increase effects of synthetic ghrelin on human gastric motor functions. Gastroenterology 131: 1431-9, 2006. PMID: 17101319.
82. Fusco A, Bianchi A, Mancini A, Milardi D, Giampietro A, Cimino V, Porcelli T, Romualdi D, Guido M, Lanzone A, Pontecorvi A, De Marinis L. Effects of ghrelin administration on endocrine and metabolic parameters in obese women with polycystic ovary syndrome. J Endocrinol Invest 30: 948-56, 2007. PMID: 18250617.
83. Akamizu T, Iwakura H, Ariyasu H, Hosoda H, Murayama T, Yokode M, Teramukai S, Seno H, Chiba T, Noma S, Nakai Y, Fukunaga M, Nakai Y, Kangawa K, FD Clinical Study Team. Repeated administration of ghrelin to patients with functional dyspepsia: its effects on food intake and appetite. Eur J Endocrinol 158: 491-8, 2008. PMID: 18362296.
84. Guido M, Romualdi D, De Marinis L, Porcelli T, Giuliani M, Costantini B, Lanzone A. Administration of exogenous ghrelin in obese patients with polycystic ovary syndrome: effects on plasma levels of growth hormone, glucose, and insulin. Fertil Steril 88: 125-30, 2007. PMID: 17276431.
85. Cecconi E, Bogazzi F, Morselli LL, Gasperi M, Procopio M, Gramaglia E, Broglio F, Giovannetti C, Ghigo E, Martino E. Primary hyperparathyroidism is associated with marked impairment of GH response to acylated ghrelin. Clin Endocrinol (Oxf). 69: 197-201, 2008. PMID: 18221398.

86. Romualdi D, De Marinis L, Campagna G, Proto C, Lanzone A, Guido M. Alteration of ghrelin-neuropeptide Y network in obese patients with polycystic ovary syndrome: role of hyperinsulinism. Clin Endocrinol (Oxf) 69: 562-7. 2008. PMID: 18248643.
87. Ashby DR, Ford HE, Wynne KJ, Wren AM, Murphy KG, Busbridge M, Brown EA, Taube DH, Ghatei MA, Tam FW, Bloom SR, Choi P. Sustained appetite improvement in malnourished dialysis patients by daily ghrelin treatment. Kidney Int 76:199-206, 2009. PMID: 19387475.
88. Kiewiet RM, van Aken MO, van der Weerd K, Uitterlinden P, Themmen AP, Hofland LJ, de Rijke YB, Delhanty PJ, Ghigo E, Abribat T, van der Lely AJ. Effects of acute administration of acylated and unacylated ghrelin on glucose and insulin concentrations in morbidly obese subjects without overt diabetes. Eur J Endocrinol 161: 567-73, 2009. PMID: 19628651.
89. Hotta M, Ohwada R, Akamizu T, Shibasaki T, Takano K, Kangawa K. Ghrelin Increases Hunger and Food Intake in Patients with Restricting-type Anorexia Nervosa: A Pilot Study. Endocr J Sep 16, 2009. PMID:19755753.
90. Loose MD, Ronnekleiv OK, Kelly MJ. Neurons in the rat arcuate nucleus are hyperpolarized by GABAB and mu-opioid receptor agonists: evidence for convergence at a ligand-gated potassium conductance. Neuroendocrinology 54: 537-44, 1991. PMID: 1664497.
91. Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50, 1714-9, 2001. PMID: 11473029.
92. Tschop M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R, Folwaczny C. Post-prandial decrease of circulating human ghrelin levels. J Endocrinol Invest 24: RC19–RC21, 2001. PMID: 11434675.
93. Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJ, Smith RG, Van der Ploeg LH, Howard AD. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res Mol Brain Res 48: 23-9, 1997. PMID: 9379845.
94. Katayama M, Nogami H, Nishiyama J, Kawase T, Kawamura K. Developmentally and regionally regulated expression of growth hormone secretagogue receptor mRNA in rat brain and pituitary gland. Neuroendocrinology 72:333–40, 2000. PMID: 11146416.
95. Zigman JM, Jones JE, Lee CE, Saper CB, Elmquist JK. Expression of ghrelin receptor mRNA in the rat and the mouse brain. J Comp Neurol 494:528–48, 2006. PMID: 16320257.
96. Naleid AM, Grace MK, Cummings DE, Levine AS. Ghrelin induces feeding in the mesolimbic reward pathway between the ventral tegmental area and the nucleus accumbens. Peptides 26:2274–9, 2005. PMID: 16137788.
97. Jerlhag E, Grøtli M, Luthman K, Svensson L, Engel JA. Role of the subunit composition of central nicotinic acetylcholine receptors for the stimulatory and dopamine-enhancing effects of ethanol. Alcohol Alcohol 41, 486-93, 2006. PMID: 16799162.
98. Kawahara Y, Kawahara H, Kaneko F, Yamada M, Nishi Y, Tanaka E, Nishi A. Peripherally administered ghrelin induces bimodal effects on the mesolimbic dopamine system depending on food-consumptive states. Neuroscience. 161:855-64. 2009. PMID: 19362120.
99. Quarta D, Di Francesco C, Melotto S, Mangiarini L, Heidbreder C, Hedou G. Systemic administration of ghrelin increases extracellular dopamine in the shell but not the core subdivision of the nucleus accumbens. Neurochem Int. 54:89-94. 2009. PMID: 19118592.
100. Tupala E, Tiihonen J. Dopamine and alcoholism: neurobiological basis of ethanol abuse. Prog Neuropsychopharmacol Biol Psychiatry 28: 1221-47, 2004. PMID: 15588749.

101. Koob GF. Neural mechanisms of drug reinforcement. Ann N Y Acad Sci 654:171-91, 1992. PMID: 1632582.
102. Wise RA. Neurobiology of addiction. Curr Opin Neurobiol 6: 243-51, 1996. PMID: 8725967.
103. Melendez RI, Rodd-Henricks ZA, Engleman EA, Li TK, McBride WJ, Murphy JM. Microdialysis of dopamine in the nucleus accumbens of alcohol-preferring (P) rats during anticipation and operant self-administration of ethanol. Alcohol Clin Exp Res 26: 318-25, 2002. PMID: 11923583.
104. Yan QS. Extracellular dopamine and serotonin after ethanol monitored with 5-minute microdialysis. Alcohol 19:1-7, 1999. PMID: 10487381.
105. Nurmi M, Ashizawa T, Sinclair JD, Kiianmaa K. Effect of prior ethanol experience on dopamine overflow in accumbens of AA and ANA rats. Eur J Pharmacol 315:277-83, 1996. PMID: 8982665.
106. Hemby SE. Neurobiology of alcoholism. In: Johnson BA, Ruiz P, Galanter M, eds. Handbook of clinical alcoholism treatment. Baltimore, MD: Lippincott Williams & Wilkins; 10-8, 2003.
107. Devlin MJ. Is there a place for obesity in DSM-V. Int J Eat Disord 40 Suppl: S83-8, 2007. PMID: 17683083
108. Volkow ND, Wang GJ, Fowler JS, Telang F. Overlapping neuronal circuits in addiction and obesity: evidence of systems pathology. Philos Trans R Soc Lond B Biol Sci 363:3191-200, 2008. PMID: 18640912.
109. Koob GF. Drugs of abuse: anatomy, pharmacology and function of reward pathways. Trends Pharmacol Sci. 1992;13:177-84.
110. Heilig M, Koob GF. A key role for corticotropin-releasing factor in alcohol dependence. Trends Neurosci. 2007;30:399-406.
111. Pandey SC, Carr LG, Heilig M, Ilveskoski E, Thiele TE. Neuropeptide y and alcoholism: genetic, molecular, and pharmacological evidence. Alcohol Clin Exp Res. 2003;27:149-54.
112. Koob GF. Brain stress systems in the amygdala and addiction. Brain Res. 2009; 13;1293:61-75.
113. Skibicka KP, Shirazi RH, Hansson C, Dickson SL. Ghrelin Interacts with Neuropeptide Y Y1 and Opioid Receptors to Increase Food Reward. Endocrinology 2012; 153: 1194-1205.
114. Naleid AM, Grace MK, Cummings DE, Levine AS. Ghrelin induces feeding in the mesolimbic reward pathway between the ventral tegmental area and the nucleus accumbens. Peptides 2005;26:2274-9.
115. Spencer SJ, Xu L, Clarke MA, Lemus M, Reichenbach A, Geenen B, Kozicz T, Andrews ZB. Ghrelin Regulates the Hypothalamic-Pituitary-Adrenal Axis and Restricts Anxiety After Acute Stress. Biol Psychiatry 2012 Apr 20. [Epub ahead of print] PMID: 22521145.
116. Clarke TK, Treutlein J, Zimmermann US, Kiefer F, Skowronek MH, Rietschel M, Mann K, Schumann G. HPA-axis activity in alcoholism: examples for a gene-environment interaction. Addict Biol. 2008 Mar;13(1):1-14
117. Jerlhag E, Egecioglu E, Dickson SL, Svensson L, Engel JA. Alpha-conotoxin MII-sensitive nicotinic acetylcholine receptors are involved in mediating the ghrelin-induced locomotor stimulation and dopamine overflow in nucleus accumbens. Eur Neuropsychopharmacol 18: 508-18, 2008. PMID: 18343642.
118. Larsson A, Edström L, Svensson L, Söderpalm B, Engel JA. Voluntary ethanol intake increases extracellular acetylcholine levels in the ventral tegmental area in the rat. Alcohol Alcohol 40, 349-58, 2005. PMID: 16043436.

119. Larsson A, Jerlhag E, Svensson L, Söderpalm B, Engel JA. Is an alpha-conotoxin MII sensitive mechanism involved in the neurochemical, stimulatory and rewarding effects of ethanol. Alcohol 34, 239-46, 2004. PMID: 15902919.
120. Schneider ER, Rada P, Darby RD, Leibowitz SF, Hoebel BG. Orexigenic peptides and alcohol intake: differential effects of orexin, galanin, and ghrelin. Alcohol Clin Exp Res 31, 1858-65, 2007. PMID: 17850217.
121. Jerlhag E. Systemic administration of ghrelin induces conditioned place preference and stimulates accumbal dopamine. Addict Biol 13: 358-63, 2008. PMID: 18358991.
122. Jerlhag E, Egecioglu E, Landgren S, Salomé N, Heilig M, Moechars D, Datta R, Perrissoud D, Dickson SL, Engel JA. Requirement of central ghrelin signaling for alcohol reward. Proc Natl Acad Sci U S A. 106:11318-23. 2009. PMID: 19564604.
123. Calissendorff J, Danielsson O, Brismar K, Röjdmark S. Inhibitory effect of alcohol on ghrelin secretion in normal man. Eur J Endocrinol 152: 743-7, 2005. PMID: 15879360.
124. Calissendorff J, Danielsson O, Brismar K, Röjdmark S. Alcohol ingestion does not affect serum levels of peptide YY but decreases both total and octanoylated ghrelin levels in healthy subjects. Metabolism 55: 1625-9, 2006. PMID: 17142135.
125. Zimmermann US, Buchmann A, Steffin B, Dieterle C, Uhr M. Alcohol administration acutely inhibits ghrelin secretion in an experiment involving psychosocial stress. Addict Biol 12: 17-21, 2007. PMID: 17407493.
126. Badaoui A, De Saeger C, Duchemin J, Gihousse D, de Timary P, Stärkel P. Alcohol dependence is associated with reduced plasma and fundic ghrelin levels. Eur J Clin Invest 38: 397-403, 2008. PMID: 18422979.
127. Kim DJ, Yoon SJ, Choi B, Kim TS, Woo YS, Kim W, Myrick H, Peterson BS, Choi YB, Kim YK, Jeong J. Increased fasting plasma ghrelin levels during alcohol abstinence. Alcohol Alcohol 40: 76-9, 2005. PMID: 15520048.
128. Kraus T, Schanze A, Gröschl M, Bayerlein K, Hillemacher T, Reulbach U, Kornhuber J, Bleich S. Ghrelin levels are increased in alcoholism. Alcohol Clin Exp Res 29: 2154-7, 2005. PMID: 16385185.
129. Cassin SE, von Ranson KM. Personality and eating disorders: a decade in review. Clin Psychol Rev 25:895-916, 2005. PMID: 16099563.
130. Hillemacher T, Kraus T, Rauh J, Weiss J, Schanze A, Frieling H, Wilhelm J, Heberlein A, Gröschl M, Sperling W, Kornhuber J, Bleich S. Role of appetite-regulating peptides in alcohol craving: an analysis in respect to subtypes and different consumption patterns in alcoholism. Alcohol Clin Exp Res 31:950-4. 2007. PMID: 17433008.
131. Wurst FM, Graf I, Ehrenthal HD, Klein S, Backhaus J, Blank S, Graf M, Pridzun L, Wiesbeck GA, Junghanns K. Gender differences for ghrelin levels in alcohol-dependent patients and differences between alcoholics and healthy controls. Alcohol Clin Exp Res 31:2006-11. 2007. PMID: 17949465.
132. Leggio L, Ferrulli A, Cardone S, Nesci A, Miceli A, Malandrino N, Capristo E, Canestrelli B, Monteleone P, Kenna GA, Swift RM, Addolorato G. Ghrelin system in alcohol-dependent subjects: role of plasma ghrelin levels in alcohol drinking and craving. Addict Biol. 2012 Mar;17(2):452-64.
133. Davis JF, Schurdak JD, Magrisso IJ, Mul JD, Grayson BE, Pfluger PT, Tschoep MH, Seeley RJ, Benoit SC. Gastric Bypass Surgery Attenuates Ethanol Consumption in Ethanol-Preferring Rats. Biol Psychiatry. 2012 Mar 21. PMID: 22444202.
134. Landgren S, Jerlhag E, Zetterberg H, Gonzalez-Quintela A, Campos J, Olofsson

U, Nilsson S, Blennow K, Engel JA. Association of pro-ghrelin and GHS-R1A gene polymorphisms and haplotypes with heavy alcohol use and body mass. Alcohol Clin Exp Res. 2008 Dec;32(12):2054-61. PMID: 18828808.
135. Rohsenow DJ, Monti PM, Rubonis AV, Sirota AD, Niaura RS, Colby SM, Wunschel SM, Abrams DB. Cue reactivity as a predictor of drinking among male alcoholics. J Consult Clin Psychol. 1994 Jun;62(3):620-6. PMID: 8063989
136. Monti PM, Rohsenow DJ, Hutchison KE, Swift RM, Mueller TI, Colby SM, Brown RA, Gulliver SB, Gordon A, Abrams DB. Naltrexone's effect on cue-elicited craving among alcoholics in treatment. Alcohol Clin Exp Res. 1999 Aug;23(8):1386-94. PMID: 10470982.
137. Monti PM, Rohsenow DJ, Hutchison KE. Toward bridging the gap between biological, psychobiological and psychosocial models of alcohol craving. Addiction. 2000 Aug;95 Suppl 2:S229-36. Review. PubMed PMID: 11002917.
138. Ramchandani VA, Bolane J, Li TK, O'Connor S. A physiologically-based pharmacokinetic (PBPK) model for alcohol facilitates rapid BrAC clamping. Alcohol Clin Exp Res. 1999;23:617-23.
139. Plawecki MH, DeCarlo RA, Ramchandani VA, O'Connor S. Estimation of ethanol infusion profile to produce specified BrAC time course using physiologically-based pharmacokinetic (PBPK) models. Conf Proc IEEE Eng Med Biol Soc. 2004;1:778-81.
140. Knutson B, Adams CM, Fong GW, Hommer D. Anticipation of increasing monetary reward selectively recruits nucleus accumbens. J Neurosci. 2001;21.
141. Bjork JM, Knutson B, Fong GW, Caggiano DM, Bennett SM, Hommer DW. Incentive-elicited brain activation in adolescents: similarities and differences from young adults. J Neurosci. 2004;24.
142. Malik S, McGlone F, Bedrossian D, Dagher A. Ghrelin modulates brain activity in areas that control appetitive behavior. Cell Metab. 2008;7:400-9.
143. Hommer DW. Functional imaging of craving. Alcohol Res Health 1999;23:187-96.
144. Gilman JM, Ramchandani VA, Davis MB, Bjork JM, Hommer DW. Why we like to drink: a functional magnetic resonance imaging study of the rewarding and anxiolytic effects of alcohol. J Neurosci. 2008;28:4583-91.
145. Yokoyama, A., Muramatsu, T., Ohmori, T., Kumagi, Y., Higuchi, S., and Ishii, H. Reliability of a flushing questionnaire and the ethanol patch text in screening for inactive aldehyde dehydrogenase-2 and alcohol-related cancer risk. Cancer Epidemiol Biomarkers Prev 6(12), 1105-7. 1997. Ref Type: Generic
146. Johnson RC, Nagoshi CT, Schwitters SY, Bowman KS, Ahern FM, Wilson JR. Further investigation of racial/ethnic differences and of familial resemblances in flushing in response to alcohol. Behav Genet. 1984;14:171-78.
147. Richardson NR, Roberts DC. Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy. J Neurosci Methods 1996;66:1-11.
148. de Wit H, McCracken SG. Ethanol self-administration in males with and without an alcoholic first-degree relative. Alcohol Clin Exp Res. 1990;14:63-70.
149. Bohn MJ, Krahn DD, Staehler BA. Development and initial validation of a measure of drinking urges in abstinent alcoholics. Alcohol Clin Exp Res. 1995;19:600-606.
150. Martin CS, Earleywine M, Musty RE, Perrine MW, Swift RM. Development and validation of the Biphasic Alcohol Effects Scale. Alcohol Clin Exp Res. 1993;17:140-146.

151. McNair, D., Lorr, M., Droppleman, L. Edits manual: Profile of mood states. San Diego: Educational and Industrial Testing Services, 1971.
152. Kampov-Polevoy AB, Garbutt JC, Janowsky DS. Association between preference for sweets and excessive alcohol intake: a review of animal and human studies. Alcohol Alcohol 34: 386-95, 1999. PMID: 10414615.
153. Garbutt JC, Osborne M, Gallop R, Barkenbus J, Grace K, Cody M, Flannery B, Kampov-Polevoy AB. Sweet liking phenotype, alcohol craving and response to naltrexone treatment in alcohol dependence. Alcohol Alcohol. 2009;44:293-300
154. Bollinger, J., Acker, J., & MacKillop, J. (2012). An Initial Validation of the Brief Behavior Social Density Assessment (BASDA) in an Undergraduate Sample. Poster presented at the 35th Annual Convention of the Behavioral Sciences, Athens, GA, March 2012
155. Carver, CS, & White, TL. Behavioral inhibition, behavioral activation, and affective responses to impending reward and punishment: The BIS/BAS scales. Journal of Personality and Social Psychology. 1994;67:319-333.
156. Johnson, SL, Turner RJ, & Iwata, N. BIS/BAS Levels and Psychiatric Disorder: An Epidemiological Study. Journal of Psychopathology and Behavioral Assessment. 2003;25:25-26
157. Franken, IHA. Behavioral approach system (BAS) sensitivity predicts alcohol craving. Personality and Individual Differences. 2002; 32:349-355.
158. Blekher T, Ramchandani VA, Flury L, Foroud T, Kareken D, Yee RD et al. Saccadic eye movements are associated with a family history of alcoholism at baseline and after exposure to alcohol. Alcohol Clin Exp Res. 2002;26:1568-73.
159. Gilman JM, Ramchandani VA, Davis MB, Bjork JM, Hommer DW. Why we like to drink: a functional magnetic resonance imaging study of the rewarding and anxiolytic effects of alcohol. J Neurosci. 2008;28:4583-91.
160. Kwo PY, Ramchandani VA, O'Connor S, Amann D, Carr LG, Sandrasegaran K et al. Gender differences in alcohol metabolism: relationship to liver volume and effect of adjusting for body mass. Gastroenterology. 1998;115:1552-57.
161. Morzorati SL, Ramchandani VA, Flury L, Li TK, O'Connor S. Self-reported subjective perception of intoxication reflects family history of alcoholism when breath alcohol levels are constant. Alcohol Clin Exp Res. 2002;26:1299-306.
162. Ramchandani VA, O'Connor S, Blekher T, Kareken D, Morzorati S, Nurnberger J, Jr. et al. A preliminary study of acute responses to clamped alcohol concentration and family history of alcoholism. Alcohol Clin Exp Res. 1999;23:1320-1330.
163. Ramchandani VA, Kwo PY, Li TK. Effect of food and food composition on alcohol elimination rates in healthy men and women. J Clin Pharmacol. 2001;41:1345-50.
164. Ramchandani VA, Flury L, Morzorati SL, Kareken D, Blekher T, Foroud T et al. Recent drinking history: association with family history of alcoholism and the acute response to alcohol during a 60 mg% clamp. J Stud Alcohol. 2002;63:734-44.
165. Ramchandani VA, O'Connor S, Neumark Y, Zimmermann US, Morzorati SL, de Wit H. The alcohol clamp: applications, challenges, and new directions--an RSA 2004 symposium summary. Alcohol Clin Exp Res. 2006;30:155-64.
166. NIAAA Guidelines: http://www.niaaa.nih.gov/research/guidelines-and-resources/administering-alcohol-human-studies
167. Cancer Therapy Evaluation Program. Common Terminology Criteria for Adverse Events v3.0 (CTCAE). 8-9-2007. DCTD, NCI, NIH, DHHS.