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# Enhanced alcohol-drinking behavior associated with active ghrelinergic and serotoninergic neurons in the lateral hypothalamus and amygdala

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ABSTRACT

Central ghrelin is required for the rewarding properties of drug abuse. We investigated whether alcohol affects ghrelinergic, dopaminergic, and serotoninergic neurons and growth hormone secretagogue receptor 1A (GHS-R1A) levels in the reward system of the brain. Alcohol-naïve C57BL/6 J mice received 2 g/kg ethanol (EtOH) intraperitoneally (i.p.). Plasma ghrelin levels decreased between 1 and 4 h. We investigated the effects of EtOH administration on plasma ghrelin levels in two different animal models at 1, 3, and 10 months of age. Plasma ghrelin levels decreased following the EtOH treatment in 1- and 3-month-old short-term (1-day) alcohol vapor-exposed (STA) mice. In contrast, EtOH administration increased plasma ghrelin levels in 1- and 3-month-old long-term (20-day) alcohol vapor-exposed (LTA) mice. *In vivo* ghrelin release in the lateral hypothalamus (LH) increased in STA and LTA mice after the i.p. administration of EtOH. EtOH increased *in vivo* dopamine (DA), but not serotonin (5-HT) release in the LH of STA mice, and increased *in vivo* DA and 5-HT release in the LH of LTA mice. *GHS-R1A* mRNA expression and GHS-R1A protein levels in the LH were increased in LTA mice. The number of GHS-R1A-immunoreactive cells was greater in the LH and amygdala of LTA mice. These results support the neurobiological correlation between the development of drinking behavior and activation of ghrelinergic and serotonergic neurons in the LH. The activation of ghrelinergic systems in the amygdala may also induce an increase in 5-HT release in the LH during long-term alcohol intake.



## 1. Introduction

The orexigenic peptide ghrelin, which is produced in the fundic mucosa of the stomach, induces the secretion of growth hormone, stimulates appetite and food intake, and controls energy balance. Ghrelin acts on growth hormone secretagogue receptor 1A (GHS-R1A) to stimulate growth hormone release (Kojima et al., 1999). Ghrelin, the endogenous ligand of GHS-R1A, also acts as an important regulator of energy balance. Ghrelin is expressed in the hypothalamus (the lateral hypothalamus [LH], arcuate nucleus, ventral medial nucleus, dorsomedial nucleus, and paraventricular nucleus (Schellekens et al., 2012; Cowley et al., 2003), mesolimbic reward area (the ventral tegmental area [VTA] and nucleus accumbens [ACC]; Egecioglu et al., 2010), and perioculomotor urocortin-containing population of neurons (Kaur and Ryabinin, 2010).

Increased plasma ghrelin levels have been reported in active drinkers, alcohol-dependent individuals, individuals with alcohol withdrawal, and individuals who abstain from alcohol (Wurst et al., 2007). In contrast, plasma and stomach ghrelin levels are reduced in alcohol-dependent patients (Badaoui et al., 2008). In healthy individuals, plasma ghrelin levels decrease after acute alcohol exposure (Zimmerman et al., 2007). However, the relationship between plasma ghrelin levels and alcohol-drinking behavior in humans remains unclear.

*Abbreviations:* (GHS-R1A), growth hormone secretagogue receptor 1A; (EtOH), ethanol; (i.p.), intraperitoneally; (STA), short-term alcohol vapor-exposed; (LTA), long-term alcohol vapor-exposed; (LH), lateral hypothalamus; (DA), dopamine; (5-HT), serotonin; [VTA], ventral tegmental area; [ACC], nucleus accumbens; (CNS), central nervous system; (ELISA), enzyme-linked immunosorbent assay; (aCSF), artificial cerebrospinal fluid; (SDS), sodium dodecyl sulfate; (EDTA), disodium-ethylenediaminetetraacetic acid; (PCR), polymerase chain reaction; (G3PDH), glycerol-3-phosphate dehydrogenase; (PB), phosphate buffer; (DIG), digoxigenin; (CCD), charge-coupled device; (ANOVA), analysis of variance; (AMY), amygdala.

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The intracerebroventricular administration of ghrelin into the VTA increases alcohol intake in mice, whereas that of GHS-R1A antagonists ((D-Lys$^3$)-GHRP-6 and JMV2959) reduces alcohol intake (Gomez et al., 2015). Ghrelin acting in the dopaminergic system is responsible for the rewarding effects of alcohol (Jerlhag et al., 2009). The dopaminergic neurons of the VTA that project to the ACC are activated by this route (Yoshimoto et al., 1992). Ghrelin signaling appears to be associated with the development of addiction, particularly to alcohol, nicotine, and stimulants.

The ghrelin-induced intake of palatable foods increases dopaminergic signaling in the reward system of the brain, which acts to reduce the deleterious effects of physical stress. Enhanced plasma ghrelin under conditions of physical stress plays an important role in stress-induced food reward behavior and food addiction (Schellekens et al., 2012).

Previous studies suggested that evidence for the involvement of ghrelin signaling in aspects of addiction is inconclusive (Panagopoulos and Ralevski, 2014). However, ghrelin was recently implicated in the regulation of the reward system of the brain: a single-nucleotide polymorphism in the *GHS-R1A* gene was found to be associated with high alcohol consumption in humans (Stevenson et al., 2016). A clearer understanding of ghrelin dynamics and the induction of monoamines such as dopamine (DA) and serotonin (5-HT) in the reward system of the brain is key to resolving complex addiction behaviors.

Although the central ghrelin signaling system is recognized as an important central nervous system (CNS) target for the development of treatments for alcohol craving, there is limited evidence for the effects of consumed ethanol (EtOH) on this signaling pathway in animal models. In the present study, we examined changes in plasma and *in vivo* brain ghrelin levels following alcohol administration, and investigated the neural relationship between ghrelin signaling and monoaminergic systems in the LH for the development of alcohol-drinking behavior.

## 2. Materials and methods

### 2.1. Animals

Male C57BL/6 J mice (4 weeks old) weighing 21–25 g purchased from CLEA Japan, Inc. (Tokyo, Japan) were used in all experiments (total: 205 mice). Each experiment was designed to limit the number of animals used and minimize animal suffering. Before the start of the experiments, all animals were housed in an environment in which temperature (21°C) and humidity (50%) were controlled with a 12-hour light–dark cycle (lights on at 06:00 h). Animals had free access to water and standard mouse chow (CE-2; CLEA Japan, Inc.). All animal experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and the protocols were approved by the Committee for the Care and Use of Laboratory Animals (A15-47), Hiroshima University, Hiroshima, Japan.

### 2.2. Experiment A: effects of alcohol on plasma ghrelin levels and lateral hypothalamic DA and 5-HT release in alcohol-naïve mice

#### 2.2.1. Alcohol administration and changes in plasma ghrelin levels over time

Male naïve C57BL/6 J mice (10 weeks old) were given 2 g/kg alcohol or saline intraperitoneally (i.p.). Mice were euthanized 0, 0.5, 1, 2, 4, and 8 h after alcohol administration ($n = 6$ for each time point). Blood samples were collected, cooled on ice, and centrifuged to separate the plasma. Plasma ghrelin concentrations were measured using an Active Ghrelin Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan). The amount of ghrelin in each sample was calculated by extrapolating values from the standard curve generated, according to the manufacturer's instructions.

#### 2.2.2. Alcohol administration and in vivo lateral hypothalamic DA and 5-HT release

A brain microdialysis guide cannula (CMA 7; CMA Microdialysis AB, Kista, Sweden) was implanted into the LH (anteroposterior: −0.8 mm anterior; lateral: −1.0 mm from the bregma; −4.5 mm from the surface of the skull) placed 1 mm above the LH using laser-polymerized fixation. A microdialysis probe (CMA7 probe: 1-mm membrane, 7-mm shaft length; CMA Microdialysis AB) was inserted through the microdialysis probe guide cannula into the LH of alcohol-naïve mice. A pilot experiment of a stimulation with EtOH (200 mM) perfusion in the LH through the microdialysis probe membrane was conducted in alcohol-naïve C57BL/6 J mice ($n = 7$). After establishing a stable baseline, 200 mM EtOH was perfused with artificial cerebrospinal fluid (aCSF) for 60 min, and the perfusion solution was then switched to aCSF alone. EtOH perfusion may directly activate dopaminergic and serotonergic neurons in the LH.

The mobile phase consisted of 2% (v/v) methanol, 0.1 M sodium dihydrogen phosphate, 500 mg/l sodium dodecyl sulfate (SDS), and 0.12 mM disodium-ethylenediaminetetraacetic acid (EDTA; pH adjusted to 6.0 with 0.1 M disodium hydrogen phosphate). A $C_{18}$ reversed-phase column (Nova-Pak C18, 150 × 2.1 mm, 4.1 μm; Waters Corp., Tokyo, Japan) was used at a flow rate of 0.8 μl/min (EP-700; Eicom Co., Ltd., Kyoto, Japan) set at a sensitivity of 0.55 nA/V with a potential of 0.65 V on the glassy carbon electrode.

### 2.3. Experiment B: effects of alcohol on plasma and lateral hypothalamic ghrelin levels and lateral hypothalamic DA and 5-HT release in short- (STA) and long-term alcohol vapor-exposed (LTA) mice

#### 2.3.1. Effects of age on plasma ghrelin levels in STA and LTA vapor-exposed mice

C57BL/6 J mice received alcohol vapor at 1, 3, and 10 months of age. As reported in previous studies (Yoshimoto et al., 2012), alcohol vapor was created by dripping 95% alcohol into a vacuum flask kept at 85 °C on a hot plate. Air was blown over the top of the flask at a rate of 1.5 l/min to vaporize the alcohol. The concentration of alcohol vapor was adjusted by varying the rate at which alcohol was pumped into the flask. The duration of alcohol vapor inhalation was controlled by a timer (Yoshimoto et al., 2012).

Mice received alcohol vapor for a maximum of 8 h per day during the dark cycle. The alcohol vapor system was turned on at 00:00 h and off at 04:00 h for a few days to acclimatize mice to alcohol vapor. Finally, vapor was delivered from 00:00 h to 08:00 h for the last 20 days to create LTA vapor-exposed mice ($n = 6$ in each month) (Yoshimoto et al., 2012). Another group of C57BL/6 J mice received alcohol vapor at 1, 3, and 10 months of age for only 8 h of 1 day to create STA vapor-exposed mice ($n = 6$ in each month) (O'Dell et al., 2004; Walker and Koob, 2007).

STA and LTA mice were confirmed using the alcohol-drinking test as modified by Yoshimoto et al. (2012). A voluntary alcohol-drinking behavior test was performed on STA and LTA mice to confirm enhanced alcohol drinking or the development of alcohol-drinking behavior. Water was removed 12 h before a 4-h period of limited access to alcohol. Mice had access to 10% (v/v) alcohol from a 20-ml graduated glass tube for a 4-h period beginning at 10:00 h. Voluntary alcohol-consumption tests were performed the day after a 1- or 20-day exposure period in alcohol-exposed and control alcohol-naïve mice. Initially, all mice had access to a 10% alcohol solution for 3 consecutive days to acclimatize them to the alcohol solution. The voluntary alcohol-drinking behavior test (alcohol preference [%], alcohol intake [g/kg/day], and water intake) was performed the day after alcohol vapor exposure (Yoshimoto et al., 2000b; Yoshimoto et al., 2012). According to the methods of Yoshimoto et al. (2012), LTA mice exhibited an increased alcohol preference from the basal alcohol preference before LTA vapor inhalation.

Plasma ghrelin concentrations were measured using the same procedure as described in Experiment A. Blood samples were collected,