

# Orexigenic Peptides and Alcohol Intake: Differential Effects of Orexin, Galanin, and Ghrelin

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**Background:** The question is which hypothalamic systems for food intake might play a role in ethanol intake and contribute to alcohol abuse. The peptide orexin was found to exhibit similar properties to galanin in its relation to dietary fat and may therefore be similar to galanin in having a stimulatory effect on alcohol intake.

**Methods:** Rats were trained to drink 10% ethanol, implanted with brain cannulas, and then injected in the paraventricular nucleus (PVN), lateral hypothalamus (LH), or nucleus accumbens (NAc) with galanin, orexin-A, and for comparison, ghrelin. Ethanol, food, and water intake were measured at 1, 2, and 4 hours postinjection.

**Results:** In the PVN, both orexin and galanin significantly increased ethanol intake, whereas ghrelin increased food intake. In the LH, orexin again induced ethanol intake, while ghrelin increased eating. In the NAc, orexin failed to influence ethanol intake but did stimulate food intake.

**Conclusions:** In ethanol-drinking rats, injection of orexin or galanin into the appropriate locus in the hypothalamus induced significant ethanol intake instead of food intake. Ghrelin, as a positive control, failed to influence ethanol intake at the same hypothalamic sites. In the NAc, as an anatomical control, orexin augmented eating but not ethanol intake. Thus orexin and galanin in the hypothalamus selectively stimulated ethanol intake at sites where other studies have shown that both ethanol and fat increase expression of the endogenous peptides. Thus, a neural circuit that evolved with the capability to augment food intake is apparently co-opted by ethanol and may serve as a potential positive feedback circuit for alcohol abuse.

**Key Words:** Ethanol, Hypothalamus, Accumbens, Galanin, Orexin.

THE NEXUS BETWEEN food intake and drug addiction has long been of interest to clinicians and researchers alike. Nowhere is this comparison more pertinent than in the case of ethanol, as it is both a drug of abuse and a source of calories. At least 28 peptides are known to be involved in food and water intake, and several of these have also been implicated in ethanol consumption. These include galanin, neuropeptide Y (NPY), melanin concentrating hormone, the opioids, and  $\alpha$ -melanocyte stimulating hormone (Bodnar and Klein, 2005; Duncan et al., 2005; Gilpin et al., 2004; Haddad, 2004; Kokare et al., 2006; Lewis et al., 2005; Navarro et al., 2005; Rada et al., 2004; Thiele et al., 2004). In addition to replicating the effects of galanin, the present report expands this

area of research to include 2 other orexigenic peptides of current interest, orexin-A and ghrelin.

The peptides orexin-A and B (hypocretin 1 and 2) have been studied in relation to feeding behavior and drug abuse, but not in terms of their effects on ethanol consumption. In addition to stimulating food intake when injected into various hypothalamic regions as well as the nucleus accumbens (NAc) (Dube et al., 1999; Thorpe and Kotz, 2005), they have also been implicated in a panoply of other behavioral phenomena, including arousal, narcolepsy, nonopioid analgesia, spatial memory, and reward (Borgland et al., 2006; Gershchenko et al., 2001; Hara et al., 2001; Harris et al., 2005; de Lecea et al., 1998; Narita et al., 2006; Sakurai et al., 1998; Yamamoto et al., 2002). A possible involvement of the orexin system in ethanol intake has recently been suggested by results obtained with systemic administration of an orexin-1 receptor antagonist (Lawrence et al., 2006). To provide more specific information on the anatomical site of the orexins' stimulation of ethanol intake, the present study examined the effects of orexin injections directly into the hypothalamus and the accumbens.

The rationale for testing the effects of orexin on ethanol intake also stems from previous work with the peptide galanin. Both galanin and orexin belong to a class of orexigenic peptides whose mRNA expression in the hypothalamus is stimulated by consumption of a high-fat diet (Leibowitz,

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2005; Wortley et al., 2003). Galanin and orexin mRNA levels also increase in response to ethanol intake (Leibowitz et al., 2003). In rats voluntarily drinking 4 to 7% ethanol, galanin injection in the lateral ventricles or directly into the hypothalamic paraventricular nucleus (PVN) increases voluntary ethanol intake in the presence as well as absence of food and water (Lewis et al., 2004; Rada et al., 2004). Interestingly, in contrast to its orexigenic effect in naïve rats, galanin injection in these ethanol-drinking rats has no significant effect on food and water intake, suggesting that its involvement in food intake may be altered by the experience of drinking ethanol. If there is a general principle that fat-stimulated peptides take on the role of inducing an appetite for ethanol in ethanol-experienced rats, then intracranial injections of orexin should also elicit the consumption of ethanol, perhaps to a greater extent than food intake.

For comparison, ghrelin, an orexigenic peptide of a different sort, was also tested. This peptide, synthesized in the gastrointestinal tract as well as in neurons in and around the arcuate nucleus of the hypothalamus (Date et al., 2000), is an endogenous ligand for the growth hormone secretagogue receptor found throughout the hypothalamus (Guan et al., 1997). It has been shown to elicit food intake when microinjected into various hypothalamic areas as well as the NAc (Naleid et al., 2005; Wren et al., 2001), and it is suggested to act in part through NPY neurons in the hypothalamic arcuate nucleus (Asakawa et al., 2005; Cowley et al., 2003; Luquet et al., 2006). Little is known about the effects of this peptide on ethanol intake or the neural mechanisms through which it operates. Studies in human alcoholics show that plasma ghrelin levels are altered and positively correlated with subjective ethanol craving (Addolorato et al., 2006; Kraus et al., 2005).

## MATERIAL AND METHODS

### *Subjects and Home Cage Conditions*

Subjects were male Sprague–Dawley rats from Taconic Farms (Germantown, NY) that weighed between 100 and 150 g at the start of the experiment. Subjects were individually housed in hanging wire cages. They were maintained on a 12:12 hour light–dark cycle with lights off at 6:00 AM. The subjects had access to LabDiet rodent chow (St Louis, MO) on an *ad libitum* basis. Water was always available, either through an automated system when the animals did not have access to ethanol or through graduated cylinders attached to the cages when ethanol was present. All procedures used in these studies were approved by the Princeton University Institutional Animal Care and Use Committee.

### *Acquisition of Oral Ethanol Intake*

Subjects were acclimated to ethanol gradually, using a variant of the 2-bottle choice procedure (Martinetti et al., 2000), including periodic deprivation (weekends off). Briefly, subjects were exposed to unsweetened ethanol and water in glass bottles *ad libitum* for 5 days. The ethanol was then removed, and for the following 2 days they received water through the automated system. Each week the concentration of ethanol was increased in the following manner: 1%, 2%, 4%, 7%, 9%, and 10% (v/v). All surgeries were performed during the fifth week when the subjects were drinking 9% ethanol. The animals' first week on 10% ethanol was also their week of recovery from

surgery. Thus, microinjections began the second week of 10% ethanol exposure. To assess baseline drinking levels induced by gradual acclimation and partial deprivation of ethanol, the daily intake of 10% ethanol was measured in 30 rats over a 5-week period. During this time, the average daily intake in these animals was 1.68 g/kg/d (SEM = 0.11). Individual ethanol intake levels were averaged across each week and ranged from 0 to 4.79 g/kg/d. Because of the variability of ethanol intake both between animals and across weeks in individual animals, the effects elicited by each peptide were compared to vehicle injections on counterbalanced consecutive days and not to other peptides presented in subsequent weeks.

### *Surgery*

Subjects were anesthetized with a combination of ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), supplemented with ketamine when necessary. Guide shafts (21-gauge stainless steel, 10 mm in length) were implanted in the PVN (B -1.8, L 0.3, V 3.2), lateral hypothalamus (LH) (B -3.0, L 1.4, V 4.5), NAc (B 1.2, L 0.8, V 4.5) with reference to bregma, the midsagittal sinus, and the level skull surface. Guide shafts aimed at the PVN and LH were implanted unilaterally, and for the NAc, bilaterally in case needed. Injectors were inserted through the guide shafts at a later date to reach the injection sites specified below.

### *Microinjection Procedures*

All solutions were delivered through concentric microinjectors made of 26 gauge stainless steel outside and fused-silica tubing inside that protruded 2.5 mm (3.0 mm in the case of the LH; 74  $\mu$ m inner diameter (ID), 154  $\mu$ m outer diameter (OD); Polymicro Technologies, Phoenix, AZ). The injector tip protruded beyond the implanted guide shaft to reach the dorsal surface of the region of interest (PVN: V 7.7, LH: V 7.5, NAc: V 9.0). Galanin (rat) 1 nmol, orexin-A (human, rat, mouse) 0.9 nmol, and ghrelin (rat, *n*-octanoylated at Ser<sup>3</sup>) 1 nmol were purchased from American Peptide Co., Inc. (Sunnyvale, CA). Drugs were dissolved in Ringer solution and prepared fresh immediately prior to microinjection. Injections were counterbalanced, so each animal received Ringer solution or peptide in randomized order on 2 days consecutively. Injections were given 4 to 5 hours in the dark cycle, and a week elapsed between each pair of injections. All injections were made using a syringe pump to infuse 0.5  $\mu$ l during 47 seconds at a flow rate of 0.6  $\mu$ l/min, with the microinjector remaining in place for another 47 seconds prior to removal to allow diffusion. The cannula size and flow rate are comparable to those published in recent literature (Amat et al., 2005; Delfs et al., 2000; Johansen and Fields, 2004). Ethanol, food, and water were weighed at 1, 2, and 4 hour intervals following injection by an experimenter blind to condition. Rats included in the PVN experiment were habituated to the injection procedure by receiving injections at least a week earlier in addition to those specified above. While rats with injectors in the LH and NAc were not habituated to the injection procedure, counterbalanced controls showed a lack of confounding effects, as described in the Results section. In all cases a week elapsed between peptide injections, to avoid interactions. Previous studies using consecutive injections of galanin and orexin spaced 24 hours apart, at doses similar to those used here, have not shown an effect of day (Rada et al., 2004; Kiwaki et al., 2004).

### *Histology and Data Analysis*

Injection sites were verified by injecting 0.5  $\mu$ l methylene blue dye (Sigma, St Louis, MO) prior to cardiac perfusion. Animals were perfused with normal saline followed by formalin. Brains were sliced in 200  $\mu$ m sections on a freezing microtome. Behavioral data from animals with injector tips in or directly dorsal to the region of interest

were included in the analysis for that region; those with probes farther from the target regions were discarded from the analysis. Ethanol, food, and water intake data were each analyzed separately using two-way repeated measures ANOVA, with 2 levels of treatment (peptide, vehicle) and time (3 levels: 1, 2, and 4 hours) as the independent variables. The effects of each peptide at each time point in the experiments were analyzed using Bonferroni-corrected pairwise comparisons, with the familywise  $\alpha$  set at 0.05, making  $p < 0.017$  for each of 3 pairwise comparisons per experiment. Partial  $\eta^2$  was used as a measure of effect size. The data for every peptide injection in each brain region at 1, 2, and 4 hours postinjection is shown in Table 1.

**Table 1.** Total Ethanol, Food and Water Intake Measured at 1, 2, and 4 hours.<sup>a</sup>

	Hour 1	Hour 2	Hour 4
PVN orexin			
Ethanol			
Orexin	0.642 ± 0.12*	0.933 ± 0.11*	1.221 ± 0.16
Vehicle	0.243 ± 0.07	0.496 ± 0.14	1.097 ± 0.21
Food			
Orexin	2.250 ± 0.50	4.675 ± 0.68	6.533 ± 0.67
Vehicle	1.717 ± 0.33	4.200 ± 0.63	7.683 ± 1.02
Water			
Orexin	0.858 ± 0.17	2.058 ± 0.50	3.025 ± 0.54
Vehicle	1.758 ± 0.40	2.308 ± 0.46	3.725 ± 0.65
PVN galanin			
Ethanol			
Galanin	0.161 ± 0.05*	0.357 ± 0.09*	0.519 ± 0.13
Vehicle	0.000 ± 0.00	0.097 ± 0.06	0.257 ± 0.10
Food			
Galanin	1.267 ± 0.20	3.544 ± 0.46	4.967 ± 0.43
Vehicle	0.689 ± 0.37	2.078 ± 0.76	5.189 ± 0.74
Water			
Galanin	1.667 ± 0.37	3.333 ± 0.99	3.889 ± 1.26
Vehicle	0.667 ± 0.29	1.778 ± 0.66	3.00 ± 1.09
PVN ghrelin			
Ethanol			
Ghrelin	0.212 ± 0.04	0.343 ± 0.05	0.545 ± 0.09
Vehicle	0.124 ± 0.04	0.325 ± 0.07	0.561 ± 0.12
Food			
Ghrelin	5.220 ± 0.83*	8.670 ± 0.95*	10.880 ± 0.66*
Vehicle	1.790 ± 0.72	3.720 ± 0.63	6.600 ± 0.67
Water			
Ghrelin	4.370 ± 1.15	8.080 ± 1.05*	9.850 ± 1.13*
Vehicle	2.120 ± 0.61	3.590 ± 0.82	5.730 ± 1.32
LH orexin			
Ethanol			
Orexin	0.367 ± 0.11*	0.673 ± 0.14*	1.185 ± 0.29
Vehicle	0.104 ± 0.02	0.261 ± 0.05	0.888 ± 0.25
Food			
Orexin	1.733 ± 0.73	4.733 ± 1.03	8.350 ± 0.52
Vehicle	1.283 ± 0.62	3.100 ± 1.12	6.983 ± 0.71
Water			
Orexin	2.788 ± 1.41	4.750 ± 2.01	7.100 ± 2.32
Vehicle	3.113 ± 1.10	5.038 ± 1.49	8.425 ± 2.39
LH galanin			
Ethanol			
Galanin	1.200 ± 0.47	2.229 ± 0.58	4.043 ± 1.04
Vehicle	0.914 ± 0.20	2.200 ± 0.43	3.286 ± 0.72
Food			
Galanin	1.729 ± 0.50	4.586 ± 0.61	8.414 ± 0.93
Vehicle	1.586 ± 0.58	3.457 ± 0.52	7.014 ± 1.19
Water			
Galanin	3.429 ± 0.57	6.086 ± 1.12	9.400 ± 1.54
Vehicle	2.286 ± 0.28	3.271 ± 0.46	7.057 ± 1.42
LH ghrelin			
Ethanol			
Ghrelin	0.096 ± 0.03	0.280 ± 0.07	0.517 ± 0.14
Vehicle	0.162 ± 0.05	0.285 ± 0.07	0.482 ± 0.08

**Table 1.** Continued

	Hour 1	Hour 2	Hour 4
Food			
Ghrelin	5.314 ± 1.27*	8.386 ± 1.60*	12.086 ± 1.91*
Vehicle	3.000 ± 0.82	4.66 ± 1.01	9.929 ± 1.57
Water			
Ghrelin	2.286 ± 0.28	3.271 ± 0.46	7.057 ± 1.42
Vehicle	4.429 ± 0.57	6.086 ± 1.12	9.400 ± 1.54
NAC orexin			
Ethanol			
Orexin	0.073 ± 0.02	0.205 ± 0.07	NA
Vehicle	0.113 ± 0.23	0.234 ± 0.05	NA
Food			
Orexin	2.770 ± 0.56	5.150 ± 0.85	NA
Vehicle	1.410 ± 0.43	2.580 ± 0.69	NA
Water			
Orexin	1.380 ± 0.45	3.820 ± 1.17	NA
Vehicle	1.450 ± 0.44	3.640 ± 1.18	NA

PVN, paraventricular nucleus; LH, lateral hypothalamus; NAC, nucleus accumbens.

<sup>a</sup>Shows all data for every peptide injection. Data are expressed as mean ± SEM.

\*Denotes a significant difference compared to the vehicle condition, after Bonferroni-correction ( $p < 0.017$ ).

## RESULTS

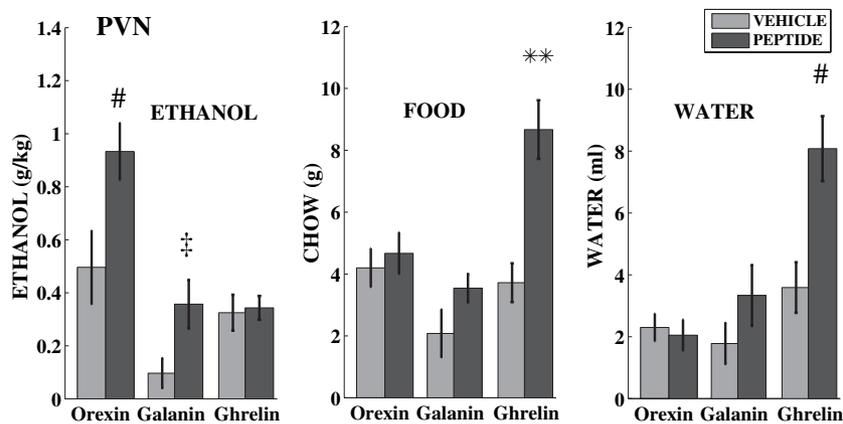
### Experiment 1: Peptide Microinjections in the PVN

When injected into the PVN, orexin-A and galanin increased ethanol intake, compared to vehicle control, whereas ghrelin's main effect was on food intake (Fig. 1). All rats in this experiment had their microinjector tips in or along the dorsal surface of the PVN, as shown in Fig. 2.

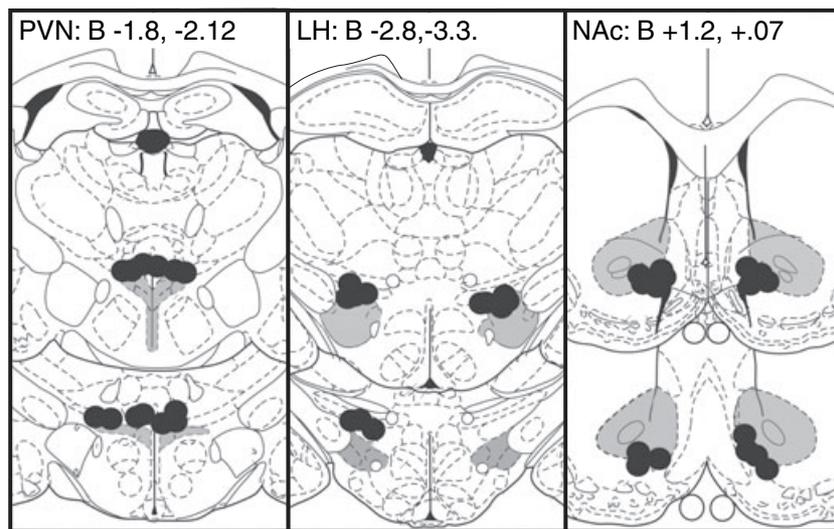
Orexin-A (0.9 nmol) increased voluntary intake of 10% ethanol during the 4 hours following injection [ $F(1,11) = 14.588$ ,  $p < 0.01$ , partial  $\eta^2 = 0.57$ ]. Post hoc pairwise comparisons determined that the means of the orexin and vehicle groups significantly differed from each other during the first and second hours, but not the fourth. Orexin-A had no significant effect on food and water intake [food:  $F(1,11) = 0.014$  (NS), partial  $\eta^2 = 0.001$ ; water:  $F(1,11) = 2.151$  (NS), partial  $\eta^2 = 0.164$ ].

Galanin (1 nmol) also increased voluntary intake of 10% ethanol during the 4 hours following injection [ $F(1,8) = 10.553$ ,  $p < 0.05$ , partial  $\eta^2 = 0.569$ ], but only the difference between the 2 means during the first hour was significant. Food intake was not significantly altered [ $F(1,8) = 0.803$  (NS), partial  $\eta^2 = 0.099$ ]. Though water intake tended to be greater when rats were injected with galanin than with vehicle, this result was not statistically significant [ $F(1,8) = 4.444$ ,  $p = 0.068$ , partial  $\eta^2 = 0.357$ ]. It is worth noting that the baseline drinking level during the first hour was lower in this experiment. However, the use of a counterbalanced design prevents this from being a confound.

Ghrelin, on the other hand, significantly increased food intake [ $F(2,9) = 31.647$ ,  $p < 0.001$ , partial  $\eta^2 = 0.779$ ] and water intake [ $F(2,9) = 28.31$ ,  $p < 0.001$ , partial



**Fig. 1.** Cumulative ethanol, food, and water consumption 2 hours after injection of orexin (0.9 nmol), galanin (1 nmol), or ghrelin (1 nmol) in the PVN (mean ± SEM, #*p* < 0.001, \*\**p* < 0.01, ‡*p* < 0.05 for main effect, but not at the second hour).



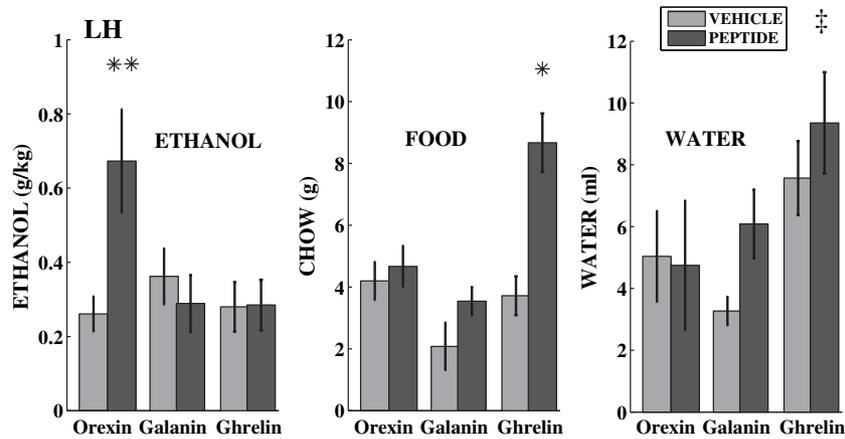
**Fig. 2.** Black dots show the injection site in the paraventricular nucleus, lateral hypothalamus, and nucleus accumbens for all animals used in the experiments. Gray shaded areas indicate the region of interest. Sections are labeled according to the distance from Bregma (B) along the rostral-caudal axis (adapted from *The Rat Brain*, compact 3rd edition, G. Paxinos and C. Watson, Figs. 13, 15, 25 26, 29 and 31, Copyright 1997, with permission from Elsevier.)

$\eta^2 = 0.759$ ]. Post hoc pairwise comparisons revealed that at each time point the mean food intake of ghrelin-injected rats was significantly higher than vehicle controls. Water intake was also significantly greater during the second and fourth hour, but not during the first hour ( $p = 0.018$ ), suggesting that this effect was postprandial. In contrast to food and water intake, ghrelin at this dosage did not produce a significant main effect on ethanol intake [ $F(1,9) = 0.239$  (NS), partial  $\eta^2 = 0.026$ ]. Although the small change in ethanol intake during the first hour would be significant if analyzed alone by paired *t*-test or one-way repeated measures ANOVA, it was not statistically significant when subjected to the same stringent conditions of analysis as the other peptides. The tendency to drink ethanol was transient and much smaller than the highly significant food and water effects, suggesting that it may reflect a general

increase in consummatory behavior rather than a specific effect on ethanol intake.

*Experiment 2: Peptide Microinjections in the LH*

In the LH, orexin-A once again increased ethanol whereas galanin did not, and ghrelin stimulated feeding (Fig. 3). The most effective sites in the LH for orexin-induced ethanol intake were generally dorsal and lateral to the fornix (Fig. 2). As the rats with cannulas in the LH and NAc were not habituated to the injection procedure, the ethanol intakes on the first and second days of injection of peptide and saline in counterbalanced order were statistically compared. With no effect of order in either the LH [ $F(1,6) = 1.9$  (NS)] or NAc [ $F(1,6) = 0.6$  (NS)], it is concluded that habituation is not a factor.



**Fig. 3.** Cumulative ethanol, food, and water consumption 2 hours after injection of orexin (0.9 nmol), galanin (1 nmol), or ghrelin (1 nmol) in the PVN (mean  $\pm$  SEM, \*\* $p < 0.01$ , \* $p < 0.05$ , † $p < 0.05$  for main effect, but not at the second hour).

Orexin-A (0.9 nmol) in the LH increased voluntary intake of 10% ethanol for 2 hours following injection [ $F(1,5) = 8.012$ ,  $p < 0.037$ , partial  $\eta^2 = 0.616$ ]. Post hoc analysis revealed that the rats drank significantly more during the first 2 hours following orexin-A injection but not during the fourth hour ( $p = 0.082$ ). In contrast, orexin-A had no significant effect on food and water intake compared to vehicle control [food:  $F(1,5) = 3.949$  (NS), partial  $\eta^2 = 0.441$ ; water:  $F(1,5) = 0.212$  (NS), partial  $\eta^2 = 0.029$ ].

Galanin (1 nmol) in the LH had no significant effect on ethanol, food, or water intake at any time point tested [ethanol:  $F(1,6) = 0.703$  (NS), partial  $\eta^2 = 0.105$ ; food:  $F(1,6) = 1.004$  (NS), partial  $\eta^2 = 0.143$ ; water:  $F(1,6) = 0.3.113$  (NS), partial  $\eta^2 = 0.342$ ].

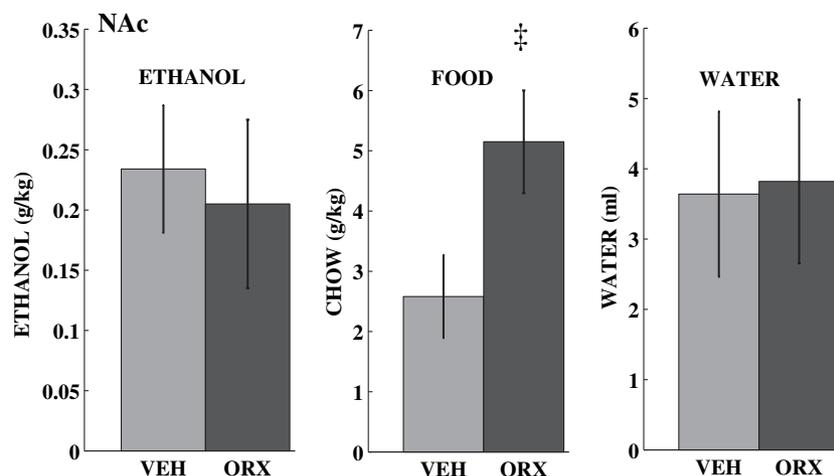
Ghrelin (1 nmol) significantly increased food [ $F(1,6) = 9.754$ ,  $p < 0.05$ , partial  $\eta^2 = 0.858$ ] and water [ $F(1,6) = 6.123$ ,  $p < 0.05$ , partial  $\eta^2 = 0.505$ ] intake during the 4 hours following injection. However, post hoc analyses showed the water effect did not achieve statistical significance

at any time point. Ghrelin at this dosage in the LH had no significant effect on ethanol intake.

#### Experiment 3: Peptide Microinjections in the NAc

Behavioral effects elicited by peptide injections in the NAc were quite different from those observed following injections in either hypothalamic region. Here orexin-A increased food intake rather than ethanol intake (Fig. 4). Galanin and ghrelin were also tested in a small subset of animals in this experiment. Neither peptide in these small groups produced any significant change in ethanol, food, or water intake; thus further animals were not tested. Histology shows the injectors for all animals in the experiment to be located in the shell of the NAc (Fig. 2).

Orexin-A (0.9 nmol) in the NAc increased food intake during the 4 hours following injection [ $F(1,9) = 6.552$ ,  $p < 0.05$ , partial  $\eta^2 = 0.421$ ]. However, no individual hour



**Fig. 4.** Cumulative ethanol, food, and water consumption 2 hours after the administration of orexin (0.9 nmol) in the NAc (mean  $\pm$  SEM, † $p < 0.05$  for main effect, but not at the second hour).

was significant using our stringent post hoc criterion ( $p = 0.038$  for both). There was no significant effect on ethanol [ $F(1,9) = 1.787$ , *ns*, partial  $\eta^2 = 0.421$ ] or water [ $F(1,9) = 0.243$ , *ns*, partial  $\eta^2 = 0.026$ ] consumption at any time point tested.

Galanin did not produce a significant increase in ethanol intake [ $F(1,2) = 0.998$ ,  $p = 0.391$ , partial  $\eta^2 = 0.25$ ] when injected into the NAc. It also did not significantly alter food [ $F(1,2) = 0.002$ ,  $p < 0.968$ , partial  $\eta^2 = 0.001$ ] or water intake [ $F(1,2) = 4.041$ ,  $p < 0.138$ , partial  $\eta^2 = 0.574$ ] during the 4 hours following injection.

Ghrelin did not significantly alter ethanol [ $F(1,3) = 1.495$ ,  $p = 0.346$ , partial  $\eta^2 = 0.428$ ], food [ $F(1,3) = 0.478$ ,  $p = 0.561$ , partial  $\eta^2 = 0.193$ ], or water intake [ $F(1,3) = 5.617$ ,  $p = 0.141$ , partial  $\eta^2 = 0.787$ ] during the 4 hours after the injection.

## DISCUSSION

The main new finding of this study is that orexin-A injected in the PVN and LH stimulates voluntary ethanol intake without significantly altering food and water intake. A similar behavioral effect was observed with galanin in the PVN, commensurate with previously published results with this peptide (Lewis et al., 2004, 2005; Rada et al., 2004). Thus, 2 peptides known to increase feeding behavior after PVN injection (Dube et al., 1999; Kyrkouli et al., 1990) were found here to stimulate the consumption of ethanol. An additional finding of particular interest is that, in contrast to naive rats, these peptides in rats trained to voluntarily drink ethanol had no effect on food intake. This suggests that chronic availability and consumption of ethanol masks the feeding-stimulatory effects of these peptides and/or supplants food as the preferred substance for consummatory behavior induced by PVN peptide injections. The mechanism for this is unknown but could well be related to the unusual positive feedback provided by ethanol or the lack of negative feedback in terms of minimal satiety factors produced by ethanol in comparison to other foods.

Orexin-A injected in the LH also increased voluntary ethanol intake, and in contrast to naive rats (Dube et al., 1999), it had no effect on food or water intake in ethanol-drinking rats, similar to the pattern of results in the PVN. The effective sites within the LH for orexin-induced ethanol intake were found to be lateral to the fornix. Interestingly, this site is similar to that found to be most effective for reinstating morphine-conditioned place preference with the Y4 agonist rat pancreatic polypeptide that activates orexin neurons (Harris et al., 2005). In contrast to orexin-A, the results obtained with galanin failed to show an effect on ethanol or food intake with LH injection. This result reveals greater site-specificity for the behavioral effects of galanin, consistent with a prior study of feeding behavior (Kyrkouli et al., 1990).

The similar magnitude, duration, and PVN locus suggests that the effects of orexin and galanin on ethanol intake may be mediated by a common mechanism, as yet to be deter-

mined. It is noteworthy that endogenous orexin and galanin are both regulated in part by diet. The RNA synthesis of these peptides is increased by fat that is eaten or systemically injected, and this effect is observed primarily in the PVN in the case of galanin and the LH in the case of orexin (Leibowitz, 2005; Leibowitz et al., 1998; Wortley et al., 2003). Galanin mRNA in the PVN is also stimulated by the consumption or injection of ethanol (Leibowitz et al., 2003). Moreover, orexin- and galanin-induced feeding responses are larger on high-fat compared to low-fat diet and in animals that show a strong preference for fat (Leibowitz, 2005). Thus, it is proposed that these peptides normally function within a positive feedback loop to stimulate consumption of a fat-rich diet. The present finding that orexin stimulates ethanol intake, together with evidence that ethanol stimulates orexin mRNA in the LH (Wortley et al., 2003), suggests a similar positive feedback loop for this peptide in the control of ethanol consumption.

The results observed with injections of ghrelin provide a control for those obtained with orexin and galanin. Injection of ghrelin into the PVN and LH in ethanol-drinking rats increased both food and water intake, but did not significantly alter ethanol intake. Ghrelin is known to stimulate food intake when injected into the PVN of naïve rats (Wren et al., 2001), and the present results show that it continues to do so in ethanol-experienced animals. Although PVN injections of ghrelin produced a small, statistically insignificant increase in ethanol intake in the first hour after injection, this effect was overshadowed by the robust stimulation of food and water intake over the 4-hour period of measurement. This provides an interesting control, showing that this feeding-stimulatory peptide in ethanol-drinking rats does not subsequently switch to the induction of ethanol intake. At this point, it is not known whether ghrelin mRNA, like orexin and galanin, is stimulated by diet or ethanol. As ghrelin continues to increase food and water intake in the presence of ethanol, this suggests that its orexigenic action might occur through a different mechanism from that of orexin and galanin. This provides further support differentiating these peptides with respect to their effects on consummatory behavior.

The results of orexin injections in the NAc are in contrast with the findings in the PVN and LH. Galanin and ghrelin did not appear to increase ethanol intake in this region. In the NAc, orexin stimulated food intake in ethanol-drinking rats but had no effect on ethanol intake at any time-point tested. Orexin has previously been shown to increase food intake when injected into the NAc (Thorpe and Kotz, 2005). The present study confirmed this result and further demonstrated that, unlike the PVN and LH, this feeding-stimulatory effect of orexin in the NAc is not altered by chronic exposure to ethanol. Thus, endogenous orexin in the NAc may have a more resilient role in controlling the consumption of food.

It is worth noting that all significant effects described herein were obtained from a species of rat that was not bred to prefer ethanol and that received no ethanol training other than

being given voluntary access to increasing concentrations of unsweetened ethanol. Thus, their baseline intake was low on average, and their drinking patterns were in some cases unstable, commensurate with a model of ethanol intake or social drinking rather than alcoholism. This suggests that the stimulatory effects obtained with peptide microinjections were not contingent on baseline ethanol preference or stability of this preference, as all effects described were significant despite this low baseline and without eliminating low-responders. In previous studies from this laboratory using 4 and 7% ethanol, the effects of galanin were significant in rats drinking > 1.5 g/kg/d (Rada et al., 2004) or rats demonstrating a preference for ethanol (Lewis et al., 2004). Additionally, in prior studies with the model of voluntary alcohol intake used here, the levels of blood alcohol achieved ( $6.3 \pm 20$  mg/dl in low alcohol drinkers and  $17.3 \pm 28$  mg/dl in high alcohol drinkers) have been shown to have strong impact on peptide mRNA expression in the PVN and also to be related to the behavioral phenomenon of fat ingestion (Chang et al., 2007). The present findings with orexin and galanin suggest that these peptides can increase ethanol intake in the absence of a clear preference for high concentrations of ethanol, consonant with the idea of evoking ethanol intake through hypothalamic feeding mechanisms. The mechanism by which these peptides stimulate ethanol consumption is not likely to be an alteration in innate taste preference, as Sprague–Dawley rats find unflavored ethanol to be very unpalatable. It is more likely that these peptides stimulate ethanol intake through an acquired taste based on the pharmacological properties of ethanol. Previous findings show that injections of galanin in the PVN can increase dopamine release in the NAc (Rada et al., 1998), an effect we have also reported with an oral stimulus that has become a conditioned taste preference (Mark et al., 1994).

In summary, orexin-A and galanin injected in the hypothalamus can stimulate ethanol intake in rats trained to drink 10% ethanol. This effect was significant with injections into the PVN for galanin and into the PVN and LH for orexin. A very different result was obtained for ghrelin in the hypothalamus, which stimulated food intake in spite of ethanol training, and also different than the 3 peptides in the NAc, which did not increase ethanol intake. The finding that galanin and orexin in the hypothalamus fail to stimulate feeding in ethanol-drinking rats suggests that these peptide systems are susceptible to being co-opted by voluntary ethanol consumption. However, it is unlikely they would ever come to exclusively control ethanol consumption, as evidenced by the fact that rats injected with orexin or galanin continue to eat a normal amount of chow while they are drinking more alcohol. Rather, this may be part of the mechanism that explains why food and ethanol intake mutually reinforce each other. This may occur through the same mechanisms that normally respond to fat or ethanol intake by synthesizing more of the peptide and theoretically stimulating further consummatory behavior. While this positive feedback would normally be counteracted by fat-induced satiety factors, this may not occur in the case of ethanol. This new evidence supports a role

for orexin in ethanol intake and also for a possible feedback mechanism that fosters ethanol abuse.

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## REFERENCES

- 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 (2006) Relationship between ghrelin levels, alcohol craving, and nutritional status in current alcoholic patients. *Alcohol Clin Exp Res* 30:1933–1937.
- Amat J, Baratta MV, Paul E, Bland ST, Watkins LR, Maier SF (2005) Medial prefrontal cortex determines how stressor controllability affects behavior and dorsal raphe nucleus. *Nat Neurosci* 8:365–371.
- Asakawa A, Inui A, Fujimiya M, Sakamaki R, Shinfuku N, Ueta Y, Meguid MM, Kasuga M (2005) Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. *Gut* 54:18–24.
- Bodnar RJ, Klein GE (2005) Endogenous opiates and behavior: 2004. *Peptides* 26:2629–2711.
- Borgland SL, Taha SA, Sarti F, Fields HL, Bonci A (2006) Orexin A in the VTA is critical for the induction of synaptic plasticity and behavioral sensitization to cocaine. *Neuron* 49:589–601.
- Chang G-Q, Karatayev O, Ahsan R, Avena NM, Lee C, Lewis MJ, Hoebel BG, Leibowitz SF (2007) Effect of ethanol on hypothalamic opioid peptides, enkephalin, and dynorphin: relationship with circulating triglycerides. *Alcohol Clin Exp Res* 31:249–259.
- Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nilni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL (2003) The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 37:649–661.
- Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M (2000) Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 141:4255–4261.
- Delfs JM, Zhu Y, Druhan JP, Aston-Jones G (2000) Noradrenaline in the ventral forebrain is critical for opiate withdrawal-induced aversion. *Nature* 403:430–434.
- Dube MG, Kalra SP, Kalra PS (1999) Food intake elicited by central administration of orexins/hypocretins: identification of hypothalamic sites of action. *Brain Res* 842:473–477.
- Duncan EA, Proulx K, Woods SC (2005) Central administration of melanin-concentrating hormone increases alcohol and sucrose/quinine intake in rats. *Alcohol Clin Exp Res* 29:958–964.
- Gerashchenko D, Salin-Pascual R, Shiromani PJ (2001) Effects of hypocretin-1 injections into the medial septum on sleep and hippocampal theta. *Brain Res* 913:106–115.
- Gilpin NW, Stewart RB, Murphy JM, Badia-Elder NE (2004) Neuropeptide Y in the paraventricular nucleus of the hypothalamus increases ethanol intake in high- and low-alcohol-drinking rats. *Alcohol Clin Exp Res* 28:1492–1498.
- Guan XM, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghi DJ, Smith RG, Van der Ploeg LH, Howard AD (1997) Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res Mol Brain Res* 48:23–29.
- Haddad JJ (2004) Alcoholism and neuro-immune-endocrine interactions: physiochemical aspects. *Biochem Biophys Res Commun* 323:361–371.
- Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, Sugiyama F, Yagami K, Goto K, Yanagisawa M, Sakurai T (2001) Genetic

- ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* 30:345–354.
- Harris GC, Wimmer M, Aston-Jones G (2005) A role for lateral hypothalamic orexin neurons in reward seeking. *Nature* 437:556–559.
- Johansen JP, Fields HL (2004) Glutamatergic activation of anterior cingulate cortex produces an aversive teaching signal. *Nat Neurosci* 7:398–403.
- Kiwaki K, Kotz CM, Wang C, Lanningham-Foster L, Levine JA (2004) Orexin A (hypocretin 1) injected into hypothalamic paraventricular nucleus and spontaneous physical activity in rats. *Am J Physiol Endocrinol Metab* 286:E551–E559.
- Kokare DM, Chopde CT, Subhedar NK (2006) Participation of alpha-melanocyte stimulating hormone in ethanol-induced anxiolysis and withdrawal anxiety in rats. *Neuropharmacology* 51:536–545.
- Kraus T, Schanze A, Groschl M, Bayerlein K, Hillemecher T, Reulbach U, Kornhuber J, Bleich S (2005) Ghrelin levels are increased in alcoholism. *Alcohol Clin Exp Res* 29:2154–2157.
- Kyrkouli SE, Stanley BG, Seirafi RD, Leibowitz SF (1990) Stimulation of feeding by galanin: anatomical localization and behavioral specificity of this peptide's effects in the brain. *Peptides* 11:995–1001.
- Lawrence AJ, Cowen MS, Yang HJ, Chen F, Oldfield B (2006) The orexin system regulates alcohol-seeking in rats. *Br J Pharmacol* 148:752–759.
- de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS II, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci USA* 95:322–327.
- Leibowitz SF (2005) Regulation and effects of hypothalamic galanin: relation to dietary fat, alcohol ingestion, circulating lipids and energy homeostasis. *Neuropeptides* 39:327–332.
- Leibowitz SF, Akabayashi A, Wang J (1998) Obesity on a high-fat diet: role of hypothalamic galanin in neurons of the anterior paraventricular nucleus projecting to the median eminence. *J Neurosci* 18:2709–2719.
- Leibowitz SF, Avena NM, Chang GQ, Karatayev O, Chau DT, Hoebel BG (2003) Ethanol intake increases galanin mRNA in the hypothalamus and withdrawal decreases it. *Physiol Behav* 79:103–111.
- Lewis MJ, Johnson DF, Waldman D, Leibowitz SF, Hoebel BG (2004) Galanin microinjection in the third ventricle increases voluntary ethanol intake. *Alcohol Clin Exp Res* 28:1822–1828.
- Lewis MJ, Rada P, Johnson DF, Avena NM, Leibowitz SF, Hoebel BG (2005) Galanin and alcohol dependence: neurobehavioral research. *Neuropeptides* 39:317–321.
- Luquet S, Phillips CT, Palmiter RD (2007) NPY/AgRP neurons are not essential for feeding responses to glucoprivation. *Peptides* 28:214–225.
- Mark GP, Smith SE, Rada PV, Hoebel BG (1994) An appetitively conditioned taste elicits a preferential increase in mesolimbic dopamine release. *Pharmacol Biochem Behav* 48:651–660.
- Martinetti MP, Andrzejewski ME, Himeline PN, Lewis MJ (2000) Ethanol consumption and the matching law: a choice analysis using a limited-access paradigm. *Exp Clin Psychopharmacol* 8:395–403.
- Naleid AM, Grace MK, Cummings DE, Levine AS (2005) Ghrelin induces feeding in the mesolimbic reward pathway between the ventral tegmental area and the nucleus accumbens. *Peptides* 26:2274–2279.
- Narita M, Nagumo Y, Hashimoto S, Narita M, Khotib J, Miyatake M, Sakurai T, Yanagisawa M, Nakamachi T, Shioda S, Suzuki T (2006) Direct involvement of orexinergic systems in the activation of the mesolimbic dopamine pathway and related behaviors induced by morphine. *J Neurosci* 26:398–405.
- Navarro M, Cubero I, Chen AS, Chen HY, Knapp DJ, Breese GR, Marsh DJ, Thiele TE (2005) Effects of melanocortin receptor activation and blockade on ethanol intake: a possible role for the melanocortin-4 receptor. *Alcohol Clin Exp Res* 29:949–957.
- Rada P, Avena NM, Leibowitz SF, Hoebel BG (2004) Ethanol intake is increased by injection of galanin in the paraventricular nucleus and reduced by a galanin antagonist. *Alcohol* 33:91–97.
- Rada P, Mark GP, Hoebel BG (1998) Galanin in the hypothalamus raises dopamine and lowers acetylcholine release in the nucleus accumbens: a possible mechanism for hypothalamic initiation of feeding behavior. *Brain Res* 798:1–6.
- Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92:573–585.
- Thiele TE, Sparta DR, Hayes DM, Fee JR (2004) A role for neuropeptide Y in neurobiological responses to ethanol and drugs of abuse. *Neuropeptides* 38:235–243.
- Thorpe AJ, Kotz CM (2005) Orexin A in the nucleus accumbens stimulates feeding and locomotor activity. *Brain Res* 1050:156–162.
- Wortley KE, Chang GQ, Davydova Z, Leibowitz SF (2003) Peptides that regulate food intake: orexin gene expression is increased during states of hypertriglyceridemia. *Am J Physiol Regul Integr Comp Physiol* 284:R1454–R1465.
- Wren AM, Small CJ, Abbott CR, Dhillon WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA, Bloom SR (2001) Ghrelin causes hyperphagia and obesity in rats. *Diabetes* 50:2540–2547.
- Yamamoto T, Nozaki-Taguchi N, Chiba T (2002) Analgesic effect of intrathecally administered orexin-A in the rat formalin test and in the rat hot plate test. *Br J Pharmacol* 137:170–176.