

# Association of Plasma Orexin A and Ethanol-Drinking Behaviors in Pregnant Rats

Kristen A Schmidt<sup>1#</sup>, Ada M-C Ho<sup>1,2#</sup>, Mark A Frye<sup>1</sup> and Doo-Sup Choi<sup>1,2\*</sup>

<sup>1</sup>Department of Psychiatry and Psychology, Mayo Clinic, Rochester, MN 55905, USA

<sup>2</sup>Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic College of Medicine, Rochester, MN 55905, USA

#Authors contributed equally to the manuscript.

\*Corresponding author: Doo-Sup Choi, Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, 200 First Street, Rochester, MN 55905, USA, Tel: +1-507-284-5602; Fax: +1-507-284-1767; E-mail: [choids@mayo.edu](mailto:choids@mayo.edu)

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## Abstract

**Aims:** A sharp reduction in gestational alcohol preference has been observed in both humans and animals. This study investigated the specific neurobiological relationship between alcohol use, pregnancy and plasma orexin A.

**Methods:** Adult female Wistar rats (n=15) were subjected to a two-bottle choice drinking paradigm (5% ethanol vs. tap water with measurement of liquid weight consumed and recorded daily) for 3 weeks prior to random assignment to mating group (n=8) vs. virgin group (n=7). Ethanol exposure was immediately resumed in the virgin group and following sperm plug observation in the mating group. Tail vein blood was collected during pre-ethanol exposure, pre-mating, and pregnancy days 12 and 19 for orexin A measurement by ELISA.

**Results:** There was no significant difference in serum orexin A levels before or after alcohol exposure. A significant reduction (p<0.05) in alcohol preference was demonstrated in pregnant rats compared to virgin rats. In the pregnant group, there was a significant positive correlation between the percentage change in plasma orexin A level and alcohol preference (Pearson's r = 0.804, p<0.05) and consumption (Pearson's r = 0.800, p<0.05).

**Conclusions:** These data suggest that pregnant rats have a significant reduction in alcohol preference compared to virgin rats exposed to alcohol for the same duration, substantiating a biological basis for diminished gestational alcohol intake. Plasma orexin A concentration during pregnancy may be associated with gestational alcohol intake.

**Short summary:** Our results demonstrate that pregnant rats have a significant reduction in gestational alcohol preference compared to virgin rats, supporting a biological basis underlying gestational alcohol reduction. The observed significant positive correlation between gestational changes in alcohol consumption, preference and circulatory orexin A concentration suggests a relationship between orexin A and gestational alcohol intake.

**Keywords:** Orexin; Ethanol; Pregnant; Rats; Alcohol

## Introduction

Over the last decade, alcohol use disorders (AUD) among women have sharply increased [1]. Women develop medical complications earlier in their course of drinking and at lower drinking thresholds compared to men [2]. Pregnancy appears to represent a unique time where even women having severe AUD exhibit reduced craving and in some cases maintain complete abstinence during pregnancy [3,4]. While it is easy to ascribe this to media and physician messaging related to the dangers of alcohol use, several studies have shown a reduced alcohol preference during pregnancy in alcohol drinking rats [5,6], macaque monkeys [7] and even in alcohol-preferring mice [8].

Orexin is one of the hormones implicated in modulating alcohol consumption patterns during pregnancy. Of note, the hypothalamic orexin expression is significantly decreased during pregnancy and lactation [9]. As orexin plays an essential role in regulating energy expenditure and reward circuitry activity, reduced orexin may be causally related with reduced alcohol drinking during pregnancy [10].

Consistently, orexin stimulates ethanol self-administration in rats [11], while the orexin 1 receptor antagonist attenuates drinking behavior [12].

Orexin is a neuropeptide hormone released from lateral hypothalamic neurons that have numerous projections [10,12], which include the adrenergic locus coeruleus, serotonergic raphe nuclei, histaminergic tuberomammillary nucleus, dopaminergic substantia nigra and ventral tegmental area [10,13,14] as well as the area postrema containing the so-called chemoreceptor trigger zone speculated to be involved with nausea and appetite aversion during pregnancy [15].

To our knowledge, however, there have not been any studies evaluating the relationship between alcohol consumption and plasma orexin A levels during pregnancy. This project will therefore be a first attempt to establish orexin A's role in gestational alcohol consumption. Our study provides a potential association of pregnancy with reduced alcohol preference in the rat along with a deficit of plasma orexin levels.

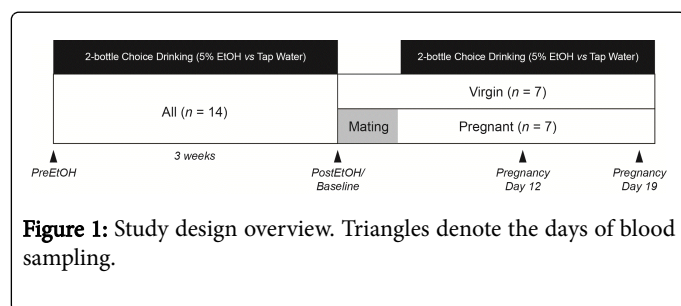
## Materials and Methods

### Animals

Twenty-four adult virgin female Wistar rats (200-230 g) were obtained from Charles Rivers Laboratory (Wilmington, MA, USA). They were paired housed and were maintained under standard animal house conditions with food and water access ad libitum (12 h light/dark cycle; light on at 6 AM;  $21 \pm 1^\circ\text{C}$ ) for a week before being separated into individual housing for the rest of the experiment. Eight rats were used for determining potential diurnal and estrous-related fluctuations in plasma orexin A levels (see Supplementary Materials); the rest were used for ethanol drinking during pregnancy investigation. Animal care and handling procedures were approved by Mayo Clinic Institutional Animal Care and Use Committee in accordance with NIH guidelines.

### Study of correlation between plasma orexin A level and ethanol consumption during pregnancy

The study design overview is presented in Figure 1. Sixteen female rats were initially allocated for this study and one was sacrificed before the experiment began due to abnormal distress behaviors of unknown cause. A baseline tail vein blood sample (200  $\mu\text{L}$ ) was collected between 11 AM and 1 PM. This time point represents a basal orexin A level as the rats were at a resting state. While previous reports have suggested diurnal and estrous-related fluctuations in central orexin A levels [16-18], we did not observe significant diurnal changes nor differences among estrous stages (see Supplementary Materials). Blood samples were collected under 2% isoflurane anesthesia. Tail vein blood was drawn and collected in tubes coated with anticoagulant EDTA (1.8 mg/mL of blood) and protease inhibitor aprotinin (0.6 TIU/mL of blood) on ice. Samples were then centrifuged at 3,000 g for 10 min at  $4^\circ\text{C}$  within 15 min. Plasma samples were frozen immediately on dry ice and were stored at  $-20^\circ\text{C}$  until analysis.



For three weeks, rats were subjected to continuous access of a two-bottle choice ethanol drinking paradigm, during which they were provided with a bottle of 5% (v/v) ethanol in tap water and a bottle of tap water for 24 h daily. This paradigm attempts to model free-access ethanol consumption in non-dependent individuals instead of maladaptive drinking behaviors. The weight of liquid consumed in each bottle was recorded and vaginal lavage for estrous cycle determination was collected daily between 11 AM and 1 PM. All female rats exhibited a normal estrous cycle. A post-ethanol exposure tail vein blood sample (200  $\mu\text{L}$ ) was collected after three weeks of ethanol drinking.

Rats were randomly allocated to the pregnant group (n=8) or the virgin (virgin; n=7) group after matching for baseline ethanol consumption (average of the last five days). The pregnant group rats

were then paired housed with an adult male Wistar rat (~5 months old) for 3-5 days. Pregnancy day 1 (PD) was marked by the observation of a mating plug or sperm present in vaginal lavage. After mating with the male rat, the two-bottle choice drinking paradigm resumed until PD20. One rat from the pregnant group was excluded from the study since it did not get pregnant (n=7 remained in the pregnant group). Body weight and ethanol/tap water consumption were observed and recorded daily. A mid pregnancy tail blood sample (200  $\mu\text{L}$ ) was collected once on PD12, and a late pregnancy blood (200  $\mu\text{L}$ ) was sampled on PD19. Rats were then sacrificed on PD21. Drinking hiatus, tail blood sampling and sacrifice were day matched for virgin rats.

### Plasma orexin A quantification

Plasma orexin A was quantified by Orexin A Extraction Free EIA kit (Phoenix Pharmaceuticals, CA, USA) as per manufacturer's instructions. Plasma samples were thawed on ice then diluted 1:1 with diluent buffer as instructed before loading. Each sample was measured in duplicates. A standard curve was established using orexin A standards between 0.01 and 100 ng/mL. Inter-plate coefficient of variation (CV) was 3.59% based on three plates. Minimal detectable concentration was 0.1 ng/mL.

### Data analysis and statistics

Ethanol preference was calculated by dividing the weight of 5% ethanol consumed by the total weight of liquid consumed. Averages of every two consecutive days were first calculated for ethanol consumption, ethanol preference, water consumption and total liquid consumption to account for side preference. These averages were then compared between pregnancy groups and across time by two-way repeated measures ANOVA during the 3-week training period and during pregnancy independently. For the pregnancy analysis, baseline measurements of these parameters were calculated by averaging the last 5 days of the training period to account for potential estrous effects.

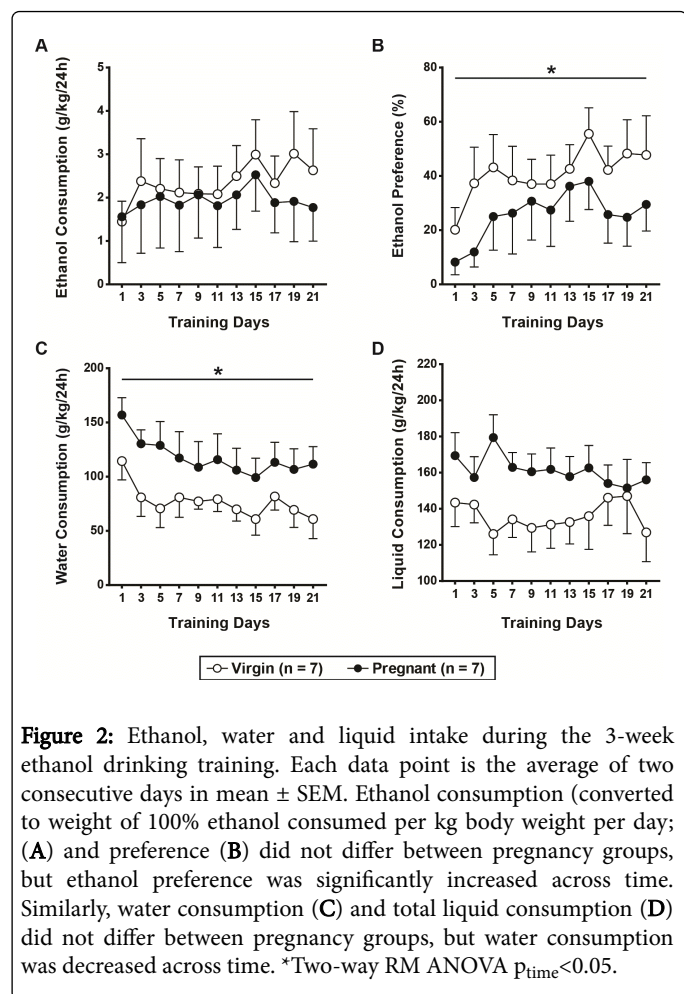
Plasma orexin A levels before and after the 3-week drinking training was compared by paired t test. To account for between-pregnant group differences in plasma orexin A level before ethanol exposure, percentage change compared to baseline level (difference between a later time point and the pre-ethanol exposure value divided by the pre-ethanol exposure value) was calculated for subsequent time points to be compared between group and across time by two-way repeated measures ANOVA.

To investigate the relationships between the change in plasma orexin A levels and those of ethanol, water and liquid consumption, percentage change to baseline was calculated. Baseline measurements of these parameters were calculated by averaging the last 5 days of the training period to account for potential estrous effects. Percentage change to baseline was calculated by dividing the difference between PD19 (or average of PD18 and PD19 for drinking measurements) and the baseline values by the baseline value. Pearson's correlation coefficient was used to assess the correlation between the change in plasma orexin A and the changes in ethanol, water and liquid consumption in each group.

## Results

### Ethanol and water intake characteristics during training

During the 3-week ethanol drinking training, no significant differences between pregnancy groups were observed in ethanol consumption, ethanol preference, water consumption and total liquid consumption. A significant increase and decrease across the training period was detected in ethanol preference ( $F_{(10,12)}=4.173$ ,  $p_{\text{time}}<0.0001$ , Figure 2B) and water consumption ( $F_{(10,12)}=5.193$ ,  $p_{\text{time}}<0.0001$ , Figure 2C).

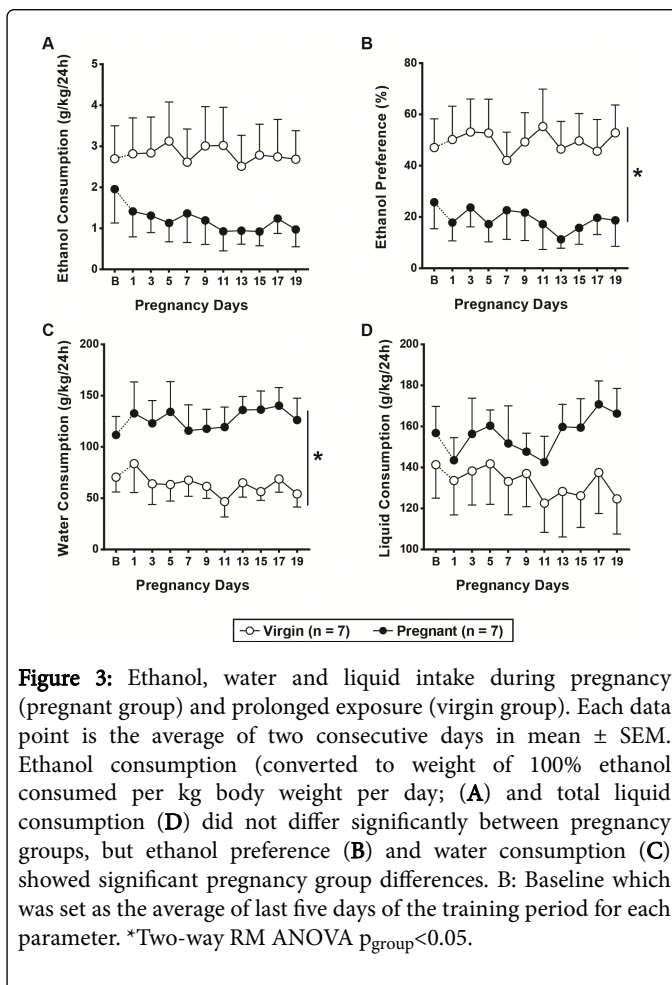


**Figure 2:** Ethanol, water and liquid intake during the 3-week ethanol drinking training. Each data point is the average of two consecutive days in mean  $\pm$  SEM. Ethanol consumption (converted to weight of 100% ethanol consumed per kg body weight per day; (A) and preference (B) did not differ between pregnancy groups, but ethanol preference was significantly increased across time. Similarly, water consumption (C) and total liquid consumption (D) did not differ between pregnancy groups, but water consumption was decreased across time. \*Two-way RM ANOVA  $p_{\text{time}}<0.05$ .

### Ethanol intake characteristics during pregnancy

Ethanol consumption and preference of the pregnant rats was gradually reduced throughout pregnancy (Figures 3A and 3B). In particular, the pregnant rats showed a significantly lower ethanol preference compared to the virgin rats ( $F_{(1,12)}=5.342$ ,  $p_{\text{group}}=0.035$ ). These reductions were most prominent during early pregnancy until mid-pregnancy (around PD13), at which time their levels were approximately half of pre-pregnancy baseline values. This reduction in alcohol intake was sustained into late pregnancy.

In contrast, ethanol consumption and preference remained relatively unchanged among virgin rats. Water consumption was significantly higher in the pregnant rats compared to the virgin rats, particularly towards the end of pregnancy ( $F_{(1,12)}=7.111$ ,  $p_{\text{group}}=0.021$ ; Figure 3C). Despite no significant group  $\times$  time interaction being detected in total liquid consumption, the pregnant group showed an apparent escalation from PD13 to PD15 (Figure 3D).



**Figure 3:** Ethanol, water and liquid intake during pregnancy (pregnant group) and prolonged exposure (virgin group). Each data point is the average of two consecutive days in mean  $\pm$  SEM. Ethanol consumption (converted to weight of 100% ethanol consumed per kg body weight per day; (A) and total liquid consumption (D) did not differ significantly between pregnancy groups, but ethanol preference (B) and water consumption (C) showed significant pregnancy group differences. B: Baseline which was set as the average of last five days of the training period for each parameter. \*Two-way RM ANOVA  $p_{\text{group}}<0.05$ .

### Plasma orexin A level after 3 weeks of ethanol consumption and during pregnancy

No significant difference in plasma orexin A levels before and after 3 weeks of two-bottle choice ethanol drinking was observed (Figure 4A). The changes in plasma orexin A level displayed opposite trends with the pregnant group showing a gradual decrease across pregnancy while the virgin group showed a gradual increase across extended exposure (Figure 4B). However, no significant group, time or interaction effect was detected.



## Discussion

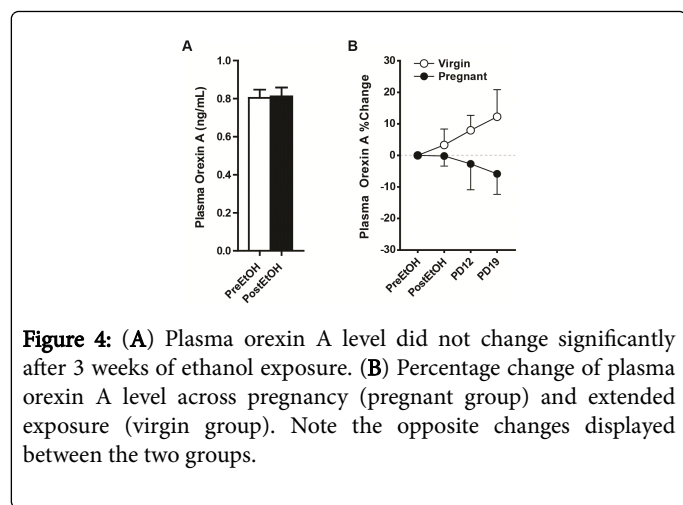
The current study is the first investigation to evaluate the relationship between alcohol intake, plasma orexin A concentration and pregnancy. In this present study, a significant reduction in alcohol preference was observed in pregnant rats compared to virgin rats. While a specious separation between the two groups emerged during the initial alcohol training period, the difference between the two groups became prominent and reached statistical significance during the extended alcohol training period once mated rats achieved gravid status. These results are consistent with previous studies [5,6] that demonstrated significant decreases in rat self-selection of alcohol during pregnancy. Similarly, prevalence rates of alcohol consumption in human populations are demonstrably lower during pregnancy, with 95% of Australian women reporting a reduction in quantity of alcohol use while pregnant or breastfeeding [19]. Another epidemiologic analysis evaluating females in the United States found that fewer than 3% of pregnant woman qualified as heavy episodic drinkers by the third month of pregnancy [20]. In that study, an ex post facto analysis was performed suggesting that excess drinking prevalence was more pervasive among those meeting criteria for alcohol dependence within the past year [20].

Diminished alcohol preference in gravid rats, accompanied by significant increases in water consumption, were demonstrated in prior studies and are supported by the present investigation as well [5,6]. It is reasonable to postulate that hormonal changes may be responsible for changes in alcohol consumption during pregnancy, as this reported phenomenon has been observed in multiple species [5-7] including alcohol preferring mice [8] and humans [3,4].

While others have evaluated the role of estrogen [5], progesterone [21] and the hypothalamic pituitary adrenal axis [5] in modulating ethanol intake during pregnancy, the hormone of interest in the current study was orexin A. To begin, we found that there was no significant difference in plasma orexin A concentration in either the pregnant group or virgin group before or after the initial three weeks of ethanol exposure. Any change that was observed in plasma orexin A concentration during pregnancy may therefore be assumed to be due to gestational state.

Our results did not demonstrate a significant difference between absolute plasma orexin A concentration levels in pregnant versus virgin rats. In contrast, other studies looking simply at the state of pregnancy and orexin A without the presence of alcohol exposure found that orexin A serum levels in rats were significantly increased on days 16 and 21 of pregnancy compared with virgin rats [22]. Prior data, however, did not evaluate the interplay between ethanol, orexin A and pregnancy. In the current study, significant and positive correlations were demonstrated between the percentage change in plasma orexin A level at late pregnancy and both the percentage change in ethanol consumption and preference. Put simply, a reduction in orexin A was significantly associated with a reduction in absolute alcohol intake and its self-selection.

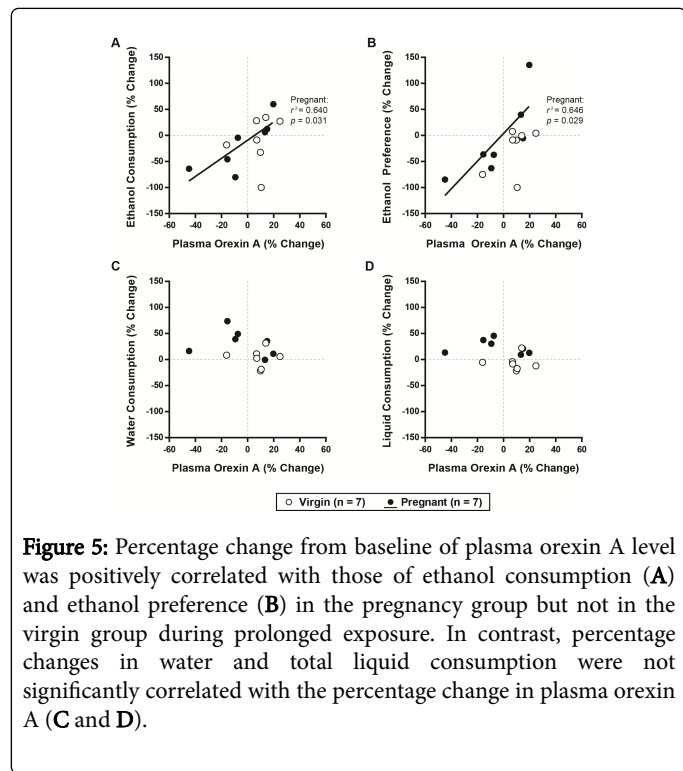
These results are consistent with previously published studies demonstrating a relationship between orexin A receptor antagonism and diminished ethanol self-administration [12]. In fact, several studies have shown that blocking orexin A reduces ethanol intake in rodent models. Recently, Olney, Navarro and Thiele demonstrated blunted binge like ethanol use and saccharin drinking in male rats with orexin A receptor inhibition [23]. Additionally, Carvajal et al. demonstrated a specific reduction in ethanol use in male C57BL/6



**Figure 4:** (A) Plasma orexin A level did not change significantly after 3 weeks of ethanol exposure. (B) Percentage change of plasma orexin A level across pregnancy (pregnant group) and extended exposure (virgin group). Note the opposite changes displayed between the two groups.

## Correlations between the change in plasma orexin A level and the change in ethanol intake during pregnancy

In the pregnant group, the percentage change in plasma orexin A was positively correlated with the percentage change in ethanol consumption (Pearson's correlation coefficient=0.800,  $p=0.031$ , Figure 5A) and ethanol preference (Pearson's correlation coefficient=0.804,  $p=0.029$ , Figure 5B). In contrast, no such correlations were observed in the virgin group. Percentage change in plasma orexin A level was not correlated with those of water consumption and total liquid consumption (Figures 5C and 5D).



**Figure 5:** Percentage change from baseline of plasma orexin A level was positively correlated with those of ethanol consumption (A) and ethanol preference (B) in the pregnancy group but not in the virgin group during prolonged exposure. In contrast, percentage changes in water and total liquid consumption were not significantly correlated with the percentage change in plasma orexin A (C and D).

mice with orexin A receptor antagonism irrespective of chow consumption, calories ingested or saccharin drinking [24]. Orexin A receptor antagonism was further shown to significantly reduce ethanol intake in female alcohol preferring rats in a two-bottle choice paradigm [25]. In this same study, Anderson and colleagues did not observe any significant effect on water consumption by orexin A receptor blockade, suggesting that orexin A may have a differential and specific effect on consummatory behavior in relation to ethanol [25]. Similarly, our study demonstrated that a percentage change in plasma orexin A level was not correlated with water consumption or total liquid consumption; orexin A change showed a specific and significant relationship with alcohol preference and consumption only. In addition to rodent investigations, a recent clinical case-control study demonstrated a significant reduction in heavy drinking among narcoleptic individuals exhibiting a deficiency in orexin A (or hypocretin-1) compared to controls [26].

It has been proposed that orexin A's role in promoting ethanol consumption originates in the perifornical lateral hypothalamus region where dopamine neurotransmission stimulates orexin expressing neurons [27]. Utilizing immunochemistry techniques, some studies have demonstrated a reduction in hypothalamic orexin A neurons during pregnancy [9,22], while others have observed an increase [28] in orexin A presence. The relationship between circulatory orexin A levels and central nervous system (CNS) orexin expression remains indeterminate and should provide another avenue for research in the future when examining alcohol intake and pregnancy.

This study has some limitations. First, the study did not evaluate central orexin A levels, thus the observed change in systemic orexin A levels in the pregnant rats might not represent the change in central orexin A levels and reflect how the central orexin system adapts during pregnancy. Second, we could not pinpoint the source of change in plasma orexin A level. Previous studies detected prepro-orexin gene expression in both the brain and peripheral tissues, including the intestine, pancreas and gonads [29], but whether orexin A production (as well as orexin receptor expression) in these peripheral tissues could be influenced by pregnancy and chronic ethanol consumption would need further investigation. Third, the relatively low ethanol concentration used and free ethanol access in this study might limit the effect size caused by pregnancy. Using higher ethanol concentrations and/or other drinking paradigms which model bingeing or dependence, such as intermittent access, may cause larger effect sizes. Fourth, a large range of ethanol consumption was observed among the rodent subjects, in turn resulting in a less sensitive detection of overall change in ethanol intake under different experimental conditions. Future studies should categorize animals into various degrees of ethanol preference, or using alcohol-preferring vs. non-alcohol-preferring rats, in order to detect maximal impacts on ethanol intake and clarify whether orexin A modulates differentially between high and low alcohol-preferring animals during pregnancy. Additionally, future studies should use other rat species, such as Sprague Dawley rats, as well as multiparous rats to confirm the present findings are not specific to Wistar rats or limited to first pregnancy. Fifth, a causal relationship between reduced plasma orexin A levels and reduced gestational ethanol intake would require additional experiments to establish and consideration may be given to manipulating orexin levels with an osmotic pump to examine causality. Finally, other hormones may play a role in and/or interact with the orexin system to modulate alcohol intake during pregnancy and should be the subject of further research.

In conclusion, our results demonstrate that pregnant rats have a significant reduction in alcohol preference compared to virgin rats that are exposed to ethanol for the same duration, supporting a biological basis underlying a change in alcohol intake during pregnancy. The observed significant positive correlation between the change in alcohol consumption and preference and the change in plasma orexin A concentration during pregnancy implies a relationship between circulatory orexin A and gestational alcohol intake warranting further investigation.

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## Conflict of Interest Statement

Dr. Frye has had grant support from Assurex, Myriad and Pfizer, and has served as an unpaid consultant for Janssen Global Services, LLC, Mitsubishi Tanabe Pharma Corporation, Myriad, Sunovion, Supernus Pharmaceuticals, and Teva Pharmaceuticals. The remaining authors declare no conflict of interest.

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