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Hypocretin-Receptor mRNA Expression in the Central Amygdala of Alcohol-Dependent and Non-Dependent Rats

Gabriel Aldridge
East Tennessee State University

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by

Gabriel Aldridge

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Dr. Brooke Schmeichel, Chair

Dr. Cerrone Foster

Dr. Patrick Bradshaw

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ABSTRACT

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Hypocretin/Orexin (HCRT) neurotransmission facilitates drug-seeking behavior. HCRT neurotransmission at HCRT-receptors 1 and 2 (HCRT-R1 and -R2, respectively) is implicated in addiction. During the shift to alcohol-dependency, adaptations in neurotransmitter systems occur in reward- and stress-related brain regions. Specifically, neurotransmission systems in the central amygdala (CeA) are modulated by alcohol drinking/exposure. Therefore, this study investigated *Hcrtr1* and *Hcrtr2* mRNA expression in the CeA of alcohol-dependent rats and in non-dependent controls during acute alcohol withdrawal. *Fos* mRNA expression in the CeA of alcohol-dependent and non-dependent rats was also determined to assess adaptations in neuronal activation. To our knowledge, this is the first study to utilize RNAscope to quantify *Hcrtr1* and *Hcrtr2* mRNA in a rodent model of alcohol dependence. However, *Hcrtr1*, *Hcrtr2*, and *Fos* mRNA levels were not found to be significantly different in alcohol-dependent rats compared to non-dependent controls, possibly due to the temporal dynamics of these neuroadaptations.

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CHAPTER 1. INTRODUCTION

The Hypocretin System

The hypocretin (HCRT) neuropeptides, also known as orexin, were independently discovered in 1998 (de Lecea et al. 1998; Sakurai et al. 1998). Proteolytic processing of a common precursor protein, prepro-HCRT, produces the biologically relevant HCRT neuropeptides, HCRT-1 and HCRT-2 (Sakurai et al. 1998). HCRT synthesis occurs exclusively in the dorsal hypothalamus, including the lateral hypothalamus (LH) proper, dorsomedial hypothalamus, and perifornical area (de Lecea et al. 1998; Peyron et al. 1998; Sakurai et al. 1998). HCRT neuronal projections target HCRT-receptor 1 (HCRT-R1) and HCRT-receptor 2 (HCRT-R2) throughout the brain. HCRT-R1 and -R2 are G-protein coupled-receptors that each exhibit different affinities for the HCRT neuropeptides. HCRT-R1 has a high affinity for HCRT-1 (20 nM IC_{50}) but has a lower affinity for HCRT-2 (420 nM IC_{50}; Sakurai et al. 1998). HCRT-R2 is a nonselective receptor for HCRT-1 (38 nM IC_{50}) and HCRT-2 (36 IC_{50}). Generally, HCRT-R1 is thought to largely mediate drug-seeking behavior because of its association with motivation and reward, whereas, HCRT-R2 is associated with arousal (Hopf 2020). Signaling at HCRT-R1 has been shown to facilitate drug-seeking behavior under conditions that require high effort and motivation for the drug (Moorman and Aston-Jones 2009; España et al. 2010; Moorman et al. 2017). Signaling at HCRT-R2 is associated with arousal, in particular, as shown by mutated or knockout HCRT-R2 rodents and dogs that display narcoleptic-like characteristics (Lin et al. 1999; Nishino et al. 2000; Peyron et al. 2000; Willie et al. 2003). It was also observed that patients with narcolepsy and cataplexy indeed present with a lack of neurons producing HCRT (Nishino et al. 2000; Peyron et al. 2000).

HCRT neurons exhibit a high-level of afferent and efferent projections. Interestingly, many reward and stress-related brain regions important to addiction neurocircuitry exhibit reciprocal connections with HCRT neurons. Brain regions with afferent projections to HCRT neurons include: the central amygdala (CeA), basolateral amygdala (BLA), bed nucleus of the stria terminalis (BNST), nucleus accumbens (NAc), ventral tegmental area (VTA), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), and dorsomedial hypothalamus (DMH) (Sakurai et al. 2005; Yoshida et al. 2006; Giardino et al. 2018; Saito et al. 2018). Efferent projections from HCRT neurons innervate: the CeA, BLA, BNST, NAc, VTA, PVN, paraventricular thalamus (PVT), and lateral hypothalamus (LH) (Peyron et al. 1998; Nambu et al. 1999). Importantly, HCRT-R expression is also observed in many of these addiction-related brain regions, such as the CeA and NAc, that also exhibit reciprocal connections with HCRT neurons (Marcus et al. 2001). Thus, the organization of the HCRT neurotransmission system facilitates its involvement in physiology and behavior implicated in reward and stress.

HCRT and Addiction

Drug addiction is a chronically relapsing disorder involving compulsive drug seeking and taking, lack of ability to control drug consumption, and the emergence of a negative affect during withdrawal. Typically, addiction is conceptualized as a cycle where an individual progresses through binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation stages. Initially, the evidence for HCRT neurotransmission's involvement in addiction was indirect and based on a combination of case and clinical studies. Interestingly, it was noted that narcoleptic patients, who have reduced numbers of HCRT neurons, rarely abuse stimulants (Akimoto 1960; Nishino and Mignot 1997). Conversely, the brains of opiate addicts contain more HCRT neurons

than control brains (Thannickal et al. 2018). The first preclinical study directly implicating the HCRT system in addiction reported that morphine withdrawal activates HCRT neurons and induces *Hcrt* gene expression, and HCRT knockout mice exhibit attenuated somatic signs of morphine withdrawal (Georgescu et al. 2003). Further research showed that HCRT neurotransmission promotes drug-seeking behavior. Specifically, extinguished morphine place preference is reinstated following chemical activation of HCRT neurons, and HCRT-R1 antagonism blocks this effect (Harris et al. 2005). Currently, it is believed that HCRT neurotransmission facilitates drug-seeking behavior, particularly, under conditions that require increased effort/motivation to take the drug. Generally, HCRT-R antagonism attenuates self-administration when reinforcement requires relatively high motivation but does not affect low-effort self-administration (Moorman 2018). Similarly, HCRT-R1 antagonism attenuates alcohol consumption, preference, and reinstatement in highly motivated rats, but not in low-alcohol-preferring rats (Moorman and Aston-Jones 2009; Lopez et al. 2016; Moorman et al. 2017). Thus, the HCRT system is particularly involved in high-motivation drug consumption.

Now, HCRT neurotransmission has been implicated in mediating drug-seeking behavior for all major drug classes, including psychostimulants (Boutrel et al. 2005; Schmeichel et al. 2018), opioids (Smith and Aston-Jones 2012; Schmeichel et al. 2015), nicotine (LeSage et al. 2010), and alcohol (Morganstern et al. 2010; Anderson et al. 2014; Moorman et al. 2017). Additionally, many animal models of addiction have been utilized to investigate the HCRT system's involvement in addiction. Conditioned place preference (Harris et al. 2005; Shoblock et al. 2011), self-administration (LeSage et al. 2010; Smith and Aston-Jones 2012; Anderson et al. 2014), and withdrawal (Georgescu et al. 2003; Lopez et al. 2016; Matzeu and Martin-Fardon 2020) paradigms have all been used in studies investigating HCRT-mediated reinforcement.

HCRT Neurotransmission Mediates Alcohol-Seeking Behavior

Many studies, using various animal models of addiction, have demonstrated that the HCRT system is involved in alcohol-seeking behavior. In an early study, treatment with a HCRT-R1 antagonist reduced cue-induced reinstatement of alcohol drinking and alcohol self-administration (Lawrence et al. 2006). In another study, alcohol consumption was increased 2 hours following HCRT-1 microinjections into the PVN or LH (Schneider et al. 2007). Thus, these studies indicate signaling at HCRT-R1 mediates alcohol-seeking behavior. Studies have also indicated a role for HCRT-R2 in alcohol-seeking behavior. Intracerebroventricular injection of a selective HCRT-R2 antagonist, TCS-OX2-29, decreased alcohol self-administration (Brown et al. 2013). Also, TCS-OX2-29 microinjections into the NAc decreased intermittent-access alcohol drinking (Brown et al. 2013). HCRT-R2 antagonism also decreases alcohol binge drinking, but the effect was observed only at a high dose (60 mg/kg) (Anderson et al. 2014). Given the role of HCRT-R2 on arousal-state, it is therefore possible that HCR-R2 antagonism indirectly reduced alcohol drinking by attenuating arousal. Collectively, the aforementioned studies implicate HCRT-R1 and -R2 in alcohol-seeking behavior, but do not address the effects of dual HCRT-R antagonism. Dual HCRT-R antagonists, such as almorexant, have demonstrated a good efficacy for attenuating alcohol drinking in rodents (Srinivasan et al. 2012; Anderson et al. 2014). In 2019, a dual HCRT-R antagonist, Lemborexant, was FDA approved to treat insomnia. Dual HCRT-R antagonists are a promising treatment for Alcohol Use Disorder (AUD) because sleep disruptions often accompany AUD. Thus, there is an increased interest in dual HCRT-R antagonists for the treatment of AUD. Dual HCRT-R antagonism may provide a comprehensive treatment for AUD by directly attenuating drug-seeking behavior (actions at HCRT-R1) and promoting healthy sleep (actions at HCRT-R2). In a recent study, intra-PVT

injection of a dual HCRT-R antagonist reduced operant responding at the active lever (resulted in an ethanol reward) without affecting activity at the inactive lever (no contingent reward), suggesting that dual HCRT-R antagonism did not affect general activity and arousal. (Matzeu and Martin-Fardon 2020). This result provides further support that dual HCRT-R antagonists are a potential treatment for AUD.

CeA in Alcohol Dependence and Withdrawal

A negative affect develops during the withdrawal stage, and irritability, stress, and emotional dysregulation are observed. The extended amygdala, consisting of the CeA, BNST, and a transition zone in the subregion of the NAc (Heimer and Alheid 1991), has been identified as an important reward/stress-related brain region involved in the development and expression of the negative affect during drug withdrawal. Currently, it is hypothesized that stress-related neurotransmitter systems are recruited in the extended amygdala during the transition to alcohol dependency (Koob and Schulkin 2019). Interestingly, there seems to be a unique role for the CeA in alcohol dependence and withdrawal. Corticotropin-releasing factor (CRF) and neuropeptide Y (NPY) neurotransmission systems within the CeA regulate alcohol drinking and alcohol withdrawal-induced stress by modulating GABAergic transmission in the CeA. In alcohol-dependent rodents, increased CRF and corticotropin-releasing factor receptor-1 (CRF1) mRNA levels are observed (Pich et al. 1995; Roberto et al. 2010). CRF1 signaling promotes increased alcohol drinking and withdrawal-induced stress/anxiety (Overstreet et al. 2004; Funk et al. 2007; Huang et al. 2010). Also, CRF increases GABAergic transmission in the CeA (Roberto et al. 2010). In contrast, NPY decreases GABAergic transmission in the CeA, withdrawal-induced stress/anxiety, and alcohol drinking in dependent rodents (Eva et al. 2008; Gilpin, Misra, et al. 2008; Gilpin et al. 2011). Thus, this evidence suggests that modulation of GABAergic

transmission in the CeA by opposing neurotransmission systems effects alcohol drinking and withdrawal induced stress in alcohol dependency. Similarly, HCRT neurotransmission mediates GABAergic transmission in the CeA of cocaine self-administering rats. Rats with extended access to cocaine (6 hrs) exhibited increased GABAergic transmission in the CeA, and this effect was blocked with intra-CeA injections of a HCRT-R1 antagonist. Thus, the HCRT system may be another important stress neurotransmission system in the CeA implicated in addiction. In conclusion, there is strong evidence of complex neurocircuitry within the CeA that regulates the development of the negative affect during the transition to alcohol dependency.

Alcohol's Regulation of Hcrt and Hcrtr Expression

As outlined above, HCRT neurotransmission mediates alcohol-seeking behavior. Thus, adaptions in the HCRT system may be involved in the development of alcohol dependence. Multiple studies have reported that chronic alcohol drinking rats exhibit increased *Hcrt* mRNA expression in the hypothalamus, as measured by PCR. For example, rats consuming 5 g/kg/day of ethanol for 70 days exhibited an increase in the area of *Hcrt* mRNA in the LH compared to alcohol-naïve and alcohol non-preferring rats (Lawrence et al. 2006). Similarly, rats consuming 3.5g/kg/day of ethanol for 7 weeks exhibited increased *Hcrt* mRNA in the perifornical lateral hypothalamus (PFLH) 30 minutes after the final alcohol session (Barson et al. 2015). In contrast, rats consuming 1.0 or 2.5 g/kg/day of ethanol for 28 days both exhibited decreased *Hcrt* mRNA in the PFLH 2 hrs following the last alcohol session (Morganstern et al. 2010). It is possible that opposing effects on *Hcrt* expression in the hypothalamus may be due to variation in the length of alcohol drinking paradigm. Additionally, differences in HCRT expression may be affected by time of tissue collection in relation to last alcohol exposure, as has been shown in corticosterone systems. For example, glucocorticoid receptor mRNA levels in the prefrontal cortex were

significantly reduced in alcohol-dependent rats compared to non-dependent controls during acute alcohol withdrawal (24 hrs after last alcohol session), but no significant difference was observed in the same region during protracted abstinence (3 weeks after last alcohol session) (Vendruscolo et al. 2012). Therefore, it is possible that opposing differences in *HCRT* expression are related to fluctuations that occur in *HCRT* expression following the last alcohol session. Interestingly, *Hcrt* mRNA expression is significantly increased in the hypothalamus of alcohol-dependent rats compared to non-dependent and naïve rats during acute alcohol withdrawal (8 hrs after last alcohol session; Matzeu and Martin-Fardon 2020). HCRT-1 peptide blood concentration has been reported to be higher in alcohol-dependent patients compared to controls. An increase in *Hcrt* mRNA and protein in alcohol-dependent subjects may contribute to increased motivation for alcohol in alcohol-dependent individuals compared to non-dependent individuals.

Alcohol drinking/exposure also regulates *Hcrtr1* and *Hcrtr2* expression. Rats receiving a single ethanol injection (2.5 g/kg) exhibited increased *Hcrtr1* mRNA levels compared to vehicle controls (Morales-Mulia 2019). In one study, chronic alcohol drinking rats exhibited increased *Hcrtr2* mRNA levels in the anterior PVT (Barson et al. 2015). Reduced *Hcrtr1* mRNA levels in the prefrontal cortex of chronic alcohol drinking rats has also been reported (Airapetov et al. 2019). The mixed results suggest that adaptions in *Hcrtr1* and *Hcrtr2* expression likely depend on the specific brain region and HCRT-R-subtype. Further, adaptations in *Hcrtr* expression are brain subregion specific. Rats consuming 3.5g/kg/day of ethanol for 7 weeks exhibited increased *Hcrtr2* mRNA levels in the anterior PVT compared to controls, but there was no significant difference in *Hcrtr2* expression in the posterior PVT (Barson et al. 2015). Importantly, experiments specifically designed to induce alcohol dependence and withdrawal have also showed significant changes in *Hcrtr1* and *Hcrtr2* expression. *Hcrtr1*and *Hcrtr2* mRNA levels

were increased in the posterior PVT of alcohol-dependent rats compared to controls during acute alcohol withdrawal (8 hrs after last alcohol session) (Matzeu and Martin-Fardon 2020). Rats 24 hrs into alcohol withdrawal exhibited reduced *Hcrtr1* mRNA in the prefrontal cortex compared to water drinking controls (Airapetov et al. 2019). A significant reduction in *Hcrtr1* mRNA was still observed 7 days into withdrawal. Thus, adaptions in *Hcrtr1* and *Hcrtr2* mRNA expression do occur in alcohol dependence and withdrawal, but the biological significance of the adaptions are not known.

RT-PCR Versus RNAscope: A Comparison

The majority of the studies outlined above use the RT-PCR technique for quantifying mRNA expression of *Hcrt* and *Hcrtr1/Hcrtr2*. RT-PCR is the standard technique for quantifying mRNA expression but has critical drawbacks. RT-PCR involves dissecting and homogenizing tissue in order to isolate RNA. In the processes of RNA isolation, the tissue context of the RNA signal is lost. Thus, it is not possible to associate RNA molecules with a specific subregion or cell type; however, the RNAscope assay is an *in situ* hybridization (ISH) assay specific for RNA molecules of interest. The RNAscope assay allows for a greater level of tissue specificity, subregion analysis, and cell type specificity. A novel probe design that hybridizes with target RNA molecules differentiates RNAscope from general RNA ISH. In typical RNA ISH, the complimentary strand that hybridizes to target RNA is a single linear RNA molecule. In RNAscope, approximately 50 Z-shaped probes hybridize to the target mRNA (Wang et al. 2012). The Z-shaped probes hybridize in pairs and create a binding site for preamplifiers and subsequent fluorescent tags. Therefore, the RNAscope technique is highly specific because fluorescent tags can only hybridize to target mRNA if double Z- hybridization is successful. Thus, the RNAscope assay utilizes a sophisticated system to enable single RNA molecule

visualization. The researcher can now easily observe the RNA signal *in situ.* In conclusion, the RNAscope technique was utilized to gain specificity and understand the signal distribution and density of *Hcrtr1*, *Hcrtr2*, and *Fos* within the CeA.

Hypothesis and Specific Goals

There is a growing amount of evidence that chronic alcohol drinking/exposure can dynamically regulate *Hcrtr1* and *Hcrtr2* expression, but the specific adaptions in *Hcrtr* expression in many reward/stress-related brain regions during acute alcohol withdrawal have not been investigated. The CeA is an important reward/stress-related brain region because it is specifically prominent in the development of the negative affect during the transition to alcohol dependence. Therefore, the goal of this study is to investigate *Hcrtr1* and *Hcrtr2* mRNA expression in the CeA of alcohol-dependent rats and in non-dependent controls during acute alcohol withdrawal. The study also investigated the interaction between alcohol dependence and neuron activation in the CeA as measured by *Fos* mRNA expression in dependent and non-dependent rats. It is hypothesized that *Hcrtr1* and *Hcrtr2* expression in the CeA of alcohol dependent rats at the time of acute alcohol withdrawal will be significantly different compared to non-dependent controls. Prior research has quantified *Hcrtr1* and *Hcrtr2* mRNA using RT-PCR, but this technique disrupts cell morphology. Therefore, there is ambiguity concerning the tissue subjected to RT-PCR. Thus, the current study also contains methodological goals because *Hcrtr1*, *Hcrtr2*, and *fos* mRNA will be quantified using RNAscope. To our knowledge, *Hcrtr1* and *Hcrtr2* mRNA has not been quantified using the RNAscope technique in a rodent model of alcohol addiction. Overall, the study aims to assess if altered *Hcrtr1* and *Hcrtr2* expression in the CeA is observed during acute alcohol withdrawal using a novel technique that maintains cell morphology and allows visualization of transcripts.

CHAPTER 2. MATERIALS AND METHODS

Animals

Adult male Wistar rats (N = 12; Charles River, Raleigh, NC), weighing between 225-275 grams at the beginning of the experiments, were housed in groups of 2-3 per cage in a temperature-controlled (22ºC) vivarium on a 12/12-hour light/dark cycle (lights on at 18:00) with *ad libitum* access to food and water. The animals were allowed to acclimate to the animal facility for at least 7 days before training. All procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care. A subset (n= 6) was used for RNAscope.

Chronic Intermittent Ethanol Vapor (CIEV)

Alcohol self-administration sessions were conducted in standard operant conditioning chambers (Med Associates, St. Albans, VT, USA). Briefly, after rats were given free-choice access to alcohol (10% w/v) and water for 1 day in their home cages, the rats were given one overnight session in the operant chambers with access to one lever that delivered water on a fixed ratio (FR1) schedule of reinforcement where one lever press resulted in 0.1 ml liquid delivery. Then, the rats were gradually transitioned to 30 min sessions with two levers available (water and alcohol; FR1; 0.1 ml liquid/press) until stable levels of 10% (w/v) alcohol intake were reached. Upon stable levels of responding for alcohol, rats were exposed to chronic intermittent ethanol vapor (CIEV) as previously described (O'Dell et al. 2004; Gilpin, Richardson, et al. 2008; Vendruscolo et al. 2012). Cycles of alcohol intoxication and withdrawal occurred daily for a minimum of 4 weeks, after which blood alcohol levels during vapor exposure ranged between 150-250 mg/dl. Over a 24-hour period, the alcohol vapor was on for 14 hours consecutively, and

operant alcohol self-administration occurred during the 10-hour period without alcohol vapor, 6-8 hours into withdrawal when brain and blood alcohol levels are negligible (Gilpin et al. 2009). In this model, rats exhibit somatic withdrawal signs and negative emotional symptoms reflected by anxiety-like responses and elevated brain reward thresholds (Schulteis et al. 1995; Roberts et al. 2000; O'Dell et al. 2004). Rats exposed to ambient air were used as non-dependent controls.

RNAscope

A subset of rats were used for RNAscope analyses (n=6). Rats were euthanized and fixed with 4% paraformaldehyde. The brains were collected 24 hours after the final ethanol vapor session and placed in formaldehyde, and then subjected to a sucrose gradient. Brains were cryosectioned (20 µm) and stored at -80°C in long-term storage buffer. Prior to the *in situ* assay the tissue was removed from the long-term storage buffer and mounted onto Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA). Slides incubated overnight at 60°C before the day of the in assay. On the day of the assay, the tissue was dehydrated in 50%, 75% and 100% ethanol. All of the necessary reagents for the RNAscope assay were included in the RNAscope Multiplex Fluorescent Reagent Kit v2 from Advanced Cell Diagnostics (320293-USM). Next, the tissue was subject to submersion in target retrieval solution at 100°C for 10 minutes. Tissue sections were treated with protease III and incubated at 40°C for 30 min. The hybridization and amplification steps were performed exactly according to the RNAscope Multiplex Fluorescent Reagent Kit v2 manual (320293-USM). Hybridization was achieved by allowing the tissue sections to incubate with hcrtr1 (444761), hcrtr2 (484571), and fos (455821) probes at 40°C for 2 h. Sections were then incubated with preamplifier and amplifier probes by applying AMP1 at 40°C for 30 min, AMP2 at 40°C for 15 min, and AMP3 at 40°C for 30 min, followed by

incubation in AMP4 ALTC 40°C for 15 min. Finally, the slides were stained with DAPI and coverslipped.

Statistical Analysis

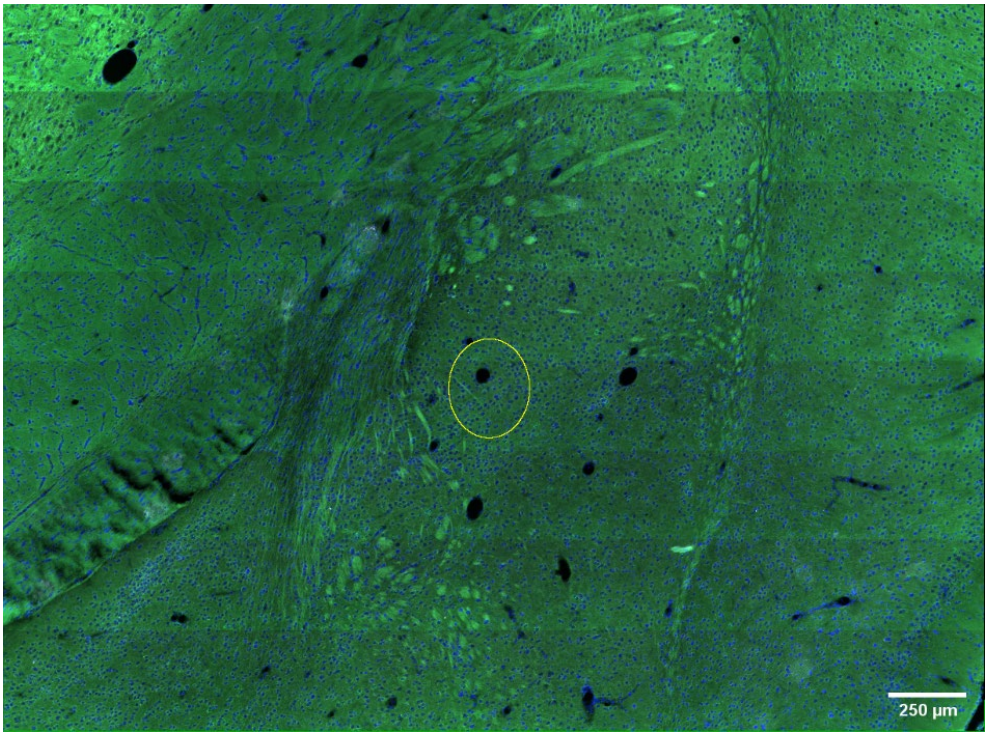
All data are expressed as means and standard errors of the mean (+SEM). ImageJ software was used to count positive signal and determine the area of the region of interest (ROI). Positive signal was verified using the RNAscope Multiplex Fluorescent Reagent Kit v2 manual from Advanced Cell Diagnostics (320293-USM). Expression of mRNA was quantified by calculating signal density (mRNA/µm^2). Student's *t*-test was used to compare signal density for each *Fos*, *Hcrtr1*, and *Hcrtr2*. Statistical analyses were performed using GraphPad Prism software version (GraphPad Software, San Diego, CA, USA). P values less than 0.05 were considered statistically significant for all tests.

CHAPTER 3. RESULTS

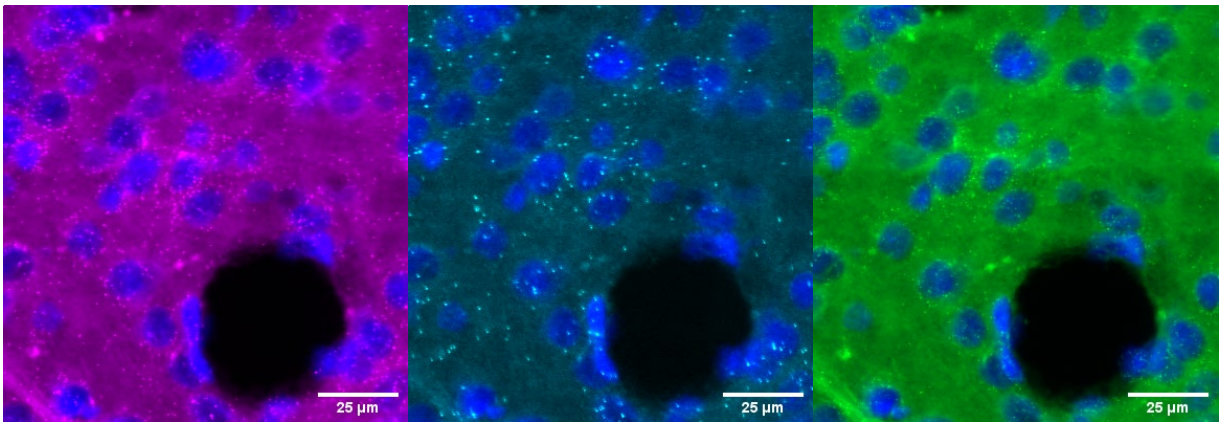
Hcrtr1 and Hcrtr2 mRNA Expression in the CeA

The central amygdala is a reward/stress-related brain region involved in the development and expression of the negative affect observed during alcohol withdrawal. Therefore, the ability of the CeA to biochemically respond to alcohol dependence was examined. *Hcrtr1*, *Hcrtr2*, and *Fos* mRNA expression in the CeA of alcohol-dependent and non-dependent rats (n=3 per group/brain region) were investigated using RNAscope. Alcohol-dependent rats were euthanized 24 hours into alcohol withdrawal and non-dependent rats were euthanized 24 hours following ambient air exposure rather than ethanol vapor. Sufficient hybridization was achieved in both alcohol-dependent and non-dependent groups for *Hcrtr1*, *Hcrtr2*, and *Fos* (Figure 1). *Hcrtr1* signal density was not significantly different in the CeA of alcohol-dependent rats compared to non-dependent rats (Figure 2; $t(4)= 0.014$, $p= 0.989$). There was no significant difference in *Hcrtr2* mRNA signal density in the CeA of alcohol-dependent rats compared to non-dependent rats (Figure 2; $t(4)= 1.669$, $p= 0.171$). There was no significant difference in *Fos* mRNA signal density in the CeA between alcohol-dependent and non-dependent rats (Figure 2; $t(4)= 0.436$, $p= 0.686$). Thus, the current findings do not support that *Hcrtr1* and *Hcrtr2* expression is significantly altered in alcohol-dependent rats compared to non-dependent rats.

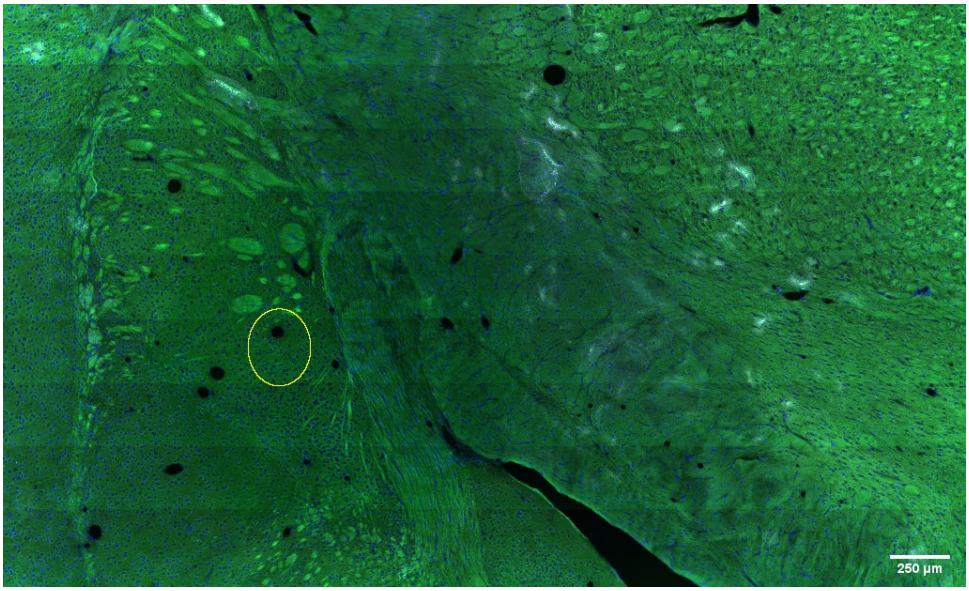
A) Alcohol-dependent



DAPI + *Hcrtr1* DAPI + *hcrtr2* DAPI + *fos*



B) Non-dependent



DAPI + *Hcrtr1* DAPI + *Hcrtr2* DAPI + *Fos*

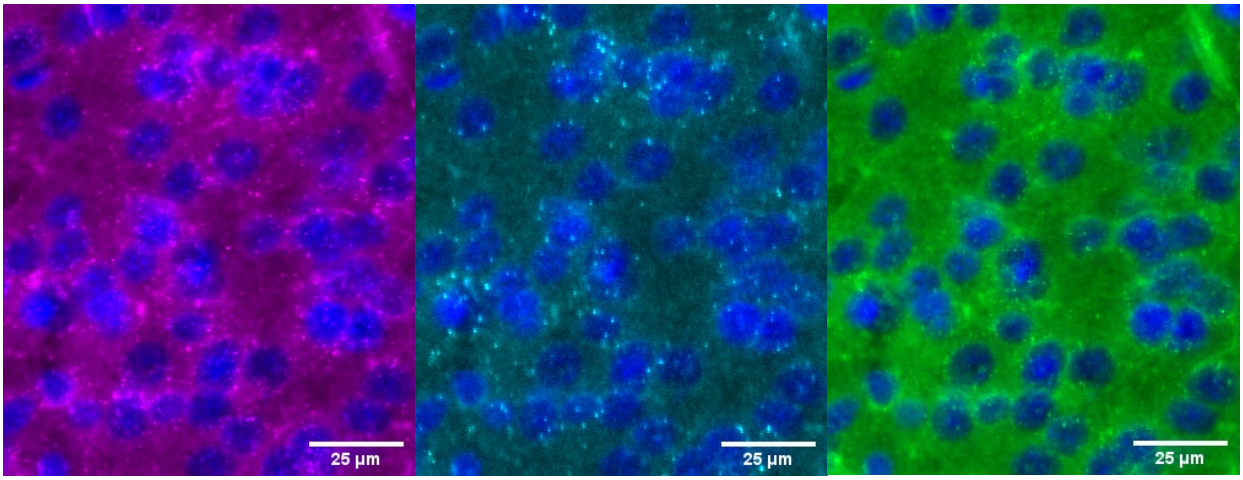


Figure 1. Representative images of *Hcrtr1, Hcrtr2*, and *Fos* mRNA hybridization in the CeA of alcohol-dependent and non-dependent rats. The RNAscope in-situ assay achieved hybridization with *Hcrtr1* (magenta), *Hcrtr2* (cyan), and *Fos* (green) mRNA in alcohol-dependent rats **(A)** and non-dependent controls **(B).** The yellow circle indicates a representative region of interest used to determine signal density. Images with the yellow circle are merged images containing signal from all three channels. The cell nuclei were identified by staining with DAPI (blue). Scale bars= 250 µm **(upper panel) or** 25 µm (lower panels).

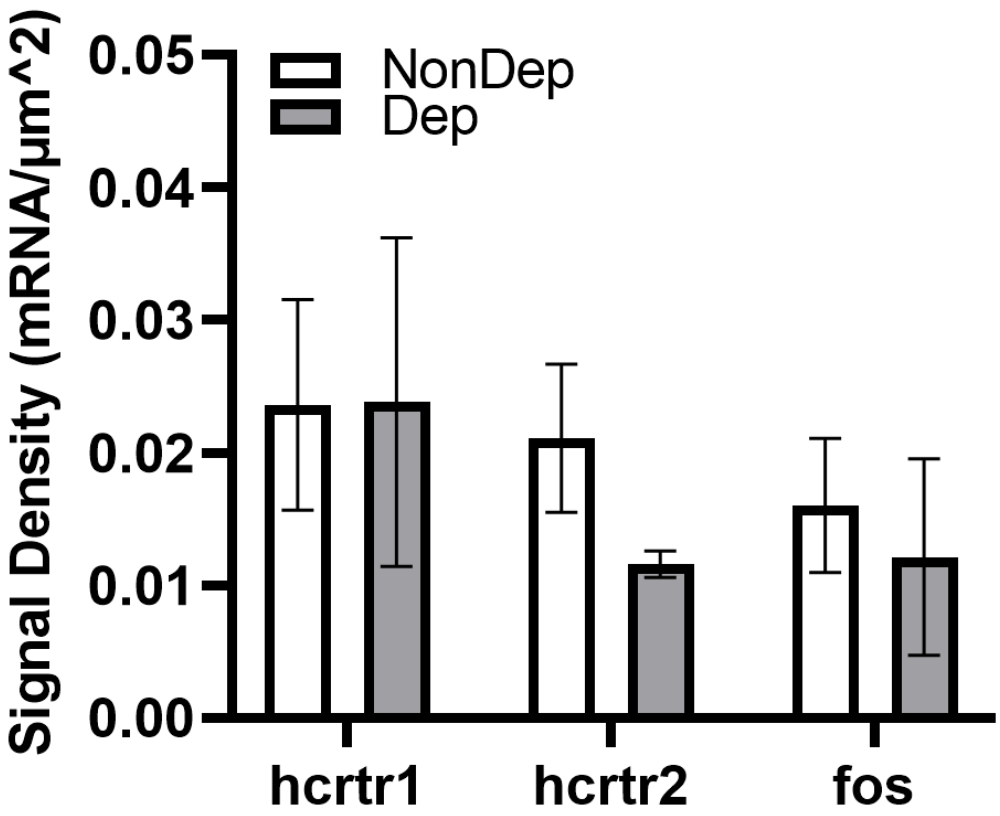


Figure 2. *Hcrtr1*, *Hcrtr2*, and *Fos* mRNA expression in the CeA of alcohol-dependent and non-dependent rats. mRNA expression of each gene is expressed as signal density (mRNA/μm^2) in the CeA of alcohol non-dependent (NonDep, n =3) and dependent (Dep, n =3) rats. There is no significant difference in *Hcrtr1*, *Hcrtr2*, or *Fos* mRNA expression in alcohol-dependent rats compared to non-dependent controls.

CHAPTER 4. DISCUSSION

Adaptions in neurotransmitter systems are observed during the transition to alcohol dependence. In this study, *Hcrtr1*, *Hcrtr2*, and *Fos* mRNA expression was investigated in the CeA of alcohol-dependent rats and in non-dependent controls during acute alcohol withdrawal. No significant difference in *Hcrtr1*, *Hcrtr2*, and *Fos* mRNA expression was observed in alcohol-dependent rats compared to non-dependent controls. Thus, it does not appear that *Hcrtr1* and *Hcrtr2* adaptations in the CeA are implicated in acute alcohol withdrawal. It is possible that the CeA does not experience adaptations in *Hcrtr1* and *Hcrtr2* expression during acute alcohol withdrawal. Similar findings have been observed for the VTA. The VTA is an important reward/stress-related brain region implicated in addiction that is innervated by HCRT neurons (Marcus et al. 2001; Koob and Schulkin 2019). *Hcrtr1* mRNA expression in the VTA was not significantly different in chronic alcohol drinking rats compared to controls (Airapetov et al. 2019). Additionally, it is possible that changes in *Hcrtr1* and *Hcrtr2* expression are not uniform within the CeA. There may be differences in *Hcrtr1* and *Hcrtr2* expression between the medial and lateral CeA. Chronic alcohol drinking induces a significant increase in *Hcrtr2* expression in the anterior PVT, but not the posterior PVT. Similarly, an experimental design that compares subregions within the CeA may reveal variation in alcohol's effect on *Hcrtr1* and *Hcrtr2* expression in the CeA. Furthermore, we collected tissue 24 hrs after the last vapor session. It is possible that a different time of tissue collection would have resulted in different findings. For example, rats euthanized 30 minutes following a 7-week chronic intermittent-drinking paradigm exhibited no change in *Hcrtr1* or *Hcrtr2* mRNA in the posterior PVT (Barson et al. 2015), but rats made dependent through CIEV euthanized 8 hours into withdrawal had significantly higher *Hcrtr1* and *Hcrtr2* mRNA in the same region compared to non-dependent controls (Matzeu and

Martin-Fardon 2020). The current study used a period of 24 hours following CIEV for brain collection. This timepoint was selected to target more stable gene expression while animals are still in acute withdrawal (Rimondini et al. 2003; O'Dell et al. 2004; Gilpin, Richardson, et al. 2008; Sommer et al. 2008). However, this timepoint might not be sufficient to capture transient changes in *Hcrtr1* and *Hcrtr2* mRNA that may have relevant implications for understanding adaptations in the HCRT neurotransmission system during acute alcohol withdrawal.

Limitations

As mentioned above, the CeA may not respond uniformly during alcohol withdrawal. Therefore, future studies should investigate differences in medial and lateral CeA *Hcrtr* expression during alcohol withdrawal. It is also important to investigate *Hcrtr1* and *Hcrtr2* expression in the CeA at various time points during acute alcohol withdrawal. Collecting tissue from alcohol-dependent rats at 4, 12, and 24 hrs into acute alcohol withdrawal would reveal potential fluctuations in *Hcrtr1* and *Hcrtr2* mRNA. Furthermore, investigating *Hcrtr1* and *Hcrtr2* mRNA expression during acute and protracted abstinence may reveal important differences that facilitate relapse. Another major limitation in the current is that *Hcrtr1*, *Hcrtr2*, and *Fos* mRNA levels were exclusively investigated. Therefore, future studies should assess if altered *Hcrtr1*, *Hcrtr2*, and *Fos* levels correspond with protein expression and assess the physiological significance of altering HCRT-R protein expression. Concerning techniques, comparative studies for RNAscope and RT-PCR need to be conducted. This would provide support and further validate RNAscope as a technique for quantifying mRNA. Lastly, the current study utilized a small sample size due to limitations in the number of animals and tissue quality. A more powerful study with a larger sample size could reveal adaptions in *Hcrtr1* and *Hcrtr2* expression within the CeA of alcohol-dependent animals.

Conclusion

The current study, reports that *Hcrtr1* and *Hcrtr2* Mrna levels are not significantly different in the CeA of alcohol-dependent rats compared to non-dependent controls at the time of acute alcohol withdrawal. There is no significant difference in *Fos* expression in the CeA of alcohol-dependent rats compared to non-dependent controls. In conclusion, the current study does not support that adaptions in *Hcrtr1* and *Hcrtr2* expression in the CeA are implicated in acute withdrawal.

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VITA

GABRIEL ALDRIDGE

Education:	M.S. Biology, East Tennessee State University, Johnson City, Tennessee, 2022
	B.A. Biology, Carson-Newman University, Jefferson City, Tennessee, 2020
Professional Experience:	Graduate Assistant, East Tennessee State University, College of Arts and Sciences, 2020-2022
Professional Presentations:	Effects of single and dual hypocretin-receptor blockade or knockdown of hypocretin amygdalar projections on alcohol drinking in dependent male rats. Appalachian Student Research Forum, Johnson City, Tennessee. April 2022.