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Authors

Aldridge, Gabriel M
Zarin, Tyler A
Brandner, Adam J
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Effects of single and dual hypocretin-receptor blockade or knockdown of hypocretin projections to the central amygdala on alcohol drinking in dependent male rats

Gabriel M. Aldridge[a], **Tyler A. Zarin**[a], **Adam J. Brandner**[b], **Olivier George**[c,d], **Nicholas W. Gilpin**[c,e], **Vez Repunte-Canonigo**[c,f], **Pietro P. Sanna**[c,f], **George F. Koob**[b,c], **Leandro F. Vendruscolo**[b,c], **Brooke E. Schmeichel**[a,b,c,*]

[a]Department of Biomedical Sciences, Quillen College of Medicine, East Tennessee State University, Johnson City, TN, 37614, USA

[b]Integrative Neuroscience Research Branch, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD, 21224, USA

[c]Committee on the Neurobiology of Addictive Disorders, The Scripps Research Institute, La Jolla, CA, USA

[d]Department of Psychiatry, School of Medicine, University of California, San Diego, San Diego, CA, USA

[e]Department of Physiology, Louisiana State University Health Sciences Center, New Orleans, LA, 70112, USA

[f]Department of Immunology and Microbiology, Scripps Research, La Jolla, CA 92037, United States

Abstract

Hypocretin/Orexin (HCRT) is a neuropeptide that is associated with both stress and reward systems in humans and rodents. The different contributions of signaling at hypocretin-receptor 1 (HCRT-R1) and hypocretin-receptor 2 (HCRT-R2) to compulsive alcohol drinking are not yet fully understood. Thus, the current studies used pharmacological and viral-mediated targeting of HCRT to determine participation in compulsive alcohol drinking and measured HCRT-receptor mRNA expression in the extended amygdala of both alcohol-dependent and non-dependent male rats. Rats were made dependent through chronic intermittent exposure to alcohol vapor and were tested for the acute effect of HCRT-R1-selective (SB-408124; SB-R1), HCRT-R2-selective

*Corresponding author: Dr. Brooke E. Schmeichel, Department of Biomedical Sciences, Quillen College of Medicine, East Tennessee State University, PO Box 70577, Johnson City, TN, 37614, Schmeichel@etsu.edu.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

(NBI-80713; NB-R2), or dual HCRT-R1/2 (NBI-87571; NB-R1/2) antagonism on alcohol intake. NB-R2 and NB-R1/2 antagonists each dose-dependently decreased overall alcohol drinking in alcohol-dependent rats, whereas, SB-R1 decreased alcohol drinking in both alcohol-dependent and non-dependent rats at the highest dose (30 mg/kg). SB-R1, NB-R2, and NB-R1/2 treatment did not significantly affect water drinking in either alcohol-dependent or non-dependent rats. Additional PCR analyses revealed a significant decrease in *Hcrtr1* mRNA expression within the central amygdala (CeA) of dependent rats under acute withdrawal conditions compared to nondependent rats. Lastly, a shRNA-encoding adeno-associated viral vector with retrograde function was used to knockdown HCRT in CeA-projecting neurons from the lateral hypothalamus (LH). LH-CeA HCRT knockdown significantly attenuated alcohol self-administration in alcohol-dependent rats. These observations suggest that HCRT signaling in the CeA is necessary for alcohol-seeking behavior during dependence. Together, these data highlight a role for both HCRT-R1 and -R2 in dependent alcohol-seeking behavior.

Keywords

Hypocretin/orexin; alcohol; central amygdala; retro-adeno-associated viral vector; alcohol-dependence; self-administration

1. Introduction

Alcohol use disorder (AUD), is a multifaceted psychiatric disorder characterized by compulsive alcohol seeking, loss of control over intake, and the emergence of a negative emotional state, including dysphoria, anxiety, depression, that occurs during alcohol withdrawal. During alcohol withdrawal, brain stress systems are dysregulated in both humans [1–4] and rodents [5–8], resulting in an increased sensitivity to stress. Exposure to alcohol vapor in rodents is an animal model of dependence that closely mimics somatic and motivational signs of AUD resembling those of the human condition [9,10]. It is hypothesized that following prolonged alcohol dependence, negative reinforcement mechanisms predominate, in which brain stress systems are recruited and the drug is taken to alleviate negative affective states associated with drug withdrawal (for review, [11]).

Hypocretin/orexin (HCRT) is a neuropeptide believed to be dysregulated in alcohol dependence (for review, [12–14]). HCRT neuropeptides have been associated with both stress and drug seeking behaviors [13–15]. The two HCRT neuropeptides, HCRT-1 and HCRT-2, are synthesized within a restricted region of the dorsal hypothalamus, including the lateral hypothalamus proper, adjacent perifornical area, and dorsomedial hypothalamus [16,17]. HCRT neurons project widely throughout the brain [18–21] and target two G-protein-coupled receptors, HCRT receptor 1 and receptor 2 (HCRT-R1 and -R2, respectively) [17]. HCRT projections include reciprocal connections to the extended amygdala and other subcortical regions that are implicated in negative reinforcement [19,20]. These brain regions, including the central amygdala (CeA) and nucleus accumbens shell (NAs), are suspected to contribute towards enhanced motivation underlying compulsive-like intake during drug and alcohol dependence [11]. HCRT-R1 exhibits a higher affinity for HCRT-1, whereas HCRT-R2 has relatively equal affinities

for HCRT-1 and −2 [17]. Antagonists targeting HCRT-Rs are either selective for a single hypocretin-receptor subtype (e.g., HCRT-R1: SB-334867, SB-408124, ACT-539313; HCRT-R2: JNJ-10394049, NBI-80713) or non-selective for dual hypocretin-receptors (e.g., almorexant, suvorexant, lemborexant, etc.), all with varying degrees of affinity and specificity for respective HCRT-R subtypes [22,23]. HCRT neurotransmission has been shown to mediate the reinforcement of drug-seeking behavior for all major drug classes, including psychostimulants [24–33], nicotine [34–38], opioids [39–41], and alcohol [42–51]. Relevant to alcohol, studies indicate a role for both HCRT-R1 and HCRT-R2 signaling in self-administration of alcohol in alcohol-preferring rats [52–55]. Additionally, antagonism of HCRT-R1 can reduce alcohol intake in mice made alcohol-dependent by chronic intermittent alcohol vapor [56]. Treatment with the dual HCRT-R1/2 antagonist, TCS1102, prevented stress-induced reinstatement in rats made dependent by chronic intermittent exposure to alcohol vapor [57]. However, little is known about the role of HCRT neurotransmission at HCRT-R1 and -R2, individually or in combination, on the compulsive-like responding in animal models of alcohol dependence.

Here, we tested the hypothesis that altered HCRT signaling at both HCRT-R1 and -R2, mediate compulsive-like alcohol intake associated with repeated withdrawal periods in an alcohol-dependent state. Thus, we examined the effects of varying HCRT-receptor-specific antagonists administered systemically on responding for alcohol in non-dependent rats and rats made dependent on alcohol by exposure to chronic intermittent ethanol vapor (CIEV). Additionally, *Hcrtr1* and *Hcrtr2* gene expression was examined in reward/stress-related brain regions of the CeA and NAs of dependent rats during withdrawal from alcohol and in non-dependent controls. Finally, a novel shRNA-encoding adeno-associated viral vector (AAV) with retrograde function was microinjected into the CeA to knockdown HCRT projections from the lateral hypothalamus (LH) to the CeA in alcohol-dependent rats. This retrograde AAV *Hcrt* silencing was expected to decrease alcohol self-administration in alcohol-dependent rats due to the decrease in HCRT presence in the LH-CeA pathway.

2. Material and Methods

2.1. Animals

Adult male Wistar rats (N = 80; 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 (22°C) 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 and Use Committee of The Scripps Research Institute (behavioral pharmacology experiments), the National Institute on Drug Abuse (AAV experiments), and at East Tennessee State University (AAV timeline experiments).

2.2. Experiment 1: Behavioral pharmacology of HCRT-R-specific antagonism

2.2.1. Alcohol and water self-administration—Alcohol and water self-administration sessions were conducted simultaneously in standard operant conditioning

chambers (Med Associates, St. Albans, VT, USA). Briefly, for the training of alcohol and water lever pressing, rats were first given free-choice access to alcohol (10% w/v) and water for 1 day in their home cages, and then were given one overnight session in the operant chamber with access to food (*ad libitum*) and one lever that delivered alcohol on a fixed ratio (FR1) schedule of reinforcement where one lever press resulted in 0.1 ml alcohol delivery. In the following days, the rats were transitioned to 30 min sessions with two levers available (alcohol or water; FR1; 0.1 ml liquid/press) until stable levels of 10% (w/v) alcohol intake were reached with sessions occurring 4–5 days per week. Upon stable levels of responding for alcohol, rats were split into two groups (alcohol-dependent [CIEV-exposed; n = 21] and non-dependent [air-exposed; n = 21]) and operant behavior was maintained in self-administration sessions 2 times per week thereafter.

2.2.2. Alcohol-dependence induction via CIEV exposure—Following operant self-administration training, half of the rats were made dependent on alcohol via daily exposure to CIEV as previously described [7,58,59]. Cycles of alcohol intoxication and withdrawal (i.e., alcohol 14h ON/10h OFF) occurred daily for a minimum of 2 and up to 4 weeks, after which blood alcohol concentration (BAC) levels during vapor exposure ranged between 150–250 mg/dL. Following dependence induction, alcohol-dependent rats were maintained at BACs of 150–250 mg/dL with daily CIEV. Behavioral testing of 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 [60]. In this model, rats exhibit somatic withdrawal signs and negative emotional symptoms reflected by anxiety-like responses and elevated brain reward thresholds [59,61–67]. Rats exposed to ambient air were used as non-dependent controls. All behavioral testing occurred 2 times per week in 30-minute sessions for both alcohol-dependent and non-dependent rats. Alcohol-dependent rats were returned to CIEV chambers following operant testing.

2.2.3. Pharmacological testing—SB-408124 (SB-R1; AdooQ Bioscience, Irvine, CA, USA), NBI-80713 and NBI-87571 (NB-R2 and NB-R1/2, respectively; Neurocrine Biosciences, Inc., San Diego, CA, USA) were dissolved in 5% dimethyl sulfoxide and 5% Cremophor EL in sterile water. NBI-87571 ((3S)-2-[2-(3,4-dimethoxyphenoxy)ethyl]-N-[(1R)-1-phenylethyl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide, MW=460.57 g/mol) is a high-affinity dual HCRT-R1/2 antagonist (Rat HCRT-R1 Ki= 3 nM; Rat HCRT-R2 Ki=10.4 nM).

All drugs were injected intraperitoneally in a volume of 3 ml/kg 20 minutes prior to behavioral testing. Doses were chosen based on previously published studies and limited pilot studies [40,68,69]. For alcohol self-administration, the animals received all doses of one antagonist type (SB-R1: 0, 3, 10, 30 mg/kg; NB-R2: 0, 7.5, 15, 30 mg/kg; or NB-R1/2: 0, 3, 10, 30 mg/kg) in a within-subject Latin-Square design. A regular FR1 alcohol self-administration session without antagonist treatment was performed between testing days.

2.3. Experiment 2: HCRT-R mRNA expression levels in the extended amygdala of alcohol-dependent rats

2.3.1. Reverse transcription and quantitative PCR—A separate cohort of rats were trained for operant alcohol self-administration, as described above. Rats were then made alcohol-dependent via CIEV (as described above; n = 9) or non-dependent (air-exposed controls; n =9) and allowed 12 operant self-administration sessions (over 3 weeks) prior to euthanasia. Brains from alcohol-dependent and non-dependent rats were collected and snap-frozen with isopentane for measurements of *Hcrtr1* and *Hcrtr2* mRNA levels during acute alcohol withdrawal (approximately 24 hours after the vapor was turned off). Importantly, 6–48 hours after the end of exposure to CIEV, rats exhibit both somatic and motivational signs of withdrawal, and the motivational signs can still be observed 3–5 weeks into alcohol abstinence [58,59,63,64,66]. Thus, the time point of 24 hours for brain collection should be well-within the period of withdrawal. Furthermore, this time point was chosen to target more stable dysregulations of gene expression and to avoid potentially transient effects caused by early withdrawal. Brains were sliced on a cryostat, and bilateral tissue punches were collected from NAs (500 μm thickness, 0.5 mm diameter) and the CeA (500 μm thickness, 1.0 mm diameter). RNA was extracted and purified from the brain punches using the PureLink RNA Mini Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. cDNA was reverse transcribed from total RNA using the Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Gene expression levels were determined by quantitative polymerase chain reaction (qPCR) using a TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Reactions were carried out as described previously [7] and cDNA concentrations of *Hcrtr1* and *Hcrtr2* were calculated according to the relative quantification (delta-delta Ct) method, corrected for differences in PCR efficiency, normalized to glyceraldehyde-3-phosphate dehydrogenase (Gapdh). Primers used were as follows: TaqMan qPCR utilized commercially available *Hcrtr1* (Rn00565032_m1), *Hcrtr2* (Rn00565155_m1) and *Gapdh* (Rn99999916_s1) primer/probe sets (Applied Biosystems Inc., Foster City, CA), with PCR conditions according to the manufacturer's protocol.

2.4. Experiment 3: Retrograde AAV knockdown of HCRT projections to CeA

2.4.1. AAV constructs—Recombinant short hairpin RNA (shRNA) encoding AAV vectors were produced using an AAV helper-free system (Stratagene, France; as described in [70]). All AAV vectors were produced by the Genetic Engineering and Viral Vector Core at the National Institute on Drug Abuse Intramural Research Program. In these vectors, the shRNA sequence is expressed under the control of the mouse ubiquitin 6 (mU6) promoter, while the enhanced green fluorescent protein (GFP) tag is expressed under the control of the cytomegalovirus (CMV) promoter to label transduced cells. The shRNA sequence targeting the *Hcrt* transcript (-HCRT; 5'-GTCTTCTATCCCTGTCCTAGT-3') was selected using the BLOCK-iT RNAi Designer algorithm (ThermoFisher). A scrambled sequence (-SCR; 5'-GCTTACTTTCGGCTCTCTACT-3') was used as negative control. Loop sequence was 5'-AGTCGACA-3' for both. The shRNA-HCRT and -SCR constructs were packaged into a retrogradely transported AAV2 serotype (AAVretro; titer of 7.4×10^{11} GU/mL). Specificity for these particular HCRT shRNA plasmid constructs [26, 71] and AAVretro-HCRTshRNA

function [71] have been previously described by our group and others, indicating no direct off-target effects of the shRNA-AAV on neurons anatomically common to the dorsal hypothalamus that co-exist with HCRT, including prodynorphin- and melanin concentrating hormone-producing neurons.

2.4.2. AAVretro microinjections—Rats were anesthetized with isoflurane (1–3%) and mounted in a stereotaxic frame (Kopf Instruments, Tujiunga, CA, USA). A stainless steel 30-gauge injector was used to microinject either the AAVretro-HCRT or -SCR vectors contra- and unilaterally (time course experiment) or bilaterally (alcohol behavioral experiment) into the CeA (AP −2.2 mm; ML ± 4.5 mm from bregma, and DV −8.3 mm from dura). Microinjections (0.5 μL/site) were made using a micro-infusion pump (Kd Scientific LEGATO 130 pump, Holliston, MA, USA) with a flow rate of 0.15 μL/min over 3.3 minutes. Injectors remained in place for 10 minutes to assure adequate diffusion of solution and prevent backflow along the injector track.

2.4.3. Immunohistochemistry and HCRT cell quantification—In AAVretro studies, rats were euthanized, perfused with normal saline followed by 4% formaldehyde at 2, 4, or 6 weeks (time course experiment) or approximately 8 weeks (alcohol behavioral experiment) following AAVretro microinjection. The brains were collected, post-fixed overnight in 4% formaldehyde, and then transferred to a sucrose solution (20–30%) the following day. Brains were cryosectioned (40 μm) and stored in 0.01M phosphate buffered saline (PBS) with 0/1% sodium azide at 4°C. For HCRT immunolabeling, sections were rinsed with 0.3% Triton X-100 in 0.01 M PBS, then quenched with 0.75% hydrogen peroxide and blocked with 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Sections were then rinsed and incubated for 24 hours at room temperature with rabbit anti-rat prepro-HCRT antibody (AB3096, 1:1000; diluted in 0.1 M PBS with 10% Triton X-100; EMD Millipore, Billerica, MA, USA). Tissue was then rinsed with 0.01 M PBS, incubated with biotinylated goat anti-rabbit secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA) for 90 minutes, rinsed with PBS-Tx, incubated in avidin-biotin complex (Elite ABC-HRP kit, Vector Laboratories) for 90 minutes, rinsed with 0.01 M PBS and then visualized with Vector SG substrate (Vector Laboratories) to yield a blue–gray precipitate. After staining, sections were mounted on microscope slides, dehydrated, cleared (Histoclear; Fisher Scientific, Itasca, IL, USA) and coverslipped.

Immunostained sections were analyzed on an AmScope Fluorescence/Light Microscope equipped with AmScopeAmLite software and a 20MP C-mount microscope camera (MU2003-BI-RU1, AmScope, Irvine, CA, USA). GFP fluorescence was used to detect the brain area within the CeA transduced by the viral vector and was confirmed for correct placement along with visualization of the most ventral point of the needle track using brightfield. Quantification of HCRT-positive cells containing a distinct soma with blue precipitate. For the time course experiment, counting was performed on two coronal sections (anterior-posterior level ranging from −2.6 to −2.9 mm from Bregma) using manual counting at a magnification of 20x by an observer blind to the experimental condition. HCRT-positive cell counts were derived from a capture window that encompassed the entire HCRT-neuronal field dorsoventrally and mediolaterally. Counts were totaled across

two sections for each hemisphere. For the alcohol behavioral experiment, counting was performed on one coronal section (anterior-posterior level −2.8 mm from Bregma) using manual counting at a magnification of 20x by an observer blind to the experimental condition. HCRT-positive cell counts were derived from a capture window that encompassed the entire HCRT-neuronal field dorsoventrally and mediolaterally. Counts were totaled across both hemispheres.

2.5. Statistical analysis

All data are expressed as means and standard errors of the mean (+SEM). Self-administration data were analyzed using a repeated-measures two-way analysis of variance (ANOVA), with group (non-dependent and dependent) as the between-subjects factor and dose (SB-R1 and NB-R1/2: 0, 3, 10, and 30 mg/kg; NB-R2: 0, 7.5, 15, and 30 mg/kg) as the within-subjects factor, with *post hoc* comparisons performed using a Holm-Sidak multiple-comparison test when appropriate. Dose-response curves for each antagonist on alcohol and water self-administration were analyzed using a repeated-measures one-way ANOVA on alcohol-dependent and non-dependent groups separately, with *post hoc* comparisons performed using a Dunnett's multiple comparison test when appropriate. A two-way mixed-factor ANOVA was used to assess the efficacy of the 30 mg/kg dose for each antagonist on alcohol and water self-administration in alcohol-dependent and non-dependent rats with group (non-dependent and dependent) as the between-subjects factor and HCRT-R antagonist (SB-R1, NB-R2, and NB-R1/2) as the within-subjects factor. When appropriate, *post hoc* comparisons were performed using a Holm-Sidak multiple-comparisons test. For quantitative PCR analyses, data are expressed as relative mean mRNA expression fold change (2^ΔΔCT) and were analyzed using a non-parametric Mann-Whitney U test for each region and genotype tested. AAVretro data were analyzed using a repeated-measures two-way ANOVA, with AAVretro-treatment group (AAVretro-HCRT and -SCR) as the between-subjects factor and time (BL, +3, +4, and +5 weeks; or +2, +4 and +6 weeks for time course experiment) as the within-subjects factor. When appropriate, *post hoc* comparisons were performed using a Dunnett's (behavioral testing) or Holm-Sidak (time course) multiple-comparison test. P values less than 0.05 were considered statistically significant for all tests.

3. Results

3.1. Experiment 1: Behavioral pharmacology of HCRT-R-specific antagonism

3.1.1. Effects of HCRT-R1 antagonism on alcohol and water self-administration—

SB-R1 (0, 3, 10, or 30 mg/kg) was used to assess the effects of HCRT-R1 antagonism on alcohol self-administration in alcohol-dependent (n=7) and non-dependent (n=7) rats. Water self-administration was assessed concurrently during the session. There was a main effect of dose and group on alcohol drinking in rats (Dose: $F_{(3,36)}$= 10.02, p< 0.01; Group: $F_{(1,12)}$= 32.34, p< 0.01; Dose × group: $F_{(3,36)}$= 1.86, p= 0.15). *Post hoc* analyses for the main effect of SB-R1 dose on alcohol drinking showed the 30 mg/kg, but not 3 or 10 mg/kg, dose significantly reduced alcohol drinking compared to 0 mg/kg vehicle controls. The 30 mg/kg dose of SB-R1 also significantly reduced alcohol drinking compared to the 3 and 10 mg/kg doses. The results of the repeated-measures one-

way ANOVA revealed that treatment with SB-R1 antagonist significantly reduced alcohol drinking in both non-dependent rats (Figure 1A; $F_{(3,18)}$= 3.37, p< 0.05) and dependent rats (Figure 1A; $F_{(3,18)}$= 6.77, p< 0.01). *Post hoc* analyses showed the 30mg/kg dose significantly decreased alcohol drinking in non-dependent (p< 0.05) and dependent rats (p< 0.01) compared to vehicle controls. For water drinking, there was a main effect of dose (Dose: $F_{(3,36)}$= 3.53, p< 0.05; Group: $F_{(1,12)}$= 0.01, p= 0.94; Dose × group: $F_{(3,36)}$= 0.29, p= 0.83). *Post hoc* analyses for the main effect of dose on water drinking showed only the 30 mg/kg dose significantly reduced alcohol drinking compared to the 10 mg/kg dose of SB-R1. Water drinking in non-dependent rats (Figure 1B; $F_{(3,18)}$= 1.98, p= 0.15) and dependent rats (Figure 1B; $F_{(3,18)}$= 1.78, p= 0.19) was not significantly reduced compared to vehicle controls. Treatment with SB-R1 significantly reduced alcohol drinking in non-dependent and dependent rats, but did not significantly reduce water drinking in either group.

3.1.2. Effects of HCRT-R2 antagonism on alcohol and water self-administration—NB-R2 (0, 7.5, 15, or 30 mg/kg) was used to assess the effects of HCRT-R2 antagonism on alcohol self-administration in a separate cohort of alcohol-dependent (n=7) and non-dependent (n=7) rats. Water self-administration was assessed concurrently during the session. There was a main effect of dose and group, and interaction of dose × group on alcohol drinking (Figure 1C; Dose: $F_{(3,36)}$= 5.25, p< 0.01; Group: $F_{(1,12)}$= 6.04, p< 0.05; Dose × group: $F_{(3,36)}$= 5.50, p< 0.01). *Post hoc* analyses for the interaction of NB-R2 dose × group on alcohol drinking showed the 30 mg/kg, but not 7.5 or 15 mg/kg, dose significantly reduced alcohol drinking compared to 0 mg/kg vehicle controls in dependent rats only. The 30 mg/kg dose of NB-R2 also significantly reduced alcohol drinking compared to the 7.5 and 15 mg/kg doses in dependent rats. There was no effect of NB-R2 on alcohol drinking in non-dependent rats. The results of the repeated-measures one-way ANOVA revealed that treatment with NB-R2 antagonist significantly reduced alcohol drinking in dependent rats (Figure 1C; $F_{(3,18)}$= 7.28, p< 0.01), but not in non-dependent rats (Figure 1C; $F_{(3,18)}$= 1.03, p= 0.40). *Post hoc* analyses showed the 30mg/kg dose significantly decreased alcohol drinking in dependent rats (p< 0.01) compared to vehicle control. For water drinking, there was no main effect of dose or group on water drinking (Figure 1D; Dose: $F_{(3,36)}$= 0.47, p= 0.70; Group: $F_{(1,12)}$= 1.25, p= 0.29; Dose × group: $F_{(3,36)}$= 1.64, p= 0.20). Water drinking in non-dependent rats (Figure 1D; $F_{(3,18)}$= 0.91, p= 0.45) and dependent rats (Figure 1D; $F_{(3,18)}$= 1.08, p= 0.38) was not significantly reduced compared to vehicle controls. Thus, the effects of NB-R2 were specific for alcohol drinking in dependent rats.

3.1.3. Effects of dual HCRT-R1/2 antagonism on alcohol and water self-administration—NB-R1/2 (0, 3, 10, 30 mg/kg) was used to assess the effects of HCRT-R1/2 antagonism on alcohol self-administration in separate cohort of alcohol-dependent (n=7) and non-dependent (n=7) rats. Water self-administration was assessed concurrently during the session. There was a main effect of dose and group on alcohol drinking (Dose: $F_{(3,36)}$= 5.85, p< 0.01; Group: $F_{(1,12)}$= 7.92, p< 0.05; Dose × group: $F_{(3,36)}$= 2.80, p= 0.05). *Post hoc* analyses for the main effect of NB-R1/2 dose on alcohol drinking showed the 10 and 30 mg/kg, but not 3 mg/kg, dose significantly reduced alcohol drinking compared to 0 mg/kg vehicle controls. The results of the repeated-measures one-way ANOVA revealed that

treatment with NB-R1/2 antagonist significantly reduced alcohol drinking in dependent rats (Figure 1E; $F_{(3,18)}$= 5.00, p< 0.05), but not in non-dependent rats (Figure 1E; $F_{(3,18)}$= 2.5, p= 0.09). *Post hoc* analyses showed the 10 (p< 0.01) and 30 (p< 0.01) mg/kg doses significantly reduced alcohol drinking in dependent rats compared to vehicle control. For water drinking, there was no main effect of dose or group on water drinking (Dose: $F_{(3,36)}$= 0.44, p= 0.73; Group: $F_{(1,12)}$= 0.36, p= 0.561; Dose × group: $F_{(3,36)}$= 0.61, p= 0.61).Water drinking in non-dependent rats (Figure 1F; $F_{(3,18)}$= 0.83, p= 0.49) and dependent rats (Figure 1F; $F_{(3,18)}$= 0.25, p= 0.86) was not significantly changed compared to vehicle control. Thus, the effects of NB-R1/2 were specific for alcohol drinking in dependent rats.

3.1.4. Comparative effects of HCRT-R antagonism—

To directly compare efficacy of HCRT-R antagonism on alcohol and water self-administration at an equal, maximally effective dose (30 mg/kg) across compounds, a mixed-factor two-way ANOVA was performed on the percentage change from vehicle (0 mg/kg dose) scores. There was a main effect of HCRT-R antagonist type and group × HCRT-R antagonist interaction on the percent change of alcohol drinking from vehicle (Figure 2A; Antagonist type: $F_{(2,36)}$= 7.01, p< 0.01; Group: $F_{(1,36)}$= 2.62, p= 0.11; Antagonist type × group: $F_{(2,36)}$= 4.75, p< 0.05). *Post hoc* analyses show that the percent change of alcohol drinking from vehicle is significantly different in non-dependent rats treated with SB-R1 compared to NB-R2 (p< 0.01) or NB-R1/2 (p< 0.05). Also, there is a significant difference in the percent change of alcohol drinking from vehicle of non-dependent rats treated with NB-R2 compared to NB-R1/2 (p< 0.05). Thus, the HCRT-R antagonists have varying degrees of efficacy in non-dependent controls. In dependent rats, the HCRT-R antagonists did not exhibit significantly different effects on the percent change of alcohol drinking from vehicle. Thus, HCRT-R antagonists have similar maximal efficacy in attenuating alcohol self-administration by approximately 50% relative to vehicle treatment in dependent rats. In water self-administration, there was no main effect of HCRT-R antagonist type or group (Figure 1E; Antagonist type: $F_{(2,36)}$= 1.12, p= 0.34; Group: $F_{(1,36)}$= 0.13, p= 0.72; Antagonist type × group: $F_{(2,36)}$= 0.26, p< 0.78).

3.2. Experiment 2: Extended amygdalar HCRT-R mRNA in alcohol-dependent rats

3.2.1. RT-PCR: HCRT-R mRNA expression in the CeA and NAs—

Hcrtr1 and *Hcrtr2* mRNA expression in two stress/reward-related brains regions of alcohol-dependent and non-dependent rats (n=5–9 per group/brain region) were investigated using reverse transcription and quantitative PCR. Alcohol-dependent rats were euthanized 24 hours into alcohol withdrawal. Non-dependent control rats were exposed to ambient air instead of alcohol vapor, but also had exposure to alcohol self-administration (as described; Section 2.2.1). *Hcrtr1* mRNA expression was significantly decreased in the CeA (Figure 3A; Non-dependent *Md*=1.00, Dependent *Md*=0.27; *U*=3, p< 0.05) but not in the NAs (Figure 3B; Non-dependent *Md*=1.14, Dependent *Md*=1.43; *U*=16, p= 0.82) of alcohol-dependent rats compared to non-dependent rats. There was no significant difference in *Hcrtr2* mRNA expression in the CeA (Figure 3A; Non-dependent *Md*=0.97, Dependent *Md*=1.32; *U*=27, p= 0.42) or NAs (Figure 3B; Non-dependent *Md*=1.15, Dependent *Md*=1.10; *U*=31.5, p= 0.69) between dependent and non-dependent rats. These data suggest a downregulation of HCRT-R1 in the CeA during withdrawal from alcohol in dependent rats.

3.3. Experiment 3: AAVretro knockdown of HCRT projections to CeA

3.3.1. Effects of AAVretro-HCRT microinjections into CeA on alcohol intake in dependent rats—

First, to characterize the time-course of HCRT knockdown of the AAVretro-HCRT vector, a separate, alcohol-naïve cohort of rats (N=12) was unilaterally microinjected with each AAVretro-HCRT and -SCR into contralateral hemispheres and HCRT-positive cells were counted in each hemisphere after 2-, 4-, or 6-weeks (n= 4/time point) following microinjection. One rat from the 4-week group was dropped due to tissue-handling damage. Microinjection of AAVretro-HCRT yielded a significant knockdown of *Hcrt* in dorsal hypothalamic HCRT-producing neurons compared to the AAVretro-SCR-treated control hemisphere (Figure 4C; AAVretro group: $F_{(1,8)}$= 22.12, p< 0.01; Time: $F_{(2,8)}$= 0.03, p= 0.97; AAVretro group × time: $F_{(2,8)}$= 0.64, p= 0.55). AAVretro-HCRT hemispheres showed an approximate 14%, 23%, and 24% retrograde knockdown of HCRT-positive neurons compared to the AAVretro-SCR treated control at 2-, 4-, and 6-weeks post-microinjection, respectively. *Post hoc* analyses showed a significant decrease of HCRT-positive neurons in the AAVretro-HCRT-treated hemisphere compared to the AAVretro-SCR-treated control hemisphere at 4- (p< 0.05) and 6-weeks (p< 0.5) following AAVretro microinjection. To confirm the importance of HCRT-R1 signaling in the CeA in alcohol drinking, we examined the knockdown of the HCRT LH-CeA pathway in alcohol-dependent rats. CIEV exposure was used to generate alcohol-dependent rats that were trained to self-administer alcohol (as described 2.2.1). The baseline number of alcohol reinforcers were recorded prior to bilateral microinjection with either AAVretro-HCRT or -SCR (n= 5 and 3, respectively) into the CeA (Figure 4E). Post-mortem analyses of the tissue showed an approximate 34% retrograde knockdown of HCRT-positive cells in the LH of AAVretro-HCRT-treated rats *versus* AAVretro-SCR-treated rats (Figure 4D; $t_{(6)}$=3.90, p<0.01). AAVretro-HCRT knockdown significantly attenuated alcohol self-administration in dependent rats *versus* AAVretro-SCR-treated rats (Figure 4F; AAVretro group: $F_{(1,6)}$= 3.49, p=0.11; Time: $F_{(3,18)}$= 2.63, p=0.08; Group × Time: $F_{(3,18)}$= 3.33, p< 0.05). *Post hoc* analysis revealed a significant decrease in alcohol self-administration in AAVretro-HCRT-treated rats at each timepoint compared to baseline self-administration, and no significant effect in AAVretro-SCR-treated rats. These data suggest that the LH-CeA HCRT-signaling pathway is critical to alcohol self-administration in dependent rats.

4. Discussion

The different contributions of HCRT-R1 and HCRT-R2 signaling in alcohol drinking are not yet fully understood. We hypothesized that altered brain stress systems, including contributions from HCRT signaling at HCRT-R1 and -R2, mediate highly-motivated alcohol intake associated with an alcohol-dependent state. Here, we investigated the extent to which selective and dual HCRT-R antagonism affects alcohol drinking in alcohol-dependent and non-dependent rats. Systemic HCRT-R1 antagonism (SB-R1), but not HCRT-R2 (NB-R2) nor -R1/2 (NB-R1/2), attenuated alcohol intake in non-dependent rats. In contrast to the effects in non-dependent rats, all three antagonists significantly reduced alcohol self-administration in dependent rats under acute withdrawal conditions, with the HCRT dual-receptor antagonist NB-R1/2 having the most selective effect on alcohol intake. To further investigate site-specific actions of HCRT-R neurotransmission, additional studies

were performed in withdrawal/stress-related regions of the CeA and NAs to determine potential HCRT neuroadaptations during acute alcohol withdrawal. PCR analysis showed that *hcrtr1* mRNA was significantly decreased in the CeA of dependent rats during acute withdrawal compared to non-dependent rats. Therefore, the contribution of HCRT signaling in the CeA of alcohol-dependent rats was investigated by using an AAV with retrograde function to knockdown *hcrt* expression in projections from the LH to the CeA. HCRT knockdown in the LH-CeA neurocircuitry significantly attenuated alcohol-seeking behavior following AAVretro-HCRT microinjection in alcohol-dependent rats compared to AAVretro-SCR controls. Combined, these results suggest a functional role for HCRT-R neurotransmission in alcohol drinking in dependent rats, and that HCRT projections from the LH to the CeA are integral to highly-motivated alcohol self-administration behavior observed in alcohol dependence.

4.1. Pharmacological blockade of HCRT signaling in alcohol-dependent rats

Previous research implicating the HCRT system in drug- and alcohol-seeking behavior largely focuses on signaling at HCRT-R1, with far fewer studies investigating HCRT-R2 signaling. Generally, HCRT-R1 is thought to be more involved in mediating drug-seeking behavior because of its association with motivation and reward, whereas, HCRT-R2 is associated with arousal and is therefore more prevalent in sleep research. More recently, there has been renewed focus by researchers on targeting dual-HCRT-Rs with the introduction of FDA-approved drugs like suvorexant and lemborexant for the treatment of insomnia. In alcohol research, the use of dual HCRT-R antagonists has also become more prevalent. Dual HCRT-R antagonism has been shown to attenuate alcohol responding in self-administration (fixed and progressive ratio schedules of reinforcement), stress-induced reinstatement, and in binge drinking models [43,45,57]. In the current study, Experiment 1 examined the effects of various HCRT-R-specific antagonists in rats under conditions of acute withdrawal during alcohol dependence and in non-dependent rats.

Treatment with the SB-R1 antagonist dose-dependently reduced alcohol self-administration in both alcohol-dependent and non-dependent rats at the highest dose (30 mg/kg). However, treatment with either NB-R2 or NB-R1/2 selectively showed reduced alcohol self-administration in dependent rats. This may suggest that signaling at HCRT-R2 is particularly sensitive under conditions of highly motivated-behavior like that of dependent alcohol-seeking. Previous research has shown a non-specific attenuation of operant, consummatory behavior of natural rewards are modulated, in part, by HCRT-R1 signaling [72–77]. In this way, the effect of SB-R1 on alcohol self-administration in both non-dependent and dependent rats may be a reduction of overall consummatory behavior and not necessarily specific to alcohol drinking. An additional or alternative explanation for the attenuating effect of the SB-R1 antagonist on alcohol self-administration is the potential sedative-like side-effect of the antagonist decreasing locomotor activity, especially at high doses. This has been shown elsewhere, particularly under conditions of low-motivated behavior during the active phase of the light-dark cycle [78]. However, there was no effect of the SB-R1 antagonist compared to vehicle-treated controls on water self-administration, suggesting that general operant and locomotor activity were unaffected.

In regard to HCRT-R2 antagonism, NB-R2 and NB-R1/2 had no effect on alcohol self-administration in non-dependent rats, but did dose-dependently reduce alcohol self-administration in alcohol-dependent rats. Although HCRT-R2 signaling is highly implicated in arousal and locomotor-producing activity [79–81], it is unlikely that effects on alcohol self-administration observed here are due to reduced arousal/activity as there was no effect on water self-administration. In alcohol-dependent rats, all three antagonists (SB-R1, NB-R2, and NB-R1/2) significantly reduced responding for alcohol during acute withdrawal. Importantly, the treatment also had no effect on water self-administration in these rats. In dependent rats, any potential confounding effects of decreased operant activity or decreased arousal level associated with HCRT-R antagonism are likely mitigated with highly-salient, goal-directed alcohol-seeking behavior. Consistent with this hypothesis, we and others have shown HCRT-R antagonists to have little to no effect on general activity in animals that exhibit strong addiction-like phenotypes with enhanced motivation, even at high doses [40,43,55,57,82].

Finally, in order to directly compared efficacy of HCRT-R antagonism on alcohol self-administration in alcohol-dependent and non-dependent rats, we specifically analyzed the most effective dose that was equal across all antagonists, 30 mg/kg. The results displayed in Figure 2 show that the HCRT-R antagonism have differential effects on alcohol self-administration in alcohol-dependent rats compared to non-dependent rats, with more variability in efficacy across HCRT-R antagonists in the non-dependent rats. Furthermore, it appears the HCRT-R antagonists have a greater overall effect on attenuation of alcohol self-administration in alcohol-dependent rats compared to non-dependent rats. Further preclinical studies are warranted to investigate the efficacy of such HCRT-R drugs on additional models of alcohol seeking and taking, and for other classes of drugs of abuse.

4.2. Contribution and neuroadaptation of the HCRT system in the extended amygdala

The pharmacological blockade of HCRT receptors in the first experiment utilized a systemic approach to demonstrate the differential effects of selective and dual HCRT-R-antagonism on alcohol drinking, particularly in alcohol-dependent rats in acute withdrawal. Additional experiments were conducted in order to understand the role HCRT signaling plays in the extended amygdala, including the CeA and NAs, distinct brain regions associated with stress- and alcohol-withdrawal related neurocircuitry [11].

Experiment 2 investigated the degree to which *Hcrtr1* and *Hcrtr2* mRNA levels are altered in alcohol-dependent animals at the time of acute withdrawal compared to non-dependent alcohol-drinking rats. PCR analysis showed that *Hcrtr1* mRNA was downregulated in the CeA in alcohol-dependent rats compared to non-dependent rats. Interestingly, HCRT has been shown to regulate, in part, the hypothalamic-pituitary-adrenal (HPA) response to stress [83–85]. A similar downregulation has been observed with other HPA axis modulators in response to alcohol dependence-related stress, namely corticosterone, corticotropin-releasing factor and glucocorticoids [7,8,86], in which the HPA axis becomes hyporesponsive. In the same way, the decrease in *Hcrtr1* mRNA observed may be a negative feedback mechanism due to a preceding increase in exogenous HCRT peptide in the CeA recruited in an alcohol-dependent state. Consistent with this hypothesis, mRNA of the HCRT peptide

precursor, prepro-HCRT, has been shown to be upregulated in rats following chronic alcohol consumption [50,54], thus suggesting an increase in HCRT signaling. However, *Hcrtr2* mRNA levels in the CeA were not significantly changed in alcohol-dependent rats, so there may be differential effects of chronic alcohol on mRNA HCRT-R subtype within regions of the CeA. The possible changes to HCRT-R1 and -R2 numbers, sensitivity, and function during acute alcohol withdrawal are important topics for future studies. There were also no significant changes in either *Hcrtr1* or *Hcrtr2* mRNA levels in the Nas of alcohol-dependent rats. Other studies have shown mixed results concerning the effect alcohol exposure on HCRT-R mRNA expression. For example, rats euthanized 30 minutes following chronic intermittent-drinking exhibited no change in *Hcrtr1* or *Hcrtr2* mRNA in the posterior paraventricular thalamus [50], whereas CIEV-exposed rats euthanized at 8 hours of abstinence had significantly higher *Hcrtr1* and *Hcrtr2* mRNA in the same region compared to alcohol-naïve and non-dependent controls [57]. Thus, length of alcohol exposure and specific time of tissue collection following alcohol exposure should be considered in the interpretation of mRNA data as levels are likely subject to state- and time-dependent dynamics. The current studies used a period of 24 hours following CIEV for brain collection, a timepoint that likely targets more stable gene expression while animals are still in acute withdrawal [58,59,63,64,66], and avoids possible transient effects to expression occurring early in withdrawal. However, it may also be that this timepoint did not capture dynamic changes to *Hcrtr2* mRNA that may otherwise have relevant implications for understanding the role of HCRT-R2 in dependent alcohol-seeking. Alternatively, or additionally, combined with the behavioral pharmacology studies, the lack of effect on *Hcrtr2* expression may also suggest that HCRT-R2 neurotransmission may modulate, at least in part, dependent alcohol-seeking in a brain region outside of the two areas considered herein. Nonetheless, the current findings demonstrate altered *Hcrtr1* mRNA expression in the CeA of alcohol-dependent rats, implicating the extended amygdala in dependence-related neurocircuitry under conditions of acute withdrawal.

Experiment 3 sought to test whether HCRT projections to the CeA are specifically involved with alcohol drinking in dependent rats. An shRNA AAV with retrograde function was used for site-specific knockdown of HCRT projections from the LH to the CeA. Alcohol self-administration was significantly decreased at 3-, 4-, and 5-weeks following AAVretro-HCRT microinjections into the CeA of rats made dependent on alcohol via CIEV exposure. There was no effect on alcohol self-administration in AAVretro-SCR-treated (control AAV) alcohol-dependent rats. AAVretro-HCRT microinjections resulted in a 34% knockdown of HCRT-positive neurons compared to AAVretro-SCR alcohol-dependent controls, as measured at 8-weeks following AAVretro microinjections. Likewise, alternative use of this same AAVretro-HCRT vector by another group showed a similar HCRT-knockdown efficacy (35%) in cocaine self-administering rats microinjected with AAVretro-HCRT into the ventral tegmental area [71]. In the time course experiment conducted in alcohol-naïve rats herein, a similar, albeit lesser knockdown of HCRT was observed at 4- and 6-weeks following AAVretro-HCRT microinjection (23% and 24% knockdown, respectively). While it is possible that the extent of knockdown at the time of behavioral testing in alcohol-dependent rats in the current studies was different from observed end-of-study values (and possibly more like that of the time course subjects), the behavioral data suggest

that the extent of knockdown at the time of testing was sufficient to affect behavior. Finally, it is also possible that the AAVretro construct uptake did not exclusively occur in terminal HCRT-CeA neuronal synaptic fibers, but also into non-terminal HCRT fibers of passage, although the retrograde vector used here (AAV2-retro) has not been stated to infect axons of passage [87]. These studies by our group and others also confirm specificity of this exact shHcrt construct, despite possible compensatory adaptations of prodynorphin (via vesicular co-release with HCRT) and/or neighboring melanin-concentrating hormone-containing neuron activity resulting from prolonged HCRT-projection knockdown following AAV transfection [26, 71]. Thus, it is possible that these concomitant peptide adaptations may have contributed to the reduced alcohol intake in AAVretro-HCRT treated rats, but this would need to be directly tested in future studies.

These AAVretro-HCRT results suggest that HCRT signaling in the CeA is necessary for alcohol drinking during dependence. These findings extend a growing literature implicating CeA in alcohol dependence-related neurocircuitry [5,88–90]. Neuronal ensemble recruitment in the CeA is associated with alcohol dependence, and our research suggests that HCRT signaling is an important aspect of this recruitment [91,92].

In conclusion, the current studies provide evidence that both selective and dual-HCRT-R antagonismsignificantly attenuates alcohol self-administration in alcohol-dependent rats without affecting water self-administration. Further, that alcohol exposure dynamically regulates HCRT-R expression in the CeA and that LH-CeA HCRT projections are necessary for alcohol drinking during dependence. Together, these findings support a role HCRT-R1 and -R2 modulation of dependent alcohol-seeking behavior and suggest HCRT-R antagonists as a target for understanding and potentially treating AUD.

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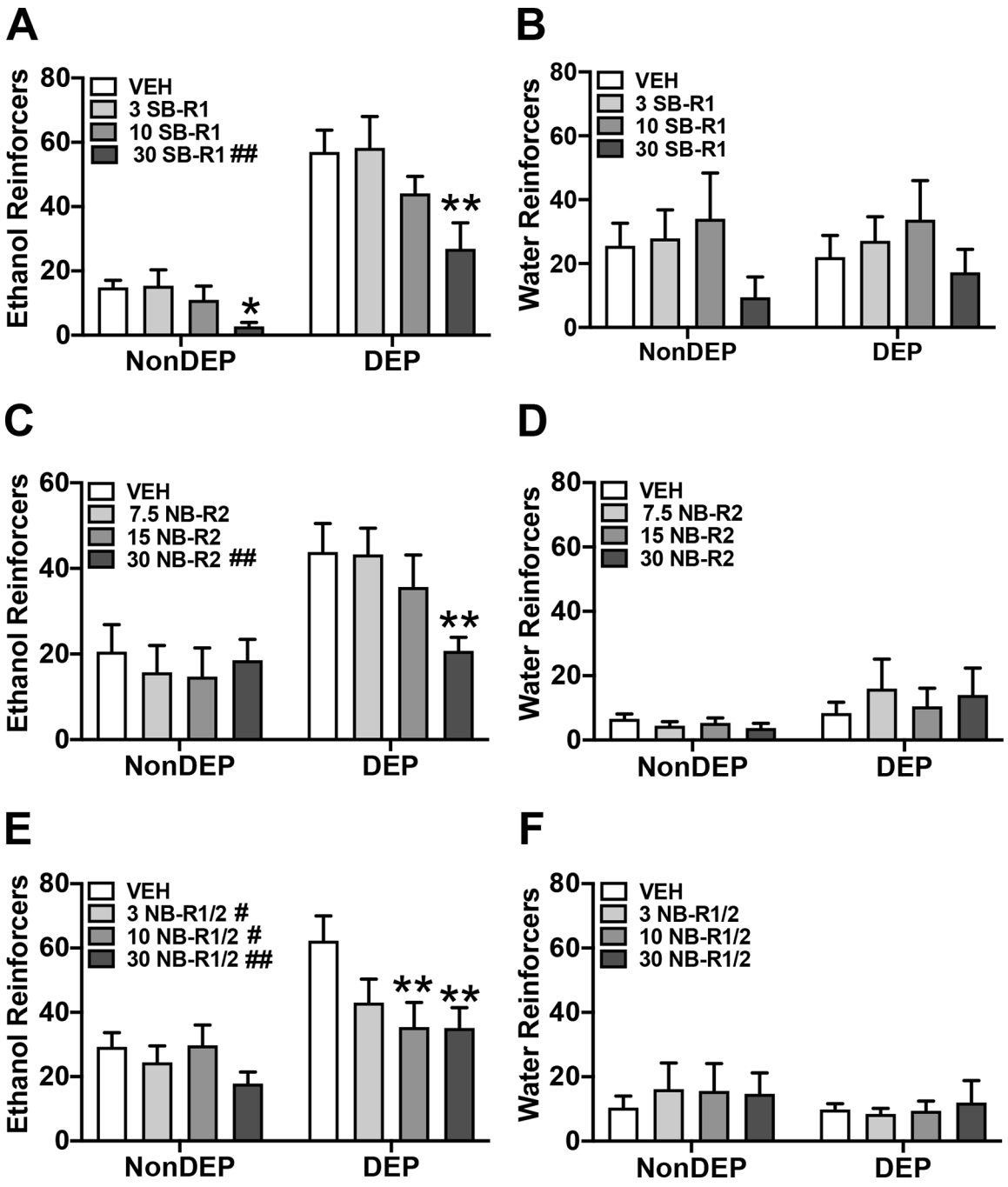

Figure 1.

HCRT-R-subtype antagonists have differential effects on alcohol self-administration. A, C, and E) Bars represent mean number (+SEM) of ethanol reinforcers per dose for SB-R1 (A), NB-R2 (C), or NB-R1/2 (E) treatment in both non-dependent (NonDEP) and alcohol-dependent (DEP) rats. A) SB-R1 (30 mg/kg) significantly decreased the number of ethanol reinforcers in both NonDEP and DEP rats. C) NB-R2 (30 mg/kg) dose significantly decreased the number of ethanol reinforcers in DEP rats. E) NB-R1/2 (10 and 30 mg/kg) significantly decreased the number of ethanol reinforcers in DEP rats. B, D, and F) Bars

represent mean number (+SEM) of water reinforcers per dose for SB-R1 (B), NB-R2 (D), or NB-R1/2 (F) treatment in both NonDEP and DEP rats. SB-R1 (B), NB-R2 (D), and NB-R1/2 (F) did not significantly change the number of water reinforcers in either NonDEP or DEP rats *versus* respective vehicle. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *versus* vehicle (analyzed using two-way ANOVA); $^{*}p<0.05$ and $^{**}p<0.01$ *versus* respective vehicle (analyzed using one-way ANOVA).

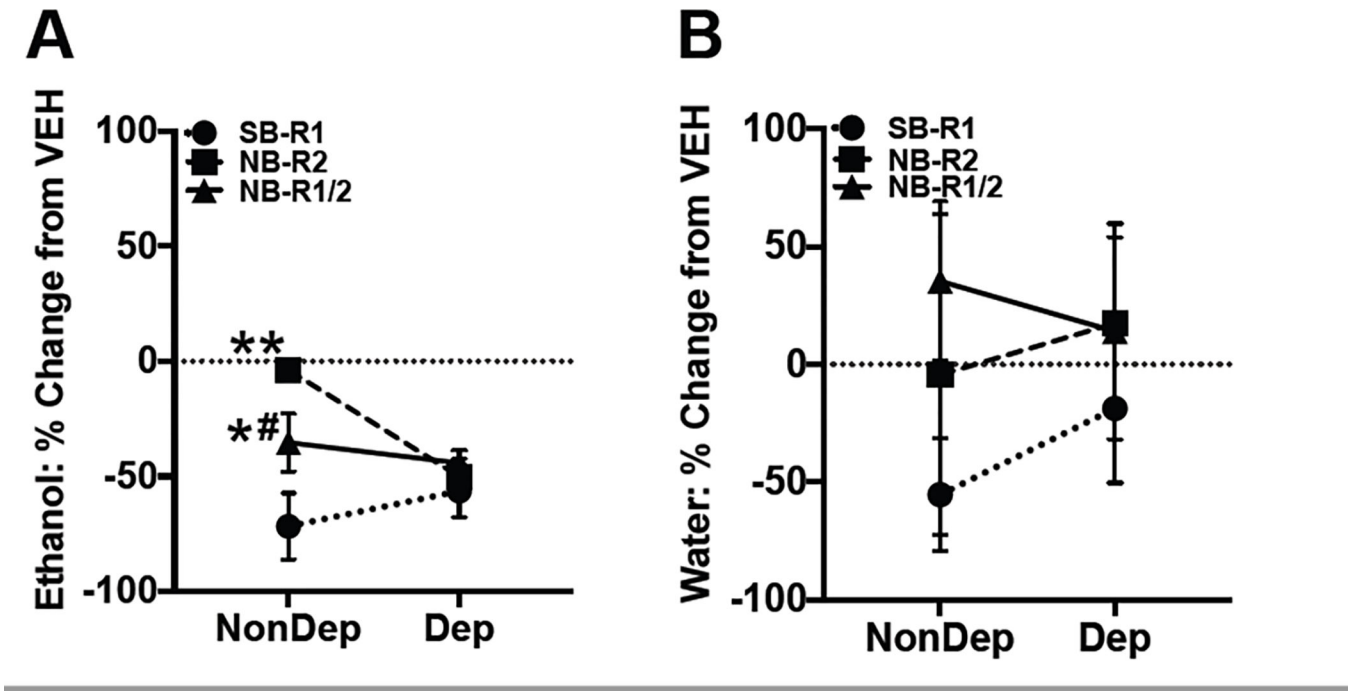

Figure 2.

Comparative effects of HCRT-R antagonism efficacy on alcohol and water self-administration. A) Markers represent the percent change in mean number (±SEM) of ethanol reinforcers from vehicle (VEH; 0 mg/kg) treatment per HCRT-R-subtype antagonist for SB-R1 (circle), NB-R2 (square), or NB-R1/2 (triangle) treatment (30 mg/kg) in both non-dependent (NonDEP) and alcohol-dependent (DEP) rats. A) In NonDEP rats, SB-R1 treatment was significantly more effective at attenuating alcohol self-administration than NB-R2 and NB-R1/2 treatment, and NB-R1/2 treatment was more effective than NB-R2 treatment. In DEP rats, all HCRT-R antagonists similarly reduced alcohol self-administration. B) Markers represent the percent change in mean number (±SEM) of water reinforcers from vehicle (VEH; 0 mg/kg) for SB-R1 (circle), NB-R2 (square), or NB-R1/2 (triangle) treatment (30 mg/kg) in both NonDEP and DEP rats. There was no significant difference in water self-administration among rats treated with either SB-R1, NB-R2, or NB-R1/2 in NonDEP or DEP rats. *$p < 0.05$ and **$p < 0.01$ *versus* SB-R1. $^{\#}p < 0.05$ *versus* NB-R2.

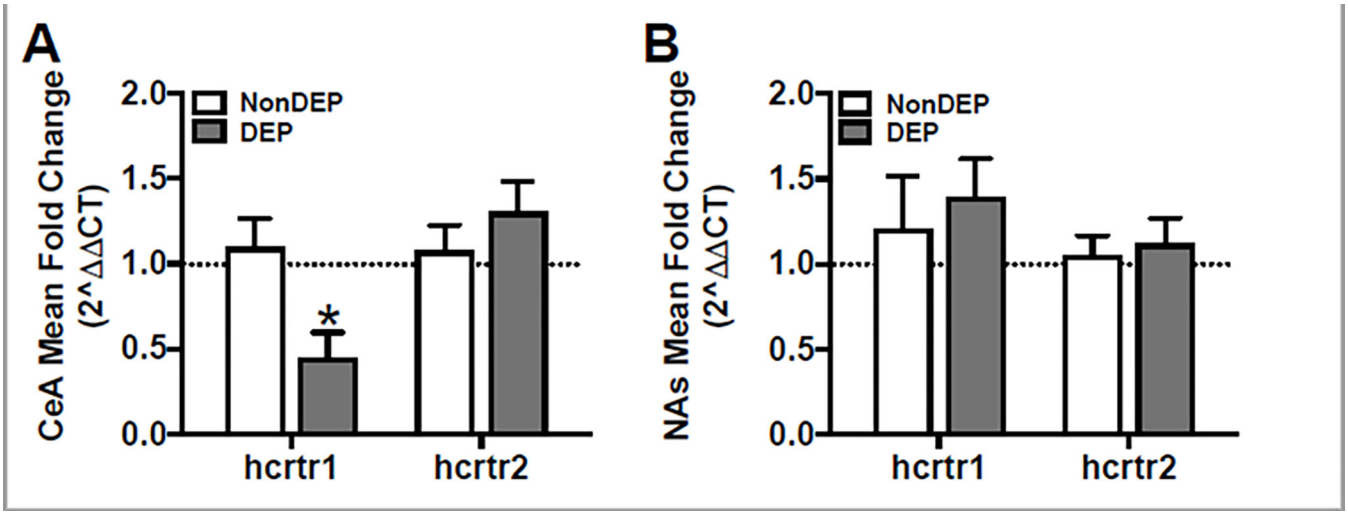

Figure 3.

Decreased hcrtr1 mRNA expression in the CeA of alcohol-dependent rats. A-B) Relative mRNA expression of each gene is expressed as mRNA expression mean (+SEM) fold change (2^ΔΔCT) in the CeA (A) and NAs (B) of alcohol non-dependent (NonDep) and dependent (Dep) rats. A) mRNA expression of *hcrtr1*, but not *hcrtr2*, was significantly decreased in the CeA of Dep (gray bars) compared to NonDep (white bars) rats. B) Neither *hcrtr1* nor *hcrtr2* mRNA expression in the NAs were significantly different between Dep and NonDep rats. *$p < 0.05$ *versus* respective NonDEP control.

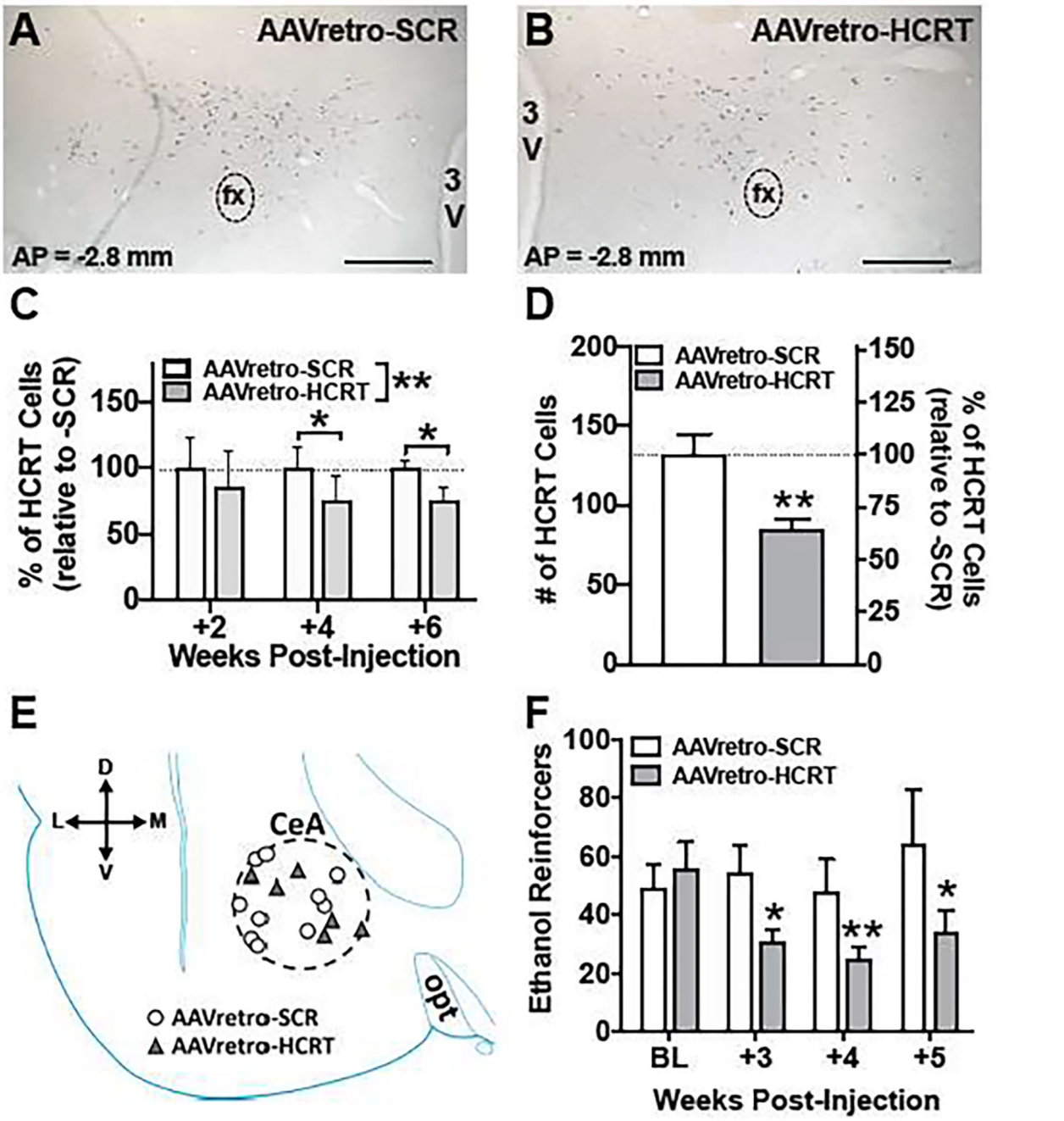

Figure 4.

AAVretro-HCRT microinjection into the CeA of alcohol-dependent rats reduces alcohol self-administration. A-B) Representative coronal sections stained with anti-preproHCRT antibody (blue/gray precipitate) to indicate HCRT-positive cells, following either AAVretro-SCR (A; scramble control) or AAVretro-HCRT (B; *hcrt*-silenced) microinjection. C) Bars represent mean percentage (+SEM) of HCRT-positive cells relative to control AAVretro-SCR treated rats at 2-, 4-, and 6-weeks following AAVretro injection in alcohol naïve rats. D) Bars represent mean number (+SEM; left) and percentage (+SEM; right; relative

to control) of HCRT-positive cells in the hypothalamus for AAVretro-SCR (control) or -HCRT treated alcohol-dependent rats. AAVretro-HCRT significantly decreased number of HCRT-positive cells. E) Schematic of AAVretro-SCR (white circles) or -HCRT (gray triangles) microinjection site locations in the CeA (dashed circle) in alcohol-dependent rats; both hemispheres are superimposed onto a diagram of the left hemisphere. F) Bars represent mean number (+SEM) of self-administered ethanol reinforcers in AAVretro-SCR or -HCRT treated alcohol-dependent rats. AAVretro-HCRT, but not AAVretro-SCR, resulted in a significant attenuation of ethanol reinforcers at 3-, 4-, and 5-weeks post-AAVretro-microinjection *versus* baseline (BL) responding. *$p<0.05$ and **$p<0.01$ *versus* respective control. Horizontal scale bar (A-B) = 500μm. 3V, third ventricle; CeA, central amygdala; D, dorsal; fx, fornix; L, lateral; M, medial; opt, optic tract; V, ventral.