Introduction

Alcohol & binge drinking

Alcohol is the most widely used psychoactive drug in the world, and alcohol use disorders (AUDs) are responsible for 6% of all deaths[1]. AUD, like most drug dependence disorders, can be conceptualized as a dysregulation between reward and stress systems, with long-term alterations in both systems[2]. The hedonic nature of drugs of abuse drives initial consumption via the reward system but drug-induced neuroplasticity leads to escalation/binge consumption and, ultimately, drug dependence[3]. Therefore, binge consumption of alcohol is a critical first step in the development of alcoholism[4–6].

Corticotropin-releasing factor system

Long-term alterations in brain stress and reward circuitry[2] that mediate the important behavioral consequences of binge alcohol drinking have been difficult to study due to the heterogeneous composition of the regions involved. One critical neural signaling molecule that bridges the gap between the stress and reward circuitry is corticotropin-releasing factor (CRF). CRF is a 41-amino acid neuromodulator involved in regulating stress and reward[7] and is also colocalized in GABA and glutamate neurons[8]. There are 2 G-protein coupled receptors for CRF, CRFR1 and CRFR2[9], with a 10-fold stronger affinity for CRFR1 vs CRFR2[10]. The amount of extracellular CRF that is available to bind is modulated by CRF-binding protein (CRF-BP), which controls extracellularly available CRF to its receptors[11,12].

CRF is believed to play a role in all three conceptual stages of drug addiction-*binge/intoxication, withdrawal/negative affect*, and *preoccupation/anticipation*[13]. CRF neurons are present throughout the brain, including the central amygdala (CeA), paraventricular nucleus of the hypothalamus (PVH), the locus coeruleus (LC), and the bed nucleus of the stria terminalis

(BNST). The CRF system in the extended amygdala is suspected to be a critical driver in the escalation of drug taking, as it is hijacked over time concomitant with the increasing strength of the aversive negative-affect-stage[14–20]. In support of this, stress-induced relapse is reduced via systemic CRF antagonist[21–23]. Stress-induced reinstatement of EtOH self-administration is attenuated by systemic CRF antagonism and is potentiated by intracerebroventricular (i.c.v.) infusion of CRF[22,23]. The CRF system may play a specific key role in regulating binge-like drinking, as systemic CRFR1 antagonism can reduce binge-like but not non-binge-like ethanol intake in rodents[24–26].

CeA, CRF, and Alcohol

CRF signaling in the CeA is critical for anxiety and fear conditioning [27] and may be a potential mediator of the negative affect stage of the opponent process theory of addiction. Binge consumption of alcohol increases immunoreactivity of CRF in the CeA immediately after drinking and persists for several days[28]. Furthermore, intra-CeA infusion of either a GABA antagonist[29] or nonspecific CRF antagonist[30] reduces alcohol consumption, and acute alcohol withdrawal activates the CRF systems in the CeA [30–33]. The effects of CRF on binge-like drinking are likely due to interactions between CeA-CRF neurons and the reward system, as CRFR1 antagonists into the CeA[28] or VTA[34] reduce binge-like drinking.

While there is an abundance of pharmacological evidence supporting the role of the CeA-CRF system, the exact role of the firing activity in CeA-CRF population, and if it increases over repeated binge drinking sessions, like CRF expression, has yet to be fully elucidated. Therefore, here, we combined optogenetics with *in vivo* electrophysiology to identify and record from CeA-CRF neurons over repeated binge-like drinking episodes to determine if CeA-CRF neuron activity is altered over time during the escalation of binge drinking. We found that CRF units dynamically encode alcohol-motivated behavior both within alcohol drinking sessions and over repeated sessions. Specifically, putative CRF units displayed either no-response/encoding

for ethanol licks or a significant increase in firing rate right before licking, which we will call a predictive response. Further, only the CRF neurons that were predictive of drinking demonstrated altered activity patterns over time as animals engaged in binge-like patterns of alcohol intake. Overall these results suggest that CRF neurons in the CeA are a heterogeneous population, with a subset of CRF neurons whose firing activity precedes the act of consuming alcohol.

Materials and Methods

Animals

$Crh^{tm1(cre)Zjh}$ mice expressing Cre recombinase under the corticotropin-releasing hormone gene (Crh) promotor were obtained from the Jackson Laboratory. They shall be referred to as corticotropin-releasing factor – cre (CRF-Cre) mice herein. All mice were heterozygous and generated by mating a male homozygous CRF-Cre mouse with a female wildtype C57. All experiments were conducted in accordance with the United States National Research Council *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

Optical-Fiber-Coupled Microelectrode arrays (OptoArrays)

Custom-made microelectrode arrays (MEA) were obtained from Innovative Neurophysiology (Durham, NC). The MEAs had 16 x 35 µm tungsten electrodes in a U-shape around a central pore for optical fibers. The MEA connector was offset from the center in a chair-like configuration to allow for the optical fiber.

Implantable optical fibers were fashioned in-house as previously described by Sparta et al (2012). Briefly, 0.48 NA 200/230 µm optical fiber was stripped and affixed with a ceramic ferrule (235 µm ID, as above) and polished. Percent transmittance (%T) of the implants were calculated using an optical power meter (PM20A, ThorLabs) and fibers with <75 %T were discarded. Fiber implants were attached to the MEA approximately 500 µm (300-800 µm) dorsal to the tips of the electrodes, at a slight angle toward the electrodes. The fibers and MEA were encased in dental cement (Industrial Grade Grip Cement, powder #675571, liquid #675572, Dentsply, York, OA).

Survival Surgeries

At 8-12 weeks of age, CRF-Cre mice (N=12) were anesthetized with isoflurane via an E-Z Anesthesia brand vaporizer (E-Z Anesthesia, Palmer, PA). A craniotomy above the CeA was centered at -1.1 mm A/P, +/- 2.95 mm L/M (from bregma) and was extended into a concentric square .+/-0.25mm A/P and +/-0.25mm L/M. An adeno-associated viral vector (serotype 5) containing a double inverted open reading frame with the ChR2(h134R)-EYFP construct (AAV-EF1a-DIO-hChR2(H134R)-EYFP-WRE-Pa was purchased from the University of North Carolina's Vector Core, with permission from Karl Deisseroth. The virus was infused into the CeA via a Hamilton 7001 1-µL syringe (Hamilton Company, Reno, NV), which was inserted into a Micro4 microinfusion pump (UMC4 controller, UMP3 pump; World Precision Instruments, Sarasota, FL). The needle was slowly lowered -4.85 mm D/V (from brain surface) into the brain and 500 nl of the virus was infused at 100 nl/min. After 5-minutes, the needle was slowly removed and the optoarrays were implanted. Optoarrays were secured to the skull using anchor screws (MF-5182 bone screws; Bioanalytical Systems, Inc, West Lafayette, IN), with the ground wire wrapped around one screw and inserted 2 mm into the cerebellum. The array was then encased in a dental cement headcap (Industrial Grade Grip Cement, powder #675571, liquid #675572, Dentsply, York, OA).

Following surgery, the mice recovered for two weeks before starting the drinking protocol. All surgical procedures were approved by the University of Maryland, Baltimore IACUC committee.

Alcohol Consumption Protocol

Mice underwent several weeks of a modified version of the drinking-in-the-dark (DID) protocol [35,36]. Briefly, 3 hours into the dark phase of their light-cycle, mice were placed into a chamber with a water bottle filled with 20% ethanol (in tap water) and food. Mice remained in the chamber for 4 hours and the number of licks and weight of alcohol consumed were analyzed. Licks were quantified by the current passed when a mouse's tongue completed the circuit between the metal floor grid and metal sipper of the ethanol bottles.

Recording Hardware

In vivo electrophysiology recordings were performed using an Omniplex-DHP system from Plexon, Inc (Dallas, TX), with a motorized commutator from NeuroTek (Toronto, Ontario, Canada). The wideband signal was acquired at 40k Hz, with an analog high-pass filter of 7.5 Hz, and a 7.5 kHz low pass filter before the signal was digitized at the headstage. The continuous spike data were extracted from the wideband signal by applying a 4-pole 500 Hz Butterworth high pass filter and a 4-pole 3000 Hz Butterworth low pass filter. Common median referencing was used for all recording sessions.

Optical Identification of Genetically-Defined Neurons (Phototagging)

Optoarray mice were tethered to a headstage and mini patch cord with ferrules on both ends plugged onto the fiber implant of the optoarray and bound to the headstage cables. The other end of the mini-patch cord remained unplugged for the duration of the 4-hour drinking session. After the 4-hour DID session, the mini patch chord attached to the mouse was then attached to the laser-attached patch chord and the phototagging procedure began. Phototagging was done in accordance with previous reports[37].

Briefly, at the beginning and end of the recording session, mice received a series of stimulation patterns at 1, 5, 10, and 20 Hz (20 4-ms long pulses each).

We defined a unit as a putative CRF neuron only when all criteria for phototagging were fulfilled: a significant increase in firing rate within 10ms after the beginning of a light pulse, when compared to the 10 ms prior to the event (as identified by a Wilcoxon signed rank test on the binned spike/sec data); the light-evoked waveforms had to have a cross-correlational coefficient R >0.9 when compared to the naturally occurring waveforms. Units were classified as light-excited, light-inhibited or no response, using Wilcoxon signed rank tests on time bins before and after the event.

Lick Response Type Classification

To classify the encoding pattern for each unit in relation to licking, we calculated peri-event raster plots and histograms for all units, from -100 ms to +100 ms, using 5 ms bins, with licks centered at time 0 sec. We analyzed the average firing rates in 50 ms periods before and after licking (Baseline: -100 ms to -50 ms; Pre-Lick: -50ms to 0 ms; Post-Lick:0 ms to +50 ms). We performed Wilcoxon rank-sign tests on these firing rates and classified the response types based on which of these comparisons were significantly different and the sign of the difference between averages. We identified four types of lick-responses: lick-excited (E), lick-inhibited (I), lick-predictive (P), and no response (NR) (see Table 1 for detailed classification criteria

TYPE	Pre-Lick - Baseline	Post-Lick – Pre-Lick
Excited (E)	No change	Sig. increase
Inhibited (I)	No change	Sig. decrease
Predictive (P)	Sig. increase	Sig. increase
No Response (NR)	No change	No change

Burst-Firing Definition and Classification

We identified bouts of burst-firing, beginning as a series of at least two spikes with an interspike-interval less than 50 ms, and ending when the interspike-interval exceeded 100 ms. Furthermore, units were classified based on their firing rate and % of spikes in bursts (%SiB), as previously reported [38,39], but with revised criteria according to the distribution of firing rate and %SiB for these neurons. We applied a cutoff of 4 Hz for the firing rate and 30% for the %SiB. We identified 4 types: low-firing/high-burst (LFHB), low-firing/low-burst (LFLB), high-firing/high-burst (HFHB), and high-firing/low-burst (HFLB).

Data Processing and Analysis

Plexon OfflineSorter 4 was used to process and separate identified units from each recording. Multiple putative units from the same electrode were considered to be different if their waveforms were significantly separated in 3D principal component space, as tested by multivariate ANOVA ($p<.05$), with L-ratios <0.05 for each cluster.

Units were classified based upon their responses to light stimulation, and licks

Outlier Time Bin Removal and Replacement

In order to eliminate any large fluctuations in noise that may have contaminated individual time bins out of the 4-hour recordings, we first calculated raw firing rate in 5-minute bins. We then used the Matlab function filloutliers to identify and replace outlier time bins, as identified using a sliding window 6 time-bins-wide (30 mins) to calculate the median, and any bins that were more than 3 scaled deviations away from the median were identified as outliers and substituted with a linear interpolation of the time bins surrounding the outlier. This was done prior to the baseline subtracted firing rate z-score calculation. The same outlier time bins were also used to remove outlier time bins for burst firing metrics from correlation analyses.

Statistics

All data are presented as means ± SEM and analyzed using GraphPad Prism 7 and Matlab 2017. Comparisons between CRF and non-CRF units were analyzed using Mann-Whitney U tests. Comparisons between 3 unit types were analyzed using Kruskal-Wallis tests with *post-hoc* Dunn's multiple comparison tests. All comparisons across hours were done using two-way repeated measures ANOVA with *post-hoc* Sidak's multiple comparison tests for between-group comparisons and *post-hoc* Tukey's multiple comparison tests. Comparisons between early and late ethanol session correlations were analyzed using Kolmogorov-Smirnov tests.

Results

Identification and electrophysiological profile of CRF vs non-CRF neurons

Here we used our opto-arrays to record from optically identified CRF neurons within the CeA during our binge drinking behavior sessions (**Fig 1 A, B**). We analyzed 149 units, of which 59 were classified as putative CRF units, 75 non-light-responsive, 4 light-excited, and 11 light-inhibited (**Fig 1C**). Herein, we refer to non-light-responsive units as non-CRF neurons and phototagged units as putative CRF neurons. Due to their higher prevalence, we focused our analyses on these two categories. We evaluated several electrophysiological parameters (**Fig 1D**) and found that CRF neurons had higher firing rates and burst rates, and smaller coefficients of variation (an index of firing regularity) when compared to non-CRF units (**Fig 1D**).

CeA-CRF neurons encode licking for ethanol, with distinct firing characteristics

We then examined whether CRF units encoded voluntary consumption of alcohol, using licking as our behavioral index. CRF-Cre mice steadily increase enhanced drinking during 4-hour sessions (**Fig 2A, left**). They also develop a robust increase in licking for ethanol over repeated sessions compared to water (**Fig 2A, right**).

We next determined if CRF unit firing activity was correlated with ethanol licking. We therefore compared CRF vs non-CRF units during early ethanol sessions (session #1-8) versus late sessions (session # 17+). We found that only CRF units significantly increased the strength of their correlation in late sessions compared to early sessions (**Fig 2B**).

To ascertain if CRF units encoded ethanol consumption, we analyzed peri-event histograms for each unit (see General Methods for lick-response-type classification). We identified four types of lick-responses: lick-excited (CRF-E), lick-inhibited (CRF-I), lick-predictive (CRF-P), and no response (CRF-NR) (**Fig 2C**). We focused our analyses on the two most

prevalent types, CRF-P and CRF-NR (which represented 86.4% of all CRF units, **Fig 2D**), and also included non-CRF non-lick responsive (non-CRF-NR) as a control group.

We found that CRF-P neurons had a significantly higher firing rate, as well as the percentage of spikes in bursts (%SiB), burst duration, burst rate, and # of spikes per burst compared to both CRF-NR or non-CRF-NR units (**Fig 2E**), suggesting that CRF-P cells were more active during alcohol consumption.

CRF-P units increase firing activity throughout drinking sessions

We then compared the change in firing rate within-sessions between CRF-P and CRF-NR neurons, using Z-scores normalized to the first 30 minutes as baseline (**Fig 3A**). We found a significant difference among CRF types, which diverged for hours 3 and 4, with CRF-P units having higher firing rates (**Fig 3B, top**). Then analyzing the effect of firing rate vs session-hour for each CRF type alone, post hoc tests revealed CRF-P units increased firing rates within-session, whereas CRF-NR neurons did not (**Fig 3B, top panels**). We also compared changes in %SiB within-session and found no effect of session-hour, but a significant difference between CRF types, with CRF-P neurons showing higher %SiB (**Fig 3B, bottom panels**).

We concluded that CRF-P units changed firing rate dynamically during ethanol sessions, while bursting activity remains consistently higher throughout sessions.

CRF lick-response types show heterogeneous changes in firing activity

When we ranked each CRF type by the change in firing rates from hour 1 to hour 4 (Δ-rate) (**Fig 3 A-C**) we found each CRF type had two sub-groups, with either increased firing rates (Δ+) or decreased (Δ-). We then split each CRF-NR and CRF-P units into two sub-groups: CRF-NR(Δ+), CRF-NR(Δ-), CRF-P(Δ+), and CRF-P(Δ-). Next, we compared the Δ-rate sub-groups CRF-NR(Δ+) vs. CRF-NR(Δ-), and CRF-P(Δ+) vs CRF-P(Δ-), revealing that the Δ-rate sub-groups were indeed significantly distinct groups for both CRF-NR and CRF-P units (**Fig 3C, left,**

orange vs blue bars). Additionally, CRF-P(Δ+) neurons increased rate significantly more than CRF-NR(Δ+) neurons, but there was no significant difference between the decrease in rates of CRF-P(Δ-) and CRF-NR(Δ-) (**Fig 3C, left**). We then separated the normalized firing rates for all 4 sub-types for further analysis (**Fig 3C, right**).

First, we compared the changes in firing rate between sub-groups over the full session, using average normalized firing rate and %SiB by hour (**Fig 3D**). We found that CRF-P(Δ+) units' firing rates increased steadily and were robustly higher by hours 3 and 4 while CRF-P(Δ-) cells showed no significant change throughout the session (**Fig 3D, top-left**)**.** While CRF-(Δ+) units dynamically changed firing rate throughout the session, they showed only modest and inconsistent changes in %SiB, as only hour 1 and hour 3 were statistically distinct (**Fig 3D, bottom-left).** On the other hand, despite the firing rate of CRF-P(Δ-) neurons was indipendent of session time, we found they markedly decreased their %SiB throughout the session (**Fig 3D, bottom-left).**

Therefore, we concluded that while CRF-P(Δ+) sub-groups showed a substantial rise in firing rate over the course of ethanol drinking sessions, accompained by a modest increase in burst firing. CRF-P(Δ-) did not change their overall firing rate across the session, but significantly decreased their bursting activity. Whereas the combined CRF-NR units did not significantly change firing rates during the session (**Fig 3B**), both sub-groups showed modest changes in firing rate and were statistically different for hours 2-4 (**Fig 3D, top-right)**. While CRF-NR(Δ+) neurons only increased rate by hour 4, CRF-NR(Δ-) units significantly decreased firing rate after hour 1 (**Fig 3D, top-right**). When we analyzed the change %SiB, we found a similar pattern, with CRF-NR(Δ+) increasing %SiB by hour 4, and (Δ-) units decreasing %SiB for the final two hours (**Fig 3D, bottom-right)**. Interestingly, the changes in rate and %SiB for CRF-NR units were to a much smaller degree than those seen in CRF-P sub-groups.

Overall, we concluded that the CRF-P units were particularly responsive over the 4-hour drinking sessions, either by increasing firing rates as in the CRF-P(Δ+) or by decreased bursting as in the CRF-P(Δ-) units. CRF-NR units changed firing rate and %SiB over the 4-hour sessions, however, these changes were modest and became evident only by separation into the CRF-NR(Δ+) and CRF-NR(Δ-) sub-groups.

CRF-P units increase firing and bursting activity over repeated drinking sessions

Finally, we examined whether the firing activity of CRF neurons changed over repeated sessions of ethanol consumption. When we compared CRF lick-response types, we found that CRF-P units' firing rates were much higher in later sessions compared to early sessions, while CRF-NR units did not display any significant change in their firing rates in later drinking sessions (**Fig 4A**). We also examined the %SiB in early vs late sessions and found similar results, with only CRF-P cells showing a substantial increase in bursting in later sessions, while CRF-NR units showed no change (**Fig 4B)**.

Altogether, this evidence further supports a relationship between prolonged/repeated ethanol consumption and the firing/burst properties of CRF neurons in the CeA. Moreover, there is a population of CeA-CRF units that fire immediately before the mouse licks for ethanol (CRF-P units), and these neurons dynamically modify their firing rate and %SiB during the 4-hour ethanol drinking sessions, as well as after repeated ethanol sessions.

DISCUSSION [Word limit of results+Discussion=1750]

Here we identified a subpopulation of CRF-expressing neurons in the CeA whose increased activity predicts (CRF-P) the mouse licking for ethanol in a binge drinking paradigm. Over repeated drinking sessions, their firing and bursting activity increases, whereas non-lick-response CRF did not. A possible explanation for heterogeneous plasticity within genetically-isolated neurons (CRF-P: 40% of CRF) may be explained, in part, in a recent co-labeling study that reports only 1/3 of CRF neurons in the CeA co-expresses Ca/CamKII (Dedic *et al*, 2018), suggesting enhanced capability .

Our findings are supported by prior pharmacological evidence on the role of CRF in binge drinking behavior (refs from intro). The reason for only a subpopulation of CRF units predict behavior may be explained by projection targets (/neurocircuitry?). CeA-CRF send projections to the ventral tegmental area (VTA), where GABAergic CRF-expressing-terminals synapse onto non-dopaminergic neurons[ref?].

The action of CRF is considered to be an excitatory one, altering the NMDA -ESPC to increase excitability of post synaptic neurons [ref]. Increased activity of the non-DA neurons may…….

Interestingly, evidence has shown that the role of CRF receptors in the VTA changes after repeated drug exposure, in this cocaine [CRF 1 vs CRF 2 from Kelly Fuge]. The increased firing rate and burst-firing of CRF neurons may be responsible for this effect, as increased activity would lead to increased CRF release, which would raise the extracellular concentrations of CRF. The [Kelly Fudge 2018]

Future studies should use retroviruses to optical isolate only VTA-projecting CeA-CRF neurons. Future studies could also use activity-dependent labeling techniques to identify where the neurons active during drinking project.

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