Neuropeptide Y in the Medial Prefrontal Cortex Modulates Ethanol Consumption in Mice

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Binge-like drinking behavior has been estimated to cause about 80,000 deaths in the United States annually and is a significant risk factor for developing ethanol dependence. This behavior is partially regulated by control of the amygdala by the medial prefrontal cortex (mPFC). A neurotransmitter called neuropeptide Y (NPY) binds to neurons in the mPFC projecting to the amygdala, thus we hypothesized that NPY regulates binge-like drinking behavior. In the mPFC, NPY has a post-synaptic receptor NPY1R, and a presynaptic receptor NPY2R. To test NPY's affect in modulating ethanol intake, we pharmacologically agonized NPY1R and pharmacologically antagonized NPY2R. The agonism of NPY's post-synaptic receptor and the antagonism of NPY's presynaptic receptor both resulted in reduced ethanol intake compared to vehicle treated animals. To further study NPY's role, we chemogenetically inhibited neurons in the mPFC expressing NPY1R through the use of a Designer Receptor Exclusively Activated by a Designer Drug (DREADD). This specific inhibition of NPY1R expressing cells in the mPFC resulted in decreased consumption of ethanol. To examine the effects of binge-like drinking on NPY activity in the mPFC, we performed immunohistochemistry and found a decrease in NPY immunoreactivity after three weeks of binge-like drinking. These results establish a role for NPY modulation of binge-like drinking behavior in mice. This study could help understand how people develop alcohol dependence and suggest therapeutic strategies.

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Neuropeptide Y's Role in Modulating Ethanol Consumption in the Medial Prefrontal Cortex in Mice

Introduction:

Binge-like drinking behavior is estimated to have led to about 80,000 deaths in the United States annually and is a significant risk factor for developing an ethanol dependence (NIAAA. 2016; Kanny, D, 2012). Previous research has identified three major elements of addiction (Koob, George Fv. 2015; Volkow, N. D., Koob, G. F., & McLellan, A. T. 2016). The first element of the addiction model is a decrease in the reward feedback due to the substance, where the substance does not offer the same stimulation as it originally did. The next element is an increase in the number of and intensity of behavioral and physical responses a person has to the substance. The last characteristic is that the regions of the brain that regulate decision-making are damaged due to continual exposure to the substance (Volkow, N. D., Koob, G. F., & McLellan, A. T. 2016;). This cycle of development and maintenance of an addiction are still being studied.

A notable effect of alcohol use is alcohol's ability to decrease anxiety and stress, which has been noted to be a factor in the development of addiction following long-term use (Frone, Michael R. Work. 2016). Due to alcohol's role in decreasing stress, the brain regions associated with modulating emotion are areas of interest to examine the effects of alcohol on the brain. One such center is the amygdala which is a regulator of mood and emotion in the limbic system (Lalumiere RT. 2014; Pleil KE, Lowery-Gionta EG, Crowley NA, Li C, Marcinkiewcz CA, Rose JH, et al..2015). One pathway of interest is the possible top-down regulation of the amygdala by the medial prefrontal cortex (mPFC). The mPFC has been shown to be a site of executive functioning and a regulator of reward seeking behavior (Ramnani, Clare. Stephen M Smith.2004). The mPFC is subdivided into the more ventral infralimbic region (IL) and more dorsally located prelimbic region (PL) (Lalumiere RT 2014). The IL region has been shown to regulate behavior associated with habitual-oriented behavior, while the PL region of the mPFC has been associated with goal-oriented behavior (Vertes RP 2004). Also, both of these regions are known to have projections that innervate the basolateral amygdala (BLA) (Barker, J. M., Taylor, J. R., & Chandler, L. J. 2014; Carballedo A, Möller HJ, et al. 2011). The BLA is a critical regulator of the extended amygdala (EA), which is associated with

emotional behavior as well as a driver of alcohol consumption (Lowery-Gionta, E., Navarro, M., Li, C., Pleil, K., Rinker, J., & Cox, B. et al. 2012). Due to the mPFC's position to regulate the amygdala's activity, and therefore anxiety and stress-related behaviors, the mPFC is of great interest when studying brains regions associated with the development of ethanol dependence.

Within the mPFC, a subpopulation of the primary inhibitory GABAergic interneurons express neuropeptide Y (NPY) (Kubota Y, Shigematsu N, Karube F, Sekigawa A, Kato S, Yamaguchi N, et al. 2011; Lalumiere RT. 2014). NPY is a 36 amino acid polypeptide chain that is part of the neuropeptide tyrosine family (Allen, Y. S., Bloom, S. R., & Polak, J. M. 1986). NPY could be an important modulator of ethanol dependence due to its role as an anti-stress neurotransmitter (Allen, Y. S., Bloom, S. R., & Polak, J. M. 1986; Robinson, Stacey L., & Thiele, Todd E. 2017). NPY is a signaling molecule that binds to a G protein-coupled receptor that causes a signal cascade within cells and inhibits the postsynaptic neuron from firing (Thiele. 2017). The GABAergic interneurons that release NPY modulate the mPFC's excitatory glutamatergic projection neurons and local inhibitory GABAergic signaling (Klein CR, et al. 2013). The NPY system includes a post-synaptic receptor NPY1R on glutamatergic and GABAergic neurons and a mostly pre-synaptic homo- and heteroreceptor NPY2R that is expressed on the GABAergic interneurons (Palmiter RD et al. 1998; Sparrow AM 2012). As a homoreceptor, NPY2R has also been implicated as a possible reuptake channel for NPY, so it's activation could affect the amount of NPY in the synaptic cleft, therefore indirectly affecting NPY1R signaling (Sparrow AM 2012).

One transgenic decreased NPY expression, which then led to increased ethanol consumption and, conversely, an over-expression of the NPY gene led to decreased ethanol consumption (Thiele TE, Palmiter RD. 1998). This earlier method of ubiquitously over-expressing NPY via transgenic means provided evidence of NPY's ability to modulate ethanol consumption, but this method did not isolate where in the brain NPY is able to target. Part of this study's goal is to target one of NPY's sites of modulation to the mPFC.

Due to NPY's position to influence ethanol intake, an in-depth analysis of NPY's role could offer insights into the biological changes that take place in the brain that lead to the transition to ethanol

dependence. The role of NPY in regulating the mPFC's projections to the amygdala has yet to be evaluated. Based on the previous research implicating NPY as a modulator of binge-like ethanol consumption, it was predicted that increasing NPY signaling in the mPFC will decrease binge-like ethanol consumption. In this study, the NPY signaling was studied through the use of pharmacological excitation of NPY's postsynaptic receptor and inhibition of NPY's presynaptic receptor. Furthermore, NPY1R expressing (NPY1R+) cells in the mPFC were chemogenetically inhibited to provide further evidence of NPY's integral role in the development of alcohol addiction. Along with these interventional experiments, the change in immunoreactivity of NPY in the mPFC was measured after binge-like exposure to ethanol. To model binge-like ethanol consumption in mice in all three experiments, the "Drinking in the Dark" (DID) method was used in this project. DID has been shown to be a useful tool in modeling binge-drinking behavior in mice, which can then be used to determine if a treatment process will alter an animal's drinking pattern (Thiele TE, Crabbe JC, Boehm SL 2014; Sparrow GM, Thiele TE 2012). This analysis of NPY's role in ethanol dependence could help understand how people transition to alcohol dependence and provide targets for therapeutics.

Methods:

Animals:

For the immunohistochemistry and pharmacological experiments, male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were obtained at about 6-8 weeks of age. For the chemogenetic studies NPY1R-Cre mice (positive for Cre-recombinase under the NPY1R promoter, determined by standard polymerase chain reaction genotyping) on a C57BL/6J background >10 weeks old at experiment start. The animals were housed individually in plastic containers in a room that was approximately 22°C and were given a week to acclimate before trials. The mice were maintained on an alternating light-dark cycle of 12 hours of darkness starting at 8am and 12 hours of light starting at 8pm. The mice had access to Prolab® RMH 3000 (Purina LabDiet®; St. Louis, MO) and water, unless otherwise stated in experiments. All procedures used in this study are in accordance with the National Institute of Health guidelines, and were approved by the University of North Carolina Institution Animal Care and Use Committee.

"Drinking in Dark" Procedures

The DID method has been used to obtain high levels of ethanol consumption in mice (Thiele TE, Crabbe JC, Boehm SL.2012). The DID procedure was done over a 4-day period. On the first 3 days, the animals' waters were removed from their cages and they were given access to ethanol for 2-hour periods, 3.5 hours into their dark cycle. On the 4^{th} day, the animals were given their drug treatment (described below) 30 minutes prior to their trial and then the animals' water bottles were switched from water to ethanol. The trials ran for 2 hours. For the immunohistochemistry experiments, the animals underwent three cycles of DID. For the pharmacological and chemogenetic studies, the animals underwent 2 cycles of DID with ethanol followed by 2 cycles of DID where sucrose (3%) took the place of ethanol in each part of the procedure. Sucrose trials are an effective control experiment because sucrose has a caloric value and is rewarding to mice, similar to ethanol. See Figure 1a for the DID procedure for the IHC experiments and

Figure 2a for the pharmacological and chemogenetic experiments. In pharmacological and chemogenetic studies tail blood samples (≈60μL) were taken immediately following DID procedure to determine BECs using the AM1 Alcohol Analyzer (Analox, London, UK).

Surgery

Before surgery, the mice were anesthetized with a mixture of xylazine (10mg/kg) and ketamine (100mg/kg) via I.P injection. For the mice in the pharmacological studies, a bilateral 26-guide cannulae was implanted in the mice's mPFC (AP: 1.7, ML: ±0.4, DV: –2.6) or BLA (AP: -1.22, ML: ±3.01, DV: –4.75) with an Angle II™ Stereotax (Leica Instruments, Buffalo Grove, IL) and the help of stereotaxic atlas. For the mice that were used in the chemogenetic studies, the mPFC was injected with 0.3-0.5 μL/side (over a 3-5 minute period) of either a Cre-dependent control vector (CON DREADD; AAV8-hSyn-DIO-mCherry) or the Cre-dependent Gi coupled Designer Drug Exclusive Activated by Designer Drug (DREADD) vector (Gi-DREADD; AAV8-hSyn-DIO-hM4d-mCherry (UNC vector core, North Carolina)). The injection needle remained in place for 10-15 additional minutes before being withdrawn. Mice recovered for >3 weeks before being used for experiments.

Drug Administration

On the DID test days for the mice, the animals in the pharmacological studies either received their respective vehicle or drug (NPY1R agonist Leu, Pro-NPY (99 pmol/0.5μl/side) or NPY2R antagonist BIIE0246 (3μg/0.5μl/side) (Tocris, Minneapolis, MN)) 30 minutes before the experiment. The drugs were administered to the mPFC cannulae at a rate of 0.3uL/min via a Hamilton syringe (Reno, NV) attached to a Harvard Apparatus PHD 2000 infusion pump (Holliston, MS). After the infusion, the injectors were kept in place for 0.5-1 minute to allow for diffusion of the drug. These trials were done with a Latin-square design where the mice were randomly assigned to receive either the drug or the vehicle on the first test day of a DID cycle and then on the second DID cycle test day the animal would receive the alternate treatment

(See Fig. 2a for diagram). Comparing the change in ethanol intake for each mouse following drug treatment to the vehicle treatment allows each mouse to act as their own control. Also, by randomly assigning animals to treatment groups, there were groups of mice that underwent drug treatment first followed by vehicle treatment, and vice versa.

The animals in the chemogenetic studies either received Clozapine-N-Oxide (CNO; intraperitoneal (I.P.) 3.0 mg/kg; microinjection to BLA 900 pmol/0.3μl/side; Sigma-Aldrich, St. Louis, MO; in 0.5% DMSO + Saline) or vehicle via an I.P. injection or microinjection to the BLA. These trials were also done using a Latin-square design.

Perfusion of Mice and Brain Slide Preparation

Mice were sacrificed with a ketamine overdose and were transcardially perfused with phosphate buffered (0.1M) saline directly followed by paraformaldehyde (4%). The brains were extracted and preserved in paraformaldehyde (4%) for 24 hours and then the brains were sliced using a vibrating microtome (Leica VT1000S; Wetzlar, Germany). The slices for the pharmacological and chemogenetic studies were mounted on slides for inspection of cannulae placement with an optical microscope (BX-51, Olympus). Any animals whose cannulae were misplaced were excluded from the study.

Immunohistochemistry

Mice used for immunohistochemistry went through three cycles of DID with ethanol, or three cycles of water DID. In the ethanol trials, after the last cycle of DID, blood samples were collected for blood ethanol concentration (BEC) analysis. Mice then went through the same perfusion and brain slicing procedure described in Perfusion of Mice and Brain Slide Preparation section. The slices of brain were then incubated in citrate buffer at 66°C for 1 hour for antigen retrieval and then used for NPY immunoreactivity detection. The slices were incubated in a 1° anti-NPY (1:1000) (produce/specified verified by ABcam, Cambridge, United Kingdom) for 72 hours. The slices were then incubated in a secondary solution for 2

hours (DyLight 488, 1:5000, Vector Laboratories, Burlingame, CA), and mounted on glass slides. The brain slices were then imaged via a digital camera (Roper Scientific), mounted on an optical microscope (BX-51, Olympus), and the amount of fluorescence was quantified using ImageI (NIH).

Open Test and Anxiety Like Behavior

A week after the animals in the pharmacological and chemogenetic studies completed the trials where they were exposed to sucrose, mice underwent behavioral testing (three weeks after DID ethanol or water testing). Animals were randomly assigned into either drug or vehicle groups in a similar manner as in the DID testing. The tests began 4-6 hours into the dark cycle of the mice. The possible change in locomotor activity due to treatments was observed by VersaMax® software program (AccuScan Instruments, Inc., Columbus, OH) (dimensions: 16.5 x 16.5 x 12 inches) over 2h period with five minute bin outputs. Time spent in the chamber center during the first five minutes of the trial period was recorded to determine any effects on anxiety-like behavior. Time spent in the center of the chamber is meant to measure the willingness the animal is to be exposed in a non-covered area, so measuring that time in the chamber provides a numerical value for anxiety-like behavior (Prut L, Belzung C. 2003).

Statistical Analysis

GraphPad Prism (GraphPad Software, Inc. La Jolla, Ca) was used to analyze and graph all data, save three-way ANOVAs which were performed in SPSS statistics (IBM Analytics, Armonk, New York). Two-way ANOVAs and Bonferroni post-hoc tests were used to determine the effect of binge cycle on NPY immunoreactivity and treatment versus time during individual DID drinking hours. T-tests were used to evaluate effect of treatment on total intake and BEC. All data are reported as the mean ± standard error of the mean and considered significant if $p<0.05$. Animals were removed from analysis if they were found to be a significant outlier as detected by a Grubbs test (Alpha=0.05) or cannulae misplacement (unilateral or bilateral) or inappropriate (lack of/incorrectly placed) DREADD expression was found. Cannula placement

was determined by locating the end of the guide cannulae and adding injector projection length (2MM in mPFC and 0.5MM in BLA). Three-way ANOVA analysis found no significant effect of treatment order in any Latin-square design experiment, therefore these data were collapsed and analyzed by two-way ANOVA. Two-way ANOVA found no significant effect of sex on any data set, therefore data from males and females were collapsed.

Results:

Three Cycles of binge-like ethanol intake decreased PL NPY immunoreactivity, but did not change IL NPY immunoreactivity

The model for addiction notes there are changes that take place in the brain as an organism begins to become dependent on ethanol, but the mechanisms by which the brain's biochemistry changes are not completely known. The following experiment looked to examine the changes in NPY expression that would take place in the early stages of binge-like ethanol consumption. To determine the change in NPY immunoreactivity one cohort of mice (N=12) went through 3 round of DID and another cohort of mice (N=10) went through water exposure. A two-way ANOVA test revealed a significant change in NPY immunoreactivity due to ethanol exposure [$F(1,20)=4.422$, $p<0.05$], but not mPFC subregion [$F(1,20)=3.613.$, $p>0.05$; interaction: $F(1,20)=0.458$, $p>0.05$] [**Fig. 1b, 1c**] on NPY expression. NPY expression was found to be significantly correlated with ethanol intake in the prelimbic (PL) [$r=0.623$, $p<0.05$] [**Fig. 1d**], but not in the infralimbic (IL) [$r=0.301$, $p>0.05$] [**Fig. 1e**] subregion. NPY expression was not found to correlate with water intake in either the PL [$r=0.163$, $p>0.05$] [**Fig. 1f**] or IL [$r=-0.52$, $p>0.05$] [**Fig. 1g**].

NPY1R Agonism in the mPFC Selectively Decreases Binge-Like Ethanol Intake

NPY has been implicated as a possible modulator of binge-like drinking due to its role as an anti-stress neuropeptide (Robinson, Stacey L., & Thiele, Todd E. 2017). Pharmacologically activating NPY1R in the mPFC would mimic the presence of NPY in the synaptic cleft and was expected to reduce ethanol consumption in mice. An ANOVA test evaluating time and cranial microinjection of NPY1R agonist Leu Pro-NPY showed significantly reduced ethanol consumption compared to vehicle treatment (N=21) [Treatment: $F(1,20)=10.62$, $p<0.01$; Time: $F(1,20)=0.997$, $p>0.05$; interaction: $F(1,20)=0.053$, $p>0.05$; Total t-test: $t=3.259$ $df=20$, $p<.01$] [**Fig. 2b**]. There was a significant reduction in the blood ethanol concentration (BEC) of drug treated animals compared to vehicle treated animals (N=18) [$t=2.223$ $df=17$, $p>0.05$] [**Fig. 2c**]. This was a specific interest because the BEC of drug treated animals where below 80 mg/dl, which is the National Institute of Alcohol Abuse and Alcoholism (NIAAA) standard for what is considered a binge of ethanol consumption (represented as dashed line) (NIAAA. 2016; Kanny, D, 2012). A caloric reinforcing liquid- sucrose (3%)- was used to determine if NPY1R agonist activity was specific to ethanol consumption or other rewarding stimuli. Two-way ANOVA test did not find a significant change in sucrose consumption between drug treated animals and vehicle treated animals (N=14) [Treatment: $F(1,13)=0.0128$, $p>0.05$; Time: $F(1,13)=0.29$, $p>0.05$; interaction: $F(1,13)=2.367$, $p>0.05$] **[Fig. 2.d**]. Mice that had cannula placements outside of the mPFC region were excluded from the study [**Fig.2.e]**.

To examine the effect of Leu Pro-NPY on locomotor activity, an open-field test tracking the mice's total distance travelled over a 2-hour period was performed. This experiment showed no significant change in locomotor activity of the mice due to Leu Pro-NPY at this dosage [Two-way ANOVA: time = $F(23, 253)=40.12$, $p<0.0001$; treatment = $F(1, 11)=1.238$, $p>0.05$; interaction: $F(23,253)=0.674$, $p>0.05$; Vehicle N=8, Leu, Pro-NPY N=5] [**Fig 3.a**]. The locomotor activity of the animal tested whether the change in decrease in ethanol consumption was due to a decrease in overall movement of the animal. To examine the effect of Leu Pro-NPY on anxiety-like behavior, the first 5 minutes of the animals in the open-field chamber

was examined. Time spent in the center of the open field chamber during the early test period is a generally accepted measure of anxiety-like behavior in rodents (Prut L, Belzung C. 2003). There was not a significant decrease in anxiety-like behavior in mice at this dose of Leu Pro-NPY ($t=0.9389$ $df=11$, $p>0.05$; Vehicle N=8; Leu, Pro-NPY N=5)[**Fig.3.b**].

NPY2R Antagonism in the mPFC Selectively Decreases Binge-Like Ethanol Intake

To further examine NPY's role of developing binge-like ethanol behavior, pharmacological inhibition of NPY's presynaptic receptor, NPY2R, was examined. NPY2R has been implicated as an important modulator of the amount of released NPY in the synaptic cleft (Sparrow AM 2012). A two-way ANOVA test was used to evaluate time and microinjection of NPY2R antagonist BIIE0246 and found significant reduction in ethanol consumption compared to vehicle treated animals (N=14) [Treatment: $F(1,13)=7.639$, $p<0.05$; Time: $F(1,13)=3.3531$, $p>0.05$; interaction: $F(1,13)=3.224$, $p>0.05$; Total t-test: $t=2.87$ $df=13$, $p<0.05$] [**Fig. 4a**]. There was a corresponding significant decrease in the BEC of drug treated animals compared to vehicle treated animals (N=10) (4 blood samples lost due to mechanical error) [$t=2.921$ $df=9$, $p<0.05$] [**Fig. 4b**]. The decrease in BEC was also below the threshold for what is considered a binge of ethanol consumption. Sucrose (3%) trials were once again used to determine if drug treatment was selectively decreasing ethanol intake or had an effect on other reinforcing substances. Two-way ANOVA test evaluating time and treatment did not find a significant change in sucrose consumption between drug treated animals and vehicle treated animals (N=14) [Treatment: $F(1,12)=0.979$, $p>0.05$; Time: $F(1,12)=4.338$, $p>0.05$; interaction: $F(1,12)=0.125$, $p>0.05$; Total t-test: $t=0.9898$ $df=12$, $p>0.05$] [**Fig.4c**]. Mice that had cannula placements outside of the mPFC region were excluded from the study [**Fig.4.d]**.

To examine the effect of the NPY2R agonist BIIE0246 on locomotor activity, an open-field test tracking the mice's total distance travelled over a 2-hour period was performed. This experiment showed no significant change in locomotor activity of the mice due to BIIE0246 at this dose [Two-way ANOVA:

time = F(23,299)=26.97, p<0.0001; treatment = F(1,13)=0.2791, p>0.05; interaction: F(23,299)=0.7306, p>0.05; Vehicle N=10, BIIE0246 N=5][**Fig.5.a**). To examine the effect of BIIE0246 on anxiety-like behavior, the first 5 minutes of the animals in the open-field chamber was examined. There was not a significant decrease in anxiety-like behavior in mice at this dose of BIIE0246 (t=0.5788 df=13, p>0.05; Vehicle N=10; BIIE0246 N=5) (**Fig.5.b**).

Chemogenetic inhibition of mPFC NPY1R neurons decreased binge-like ethanol consumption

NPY binding to NPY1R (a Gi/o-coupled receptor) generates an inhibitory postsynaptic potential, and so chemogenetically inhibiting through a Gi-coupled DREADD NPY1R+ cells in the mPFC was a direct way to target neurons expressing NPY1R and mimic the effect caused by NPY (Anthony N. van den Pol. 2012). In NPY1R-cre mice, mPFC neurons that were injected by the DREADD virus expressed Gi-coupled DREADDS. These receptors were chemogenetically inhibited via an I.P CNO injection. CNO treatment significantly reduced binge-like ethanol consumption compared to vehicle treatment after 2 hours of ethanol exposure and this was analyzed using two-way ANOVA (treatment vs. time) (N=12) [treatment: F(1,11)=6.684, p<0.05; time: F(1,11)=0.004, p>0.05; interaction: F(1,11)=2.114, p>0.05; Total t-test: t=2.585 df=11, p<0.05] [**Fig. 6a**]. There was not a significant decrease in BEC between CNO and vehicle treatment expressing Gi-coupled DREADDS in NPY1R+ mPFC cells (N=10) (2 blood samples lost due to mechanical error) [t=1.546 df=9, p>0.05] [**Fig.6b**]. There was also no significant change in consumption of 3% sucrose solution [treatment: F(1,9)=0.123, p>0.05; time: F(1,9)=6.028, p<0.05; interaction: F(1,9)=0.003, p>0.05] [**Fig. 6c**].

To verify that there were no off-target effects of CNO administration on the mice's ethanol consumption mice were injected with a control (CON) DREADD virus in the mPFC and run through the same procedure previously described. These CON DREADD treated animals would not express a receptor

that CNO would bind to when administered. These mice were not found to have a significant impact on ethanol consumption after administration of CNO using ANOVA analysis (N=9) [treatment: $F(1,8)=0.0916$, $p>0.05$; time: $F(1,8)=1.326$, $p>0.05$; interaction: $F(1,8)=0.2158$, $p>0.05$; Total t-test $t=0.3027$ $df=8$, $p>0.05$][**Fig.6d**]. The CON DREADD animals also did not show a significant change in BEC after an administration of CNO [t-test $t=0.3153$ $df=8$, $p>0.05$] [data not shown]. The CON DREADD animals also did not show a significant change in intake of 3% sucrose solution after an administration of CNO (N=9) [treatment: $F(1,8)=1.142$, $p>0.05$; time: $F(1,8)=1.032$, $p>0.05$; interaction: $F(1,8)=0.495$, $p>0.05$; Total t-test $t=1.069$ $df=8$ $p>0.05$] [**Fig. 6e**]. Brains that did not have expression of DREADD receptors (tagged in red) were excluded from the study [**Fig. 6f].**

Chemogenetic inhibition of NPY1R+ mPFC-BLA projection neurons decreased binge-like ethanol consumption

The mPFC's top-down regulation of the amygdala was specifically investigated by performing a similar chemogenetic inhibition used above, with an injection of Gi-DREADD or CON-DREADD for the respective trials. In the NPY1R-cre mice, mPFC neurons projecting to the BLA expressing CON or Gi DREADDS were chemogenetically inhibited via a BLA microinjection of CNO. The administration of CNO directly to the BLA is expected to specifically activate the DREADDs in that region of the brain, rather than an I.P. injection which would activate DREADDs wherever they are expressed. CNO treated animals significantly reduced binge-like ethanol consumption compared to vehicle treated animals and this was analyzed using two-way ANOVA (treatment vs. time) (N=6) [treatment: $F(1,5)=21.73$, $p<0.01$; time: $F(1,5)=1.178$, $p>0.05$; interaction: $F(1,5)=0.2375$, $p>0.05$; Total t-test $t=4.661$ $df=5$, $p<0.01$] [**Fig. 7a**] There was a significant decrease in BEC between CNO treatment animals and vehicle treated animals expressing Gi-coupled DREADDS in NPY1R+ mPFC cells (N=6) BEC [$t=2.571$ $df=5$, $p<0.05$] [**Fig. 7b**]. There was also no significant change in consumption of 3% sucrose [treatment: $F(1,6)=0.1932$, $p>0.05$; time: $F(1,6)=10.03$, $p<0.05$; interaction: $F(1,6)=2.625e\text{-}005$, $p>0.05$; Total t-test: $t=0.4396$ $df=6$, $p>0.05$][**Fig. 7c**].

To verify that there were no off-target interactions of CNO administration to the mice's ethanol consumption mice were treated with a CON DREADD virus and run through the same procedure previously described. CNO was not found to have a significant impact on ethanol consumption in these mice by Two-way ANOVA analysis (N=7) [treatment: $F(1,6)=0.9556$, $p>0.05$; time: $F(1,6)=0.1854$, $p>0.05$; interaction: $F(1,6)=0.09302$, $p>0.05$; Total t-test: $t=0.9776$ $df=6$, $p>0.05$] [**Fig. 7d**]. The CON DREADD animals also did not show a significant change in BEC after an administration of CNO [$t=0.3039$ $df=6$, $p>0.05$][**Fig.7e**]]. The CON DREADD animals also did not show a significant change in intake of 3% sucrose solution after an administration of CNO (N=9) [treatment: $F(1,8)=1.142$, $p>0.05$; time: $F(1,8)=1.032$, $p>0.05$; interaction: $F(1,8)=0.495$, $p>0.05$; Total t-test $t=1.069$ $df=8$ $p>0.05$] [**Fig. 7f**]. Mice that did not show expression of DREADD receptor in the BLA (fluoresce red) were excluded from the study [**Fig. 7g]**

Discussion

In the characteristics of addiction presented by previous research, it has been noted that there are certain physiological changes that take place in the brain after chronic exposure to alcohol (Volkow, N. D., Koob, G. F., & McLellan, A. T. 2016). In people, some of these changes after chronic alcohol exposure include cortical atrophy, abnormalities in mPFC functioning, and overall impairment of the mPFC's role in decision making (Kim MJ. 2011; Seo, S., Beck et al .2018). Along with this, the evidence of overexpressing NPY leading to an increase in ethanol intake highlighted NPY as a possible important factor in the binge-like ethanol intake cycle (Thiele TE, Palmiter RD. 1998). This study has provided evidence that NPY signaling in the mPFC has the capability to modulate binge-like ethanol consumption. The IHC experiments suggests that NPY is activated by binge-like ethanol intake and with repetitive binge-like ethanol consumption, there is a reduction of NPY signaling within the mPFC. Another interesting finding was that for the PL region of the mPFC, the decrease in NPY immunoreactivity were seen to correlate with the amount of ethanol consumed while it did not correlate for the IL region. This result provides evidence that the goal-orientated region of the mPFC, the PL region, is more likely to experience more direct physiological changes in the early stages of binge-like drinking behavior. An early change in the goal-

directed region of the mPFC supports the concept that the early stages of binge-like drinking are more directly regulated by a goal-direction rather than habitual direction (Barker, J. M., Taylor, J. R., & Chandler, L. J. 2014).

In our pharmacological experiments, administration of a NPY1R agonist or a NPY2R antagonist both reduced binge-like ethanol drinking in mice without significantly impacting sucrose intake, anxiety-like behavior, or general locomotor behavior. This observation of NPY signaling affecting binge-like ethanol intake was paralleled by the chemogenetic studies. Since NPY has been shown to act as an inhibitory transmitter on Gi-coupled receptors, the use of DREADD technology in NPY1R-cre mice provides a means to chemogenetically silence NPY1R+ cells in the mPFC. When the mPFC NPY1R+ cells were chemogenetically silenced, there was a decrease in ethanol consumption. Furthermore, when the mPFC NPY1R+ projections to the BLA were chemogenetically silenced the same decrease in binge-like ethanol consumption was observed, validating the idea that NPY in the mPFC is able to modulate ethanol intake through top-down regulation of the amygdala. While the administration of CNO to Gi-DREADD animals decreased ethanol consumption, administration of CNO to CON-DREADD animals did not decrease binge-like ethanol consumption in mice compared to vehicle treated mice. This demonstrated that there were no significant off-target effects of the CNO administration in the experiments.

In the pharmacological analysis of the NPY system, the introduction of a NPY1R agonist showed a decrease in the mice's overall ethanol intake compared to vehicle treated mice. The agonist acted as an activator of NPY1R, one of NPY's primary post-synaptic receptor, which has been shown to induce an inhibitory signal in the postsynaptic neuron (Sparrow AM. 2012). The administration of a NPY2R antagonist, once again decreased overall intake of ethanol compared to vehicle treated mice. The introduction of the NPY2R antagonist was expected to decrease the activity of the presynaptic receptor, thus increasing further NPY release into the synapse (Robinson, Stacey L., & Thiele, Todd E. 2017). Blocking this receptor, should therefore increase endogenous NPY levels in the synapse, which would then activate NPY1R. The method of chemogenetically inhibiting the NPY1R+ cells was a way to specifically modulate the cell population that would normally be acted on by NPY. The chemogenetic inhibition of cells

in the mPFC in general and then specifically mPFC cells projecting to the BLA, both showed decreased binge-like ethanol consumption.

The lack of change in sucrose consumption in both the pharmacological and chemogenetic studies showed that the changes to NPY signaling was for ethanol consumption and not all reward-related behavior. However, it is possible that this pathway may play a role in other substances of abuse, but further analysis would need to be made to examine that claim. The lack of change in locomotor activity of the mice after treatment with the NPY1R agonist and NPY2R antagonist was also of note to verify that the change in ethanol consumption was not due to the mice's decreased overall activity. This showed that the mice did not drink less because they were lethargic due to the dose administered. Furthermore, the lack of change in anxiety-like behavior showed that the dose administered of the drug did not alter other behavioral aspects of the mice. However, since a dose-response curve was not made for the drugs used, then there is no proof that at a higher dose there may be changes in locomotor and anxiety-like behavior. In the future, a study examining the effects of Leu Pro-NPY and BIIE0246 at multiple doses could verify the results observed in this experiment. Also, this study has used methods to increase the NPY signaling pathway, in the future methods to inhibit the NPY signaling process could be observed to see if there is a reduction in binge-like ethanol consumption. This could be done by the use of an NPY1R antagonist or a NPY2R agonist.

Since NPY1R is expressed in excitatory glutamatergic cells and inhibitory GABAergic interneurons in the mPFC, both were targeted by the pharmacological and chemogenetic approaches. A further study that would individually target each of these cell populations is still required to parse out the role of each of these groups in the top-down regulation of the amygdala. The experiment where CNO was administered directly to the BLA in DREADD expressing mice showed that the excitatory glutamatergic projections of the mPFC play a role in the mPFC's regulation of binge-like ethanol consumption. However, the role of the mPFC neurons in the regulation of the amygdala should still be further evaluated. Also, while the mPFC cells that expressed NPY1R were targeted and shown to have projections to the BLA, there are also likely mPFC neurons that express NPY1R that have projections to other parts of the brain. These projections could also play a role in the binge-like drinking behavior and projects identifying and analyzing

the role of these other regions could provide a more comprehensive picture of the mPFC's ability to modulate ethanol consumption.

In conclusion, this study has shown the importance of one of the molecular players in the development of alcohol addiction. Studying some of the biological factors that contribute to the development of addiction build a further understanding of the cyclic pattern of addiction and how the brain changes with continual ethanol exposure. The role of NPY in the top down regulation of the amygdala is only one of the pieces that is involved in the ethanol dependence. However, this work has shown that the release of NPY in the mPFC could be a powerful target for modulation of ethanol intake in people. The use of specific targets for the release of NPY in the mPFC could provide another means of attempting to reduce ethanol intake in the early stages of binge-like drinking behavior. With continued effort in discovering more of the mechanisms in the development of ethanol dependence, more effective treatments can be created to combat this debilitating disease.

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Figures

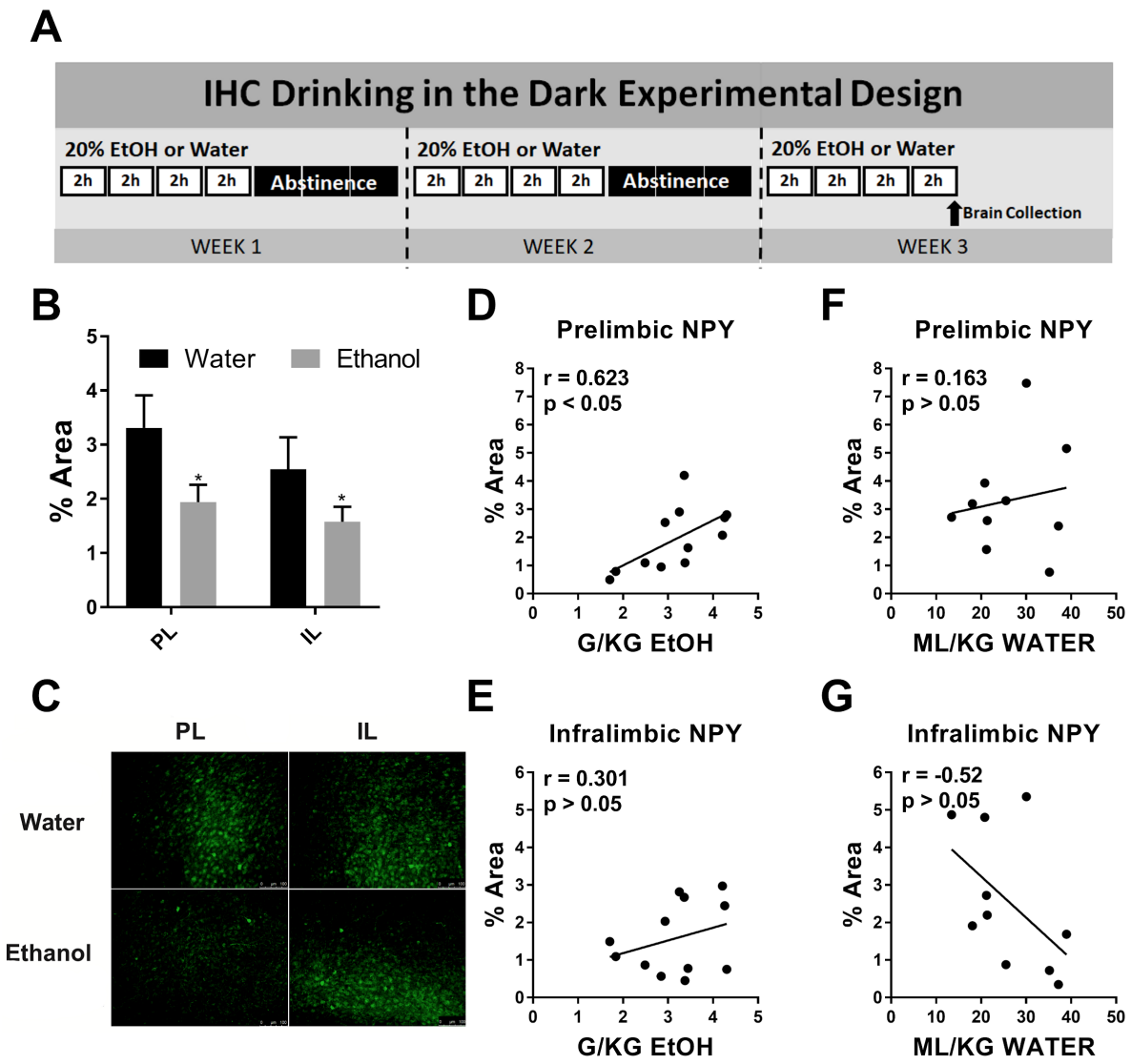

Figure 1. NPY Immunoreactivity decreased in the PL of the mPFC after 3 cycles of DID

A) Timeline of the 3 cycles of DID ethanol or water exposure the mice went through until brain extraction. B) Mice that went under 3 cycles of DID showed a decrease in NPY immunoreactivity in both the PL and IL of the mPFC compared to water exposed animals. C) Exemplar images of NPY IHC in the PL and IL in each treatment group. D) NPY immunoreactivity and total binge-like ethanol consumption on the final day of cycle 3 was found to be significantly correlated in the PL. E) NPY immunoreactivity and total binge-like

ethanol consumption on the final day of cycle 3 was not found to be significantly correlated in the IL. F) NPY immunoreactivity and total water consumption on the final day of cycle 3 did not correlate in the PL. G) NPY immunoreactivity and total water consumption on the final day of cycle 3 did not correlate in the IL.(* = main factor: liquid $p<0.05$) (scale bar = 100µm).

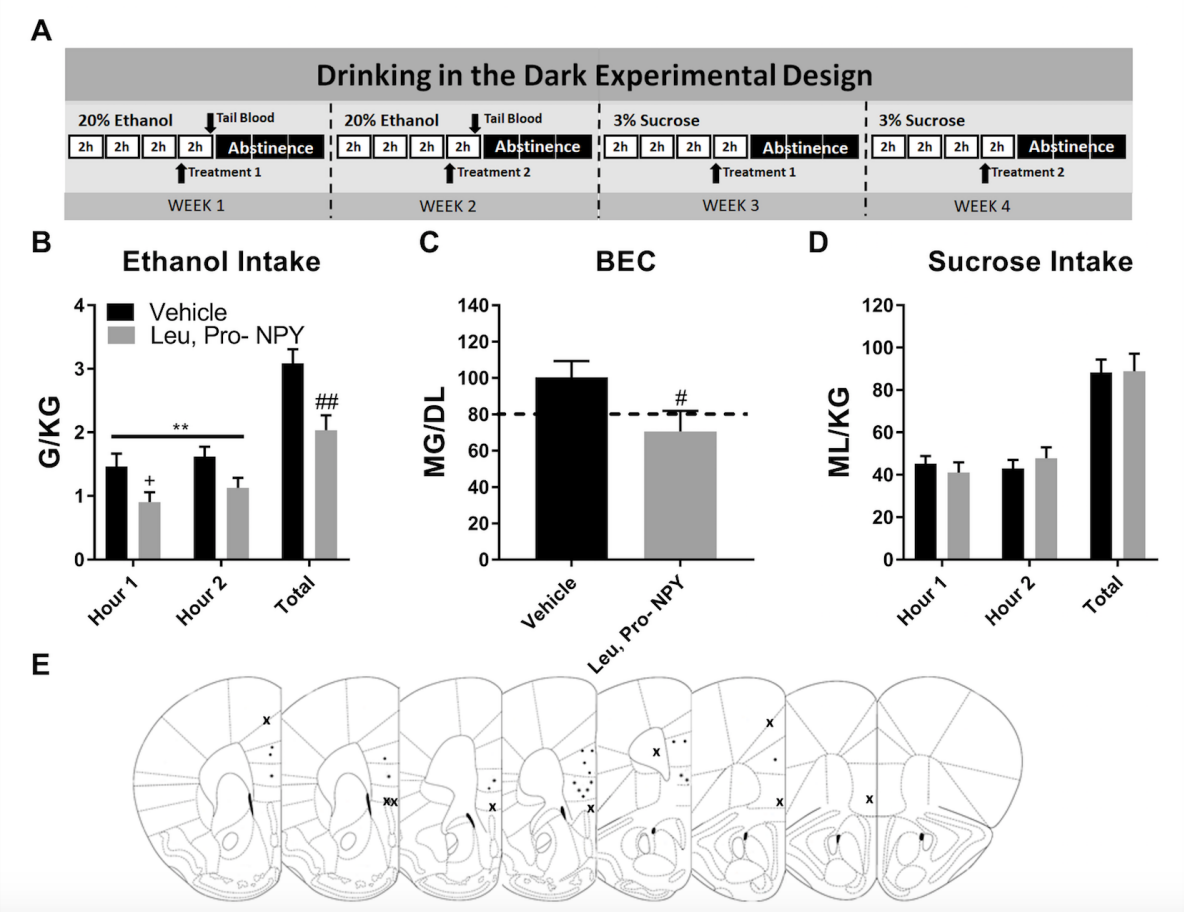

Figure 2: Pharmacological activation of NPY1R in the mPFC significantly reduced binge-like ethanol intake without altering sucrose intake.

A) Timeline of the mice's 3 cycles of DID ethanol or water exposure with treatment times and tail blood collections, followed by the mice's sucrose trials. B) Administration of NPY1R agonist Leu, Pro-NPY into the mPFC significantly reduced ethanol intake over a 2-hour trial period. C) NPY1R agonist Leu, Pro-NPY treated animals showed a significant decrease in BEC compared to the vehicle treated animals. D)

The NPY1R agonist Leu, Pro-NPY treated animals did not alter sucrose (3%) intake compared to vehicle treated animals. E) Cannulae placement checks with each mark representing a single animal (x = misplacement; • = hit). (** = main treatment effect $p<0.01$; + = posthoc effect $p<0.05$; # = paired t-test $p<0.05$; ## = paired t-test $p<0.01$).

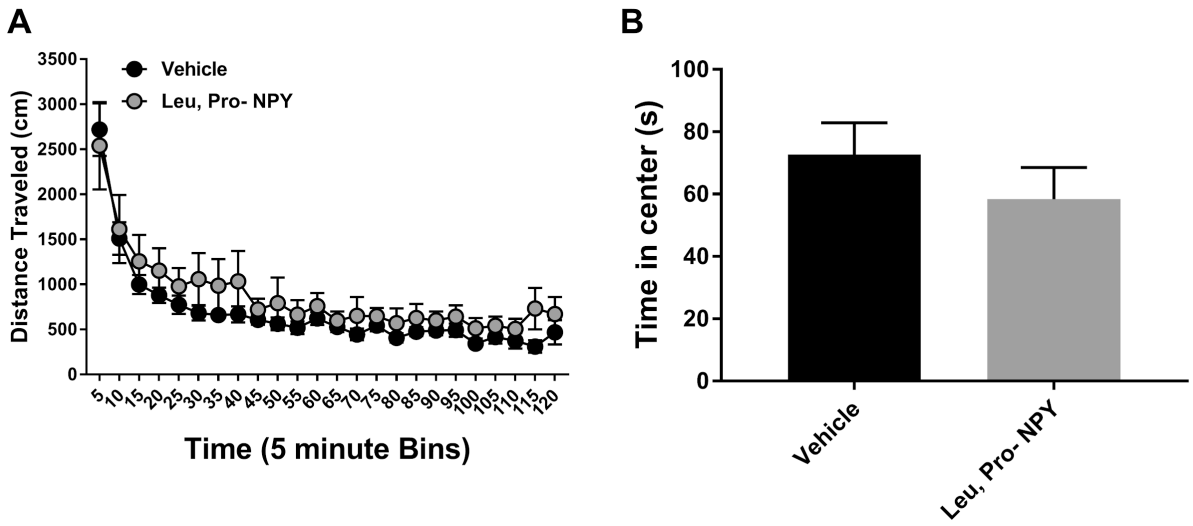

Figure 3: Pharmacological activation of NPY1R in the mPFC did not significantly alter locomotor activity or anxiety-like behavior.

A) Administration of NPY1R agonist Leu, Pro-NPY did not significantly change locomotion of the mice across a 2-hour trial period. B) Administration of NPY1R agonist Leu, Pro-NPY did not affect anxiety like behavior in center time test in locomotion chamber.

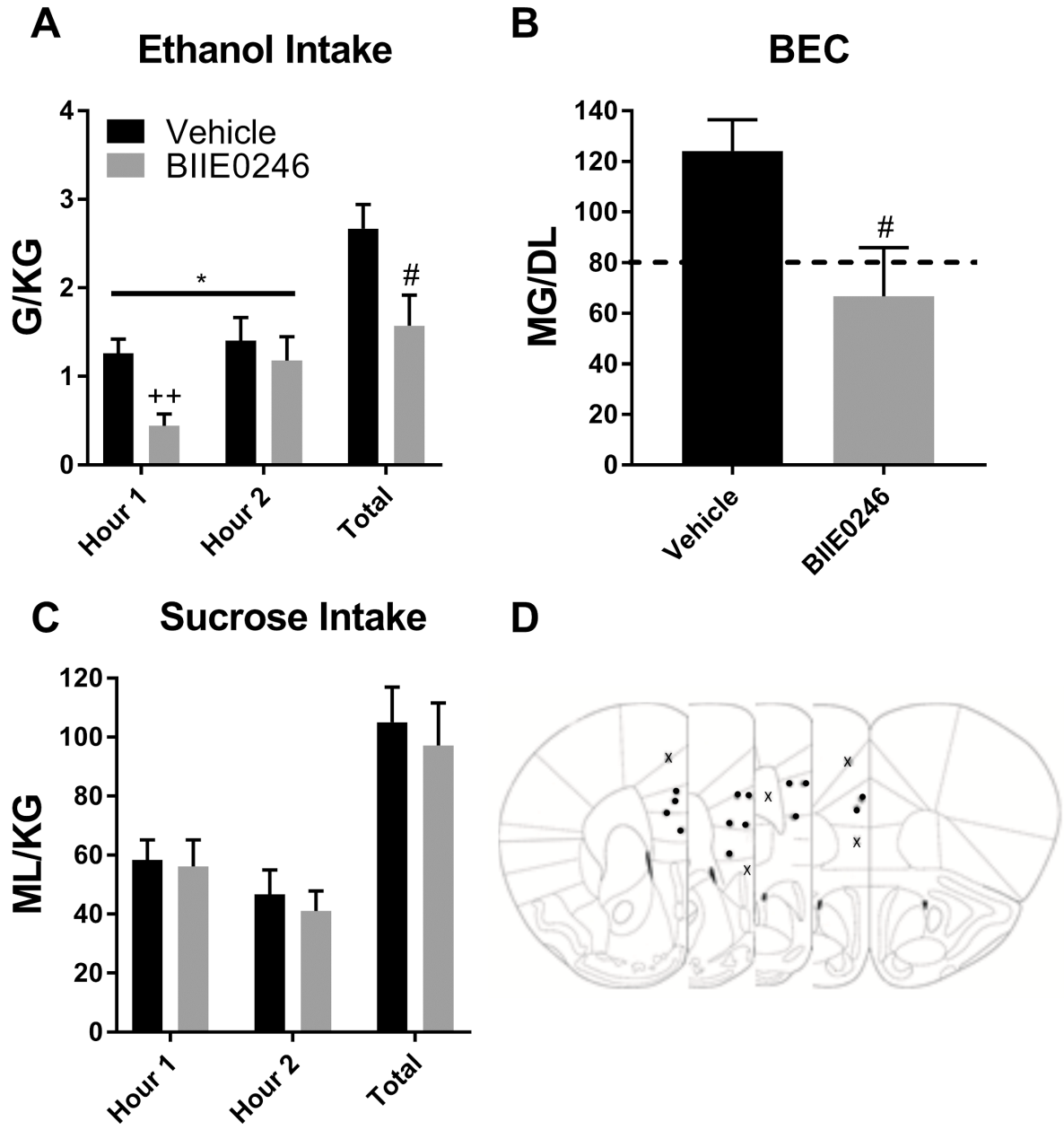

Figure 4: Pharmacological inactivation of NPY2R in the mPFC significantly reduced binge-like ethanol intake without altering sucrose intake.

A) Administration of NPY2R antagonist BIIE0246 into the mPFC significantly reduced ethanol intake across the 2-hour trial period. B) The NPY2R antagonist BIIE0246 treated animals showed a significant decrease in BEC compared to the vehicle treated animals C) The NPY2R antagonist BIIE0246 treated

animals did not alter sucrose (3%) intake compared to vehicle treated animals. D) Cannulae placement checks with each mark representing a single animal (x = misplacement; • = hit). (* = main treatment effect $p<0.05$; ++ = posthoc effect $p<0.01$; # = paired t-test $p<0.05$).

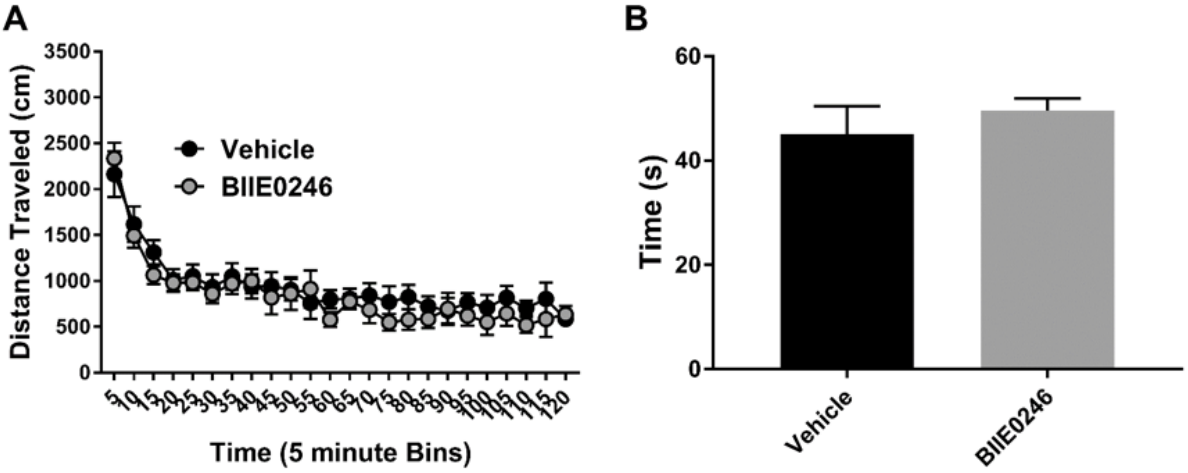

Figure 5. Pharmacological inactivation of NPY1R in the mPFC did not significantly alter locomotor activity or anxiety-like behavior.

A) Administration of NPY2R antagonist BIIE0246 into the mPFC through a cannulae significantly reduce ethanol intake across the 2-hour trial period [Two-way ANOVA: time = $F(23,299)=26.97$, $p<0.0001$; treatment = $F(1,13)=0.2791$, $p>0.05$; interaction: $F(23,299)=0.7306$, $p>0.05$; Vehicle N=10, BIIE0246 N=5]. B) The NPY2R antagonist BIIE0246 treated animals did not alter sucrose (3%) intake compared to vehicle treated animals ($p>0.05$, N=9). C) The NPY2R antagonist BIIE0246 treated animals the showed a non-significant decreased in BEC compared to the vehicle treated animals ($t=0.5788$ $df=13$, $p>0.05$; Vehicle N=10; BIIE0246 N=5).

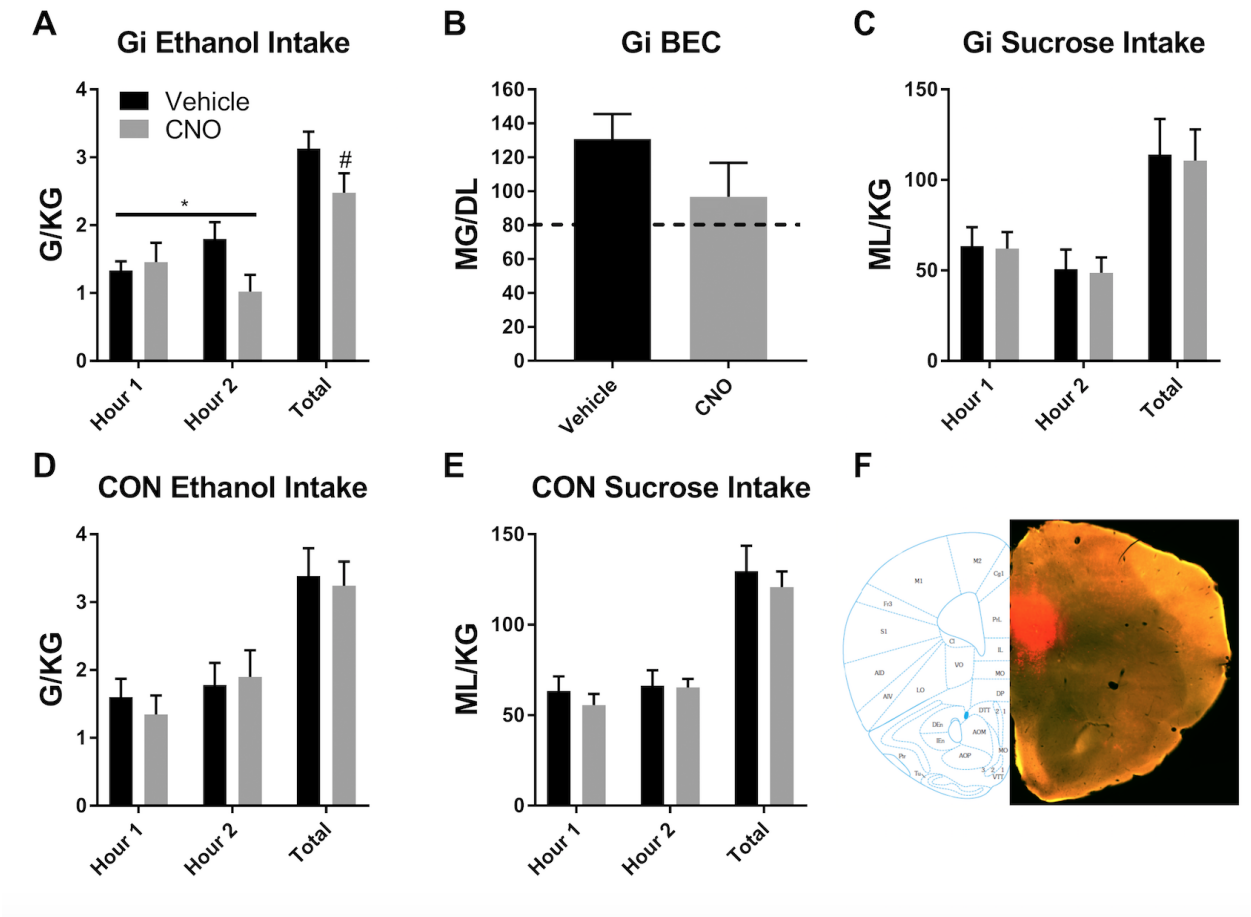

Figure 6. Chemogenetic inhibition of mPFC NPY1R+ neurons reduced binge-like ethanol intake without altering sucrose intake.

A) Administration of CNO via IP injection significantly reduced ethanol intake in NPY1R+ neurons in the mPFC expressing Gi-DREADD. B) CNO administration did not significantly reduce BEC relative to vehicle treated animals. C) CNO administration did not alter sucrose intake compared to vehicle treated animals. D) CNO administration did not significantly change ethanol intake for CON DREADD animals. E) CNO administration did not significantly change sucrose intake for CON DREADD animals. F) Exemplar image of Gi-DREADD expression in the mPFC. (* = main treatment effect $p<0.05$; # = paired t-test $p<0.05$).

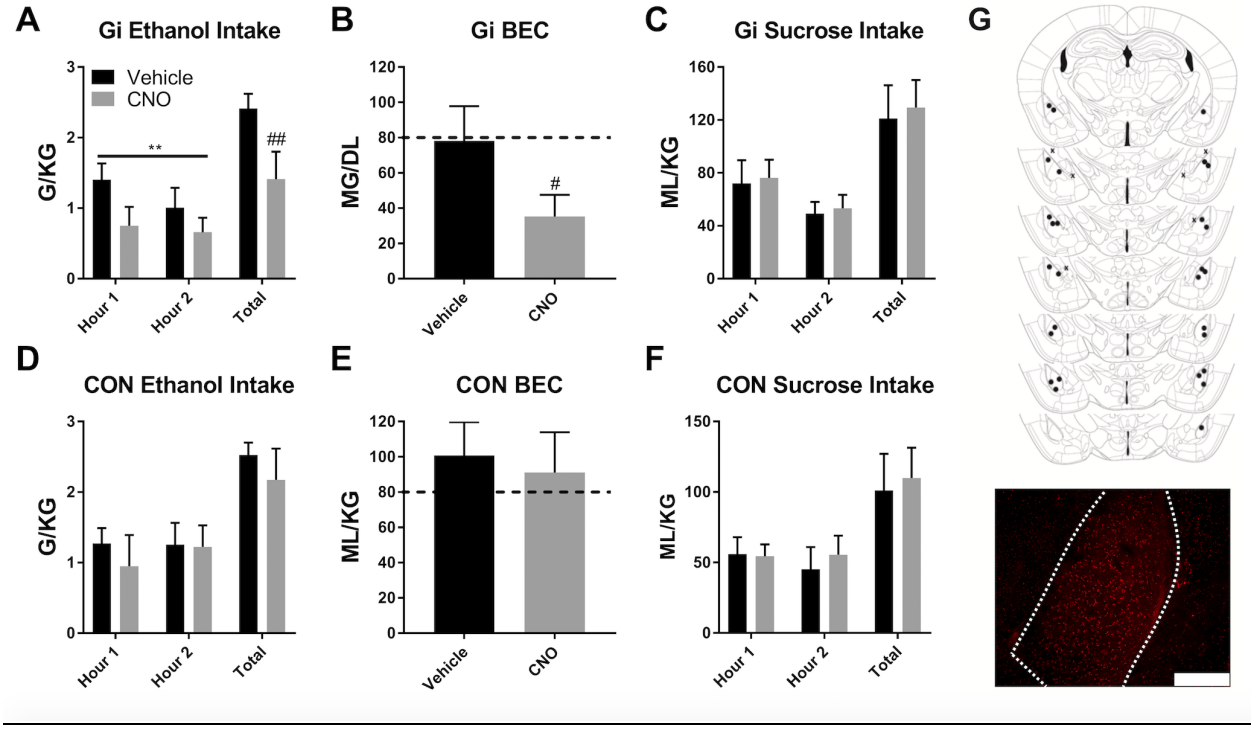

Figure 7. Chemogenetic inhibition of mPFC to BLA NPY1R+ neurons significantly reduced binge-like ethanol intake without altering sucrose intake.

A) Administration of CNO via IP injection significantly reduced ethanol intake for mice with NPY1R+ neurons in the mPFC projecting to the BLA expressing Gi-DREADD. B) CNO administration significantly reduced BEC relative to vehicle treated animals. C) CNO administration did not significantly change sucrose intake in Gi-DREADD animals. D) Administration of CNO did not significantly reduce ethanol intake for mice with NPY1R+ neurons in the mPFC projecting to the BLA expressing CON DREADD. E) CNO administration did not significantly change BEC in CON DREADD animals. F) CNO administration did not significantly change sucrose intake in CON DREADD animals. G) TOP: Cannulae placement checks with each mark representing a single cannulae (2 per animal) (x = misplacement; • = hit); BOTTOM: exemplar image of mPFC NPY1R+ terminal fluorescence in the BLA of a NPY1R-cre mouse (scale bar = 250μm). (** = main treatment effect $p<0.01$; # = paired t-test $p<0.05$; ## = paired t-test $p<0.01$)