Delineation of dynorphin and kappa opioid receptor circuit elements in alcohol consumption

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Abstract

Alcohol use disorder is complex and multi-faceted, involving the engagement of multiple signaling systems across numerous brain regions to drive pathological behavior. Previous work has indicated that both the insular cortex and dynorphin (DYN)/Kappa opioid receptor (KOR) systems contribute to excessive alcohol use. More recently, we identified a microcircuit in the medial aspect of the insular cortex that signals through DYN/KOR. Here, we explored the role of insula DYN/KOR circuit elements on alcohol intake in a long-term intermittent access (IA) procedure. Using a combination of conditional knockout strategies and site-directed pharmacology, we discovered distinct and sex-specific roles for insula DYN and KOR systems in alcohol drinking and related behavior. Our findings show that insula DYN deletion blocked escalated consumption and decreased overall intake of and preference for alcohol in male and female mice. This effect was specific to alcohol in male mice, as DYN deletion did not impact their sucrose intake. In male mice, insula KOR antagonism reduced alcohol intake and preference during the early phase of IA only. Alcohol consumption as not affected by insula KOR knockout. In addition, we found that long-term IA decreased the intrinsic excitability of DYN and deep layer pyramidal neurons (DLPN) in the insula of male mice. Excitatory synaptic transmission was also impacted by IA, as it drove an increase in excitatory synaptic drive in both DYN neurons and DLPN. Combined, our findings suggest that there is a dynamic interplay between excessive alcohol consumption and insula DYN/KOR circuit elements.

Introduction

Excessive alcohol consumption is a major public health burden that generates extreme costs on personal and economic fronts (Sacks et al., 2015). Because of this high cost, a large effort has been devoted to identifying the neural mechanism that underlie escalated drinking.

Preclinical animal models have provided vast insight into the brain structures and signaling systems that can drive excessive alcohol consumption. One neurochemical target that has shown great promise as a potential site of intervention for alcohol use disorder (AUD) is the kappa opioid receptor (KOR) and its endogenous ligand dynorphin (DYN) (Crowley and Kash, 2015). Previous work using PET imaging has shown that human AUD subjects have reduced insular KOR binding compared to controls, which suggests that there may be increased DYN tone in the insula of individuals with AUD (Vijay et al., 2018). In an exploratory analysis across the brain, the authors identified the insular cortex as a region with reduced KOR binding. The insular cortex is a brain region that plays a critical role in the integration of internal and external states (Gehrlach et al., 2020). Human imaging studies have implicated the insula as a region altered in AUD (Claus et al., 2011; Ihssen et al., 2011; Grodin et al., 2017), with findings supported by multiple rodent studies (Seif et al., 2013; Jaramillo et al., 2018a; Centanni et al., 2019; Chen and Lasek, 2020). To date, there has been no exploration of how DYN/KOR signaling in the insula can impact alcohol consumption. In this manuscript we explore the role of the insula DYN/KOR system in both male and female mice using genetic and pharmacological approaches. In addition, we use slice physiology to determine how long-term alcohol consumption can impact insula neuronal function.

Methods

Mice

Male and female mice (>8 weeks of age) were singly housed and maintained on a reverse light cycle (12:12 hr light-dark) in a temperature-controlled colony room. Throughout the experimental procedures, mice were provided continuous access to food and water. In addition to $Pdyn^{IRES\text{-}Cre}$ and $Pdyn^{IRES\text{-}Cre}$ x $Gt(ROSA26)Sor^{loxSTOPlox\text{-}L10\text{-}GFP}$($Pdyn^{GFP}$) (Krashes et al., 2014) mice, $Pdyn^{lox/lox}$ (Bloodgood et al., 2021) and $Oprk1^{lox/lox}$ (Crowley et al., 2016) conditional-

knockout mice were generated as previously described and bred in-house. Wild-type C57BL/6J mice were purchased from The Jackson Laboratory.

Alcohol and tastant drinking procedures

For drinking experiments, mice were isolate housed and maintained on an Isopro RMH 3000 (LabDiet, St. Louis, MO, USA) diet, which has been shown to produce high levels of alcohol consumption (Marshall et al., 2015). To evaluate the effect of genetic knockout and KOR antagonism on alcohol consumption and preference, an intermittent access to alcohol (IA) procedure was run as previously described (Bloodgood et al., 2021). Briefly, mice were given 24 h home-cage access to a bottle containing a 20% w/v alcohol solution alongside a water bottle. Alcohol bottles were introduced 3 h into the dark cycle and bottles were weighed at the start and end of each 24 h session. Drinking sessions occurred three times per week with no less than 24 h and no more than 48 h between sessions (e.g., Mon, Wed, Fri). Drip values for alcohol and water bottles were calculated separately and total consumption was normalized to these values. To prevent the development of a side bias, the placement of alcohol bottles (left or right) was counterbalanced across sessions. To determine the selectivity of treatment effects on alcohol consumption, mice were given a sucrose challenge where they received 2 h access to a bottle of 3% sucrose in addition to water.

Elevated plus maze

To assay anxiety-related behavior, an elevated plus maze (EPM) task was used. The EPM apparatus consisted of two open (75 × 7 cm) and two closed (75 × 7 × 25 cm) arms arranged in a plus configuration wherein the arms each type (open or closed) are positioned opposite of one another and connected via a central open zone (7 × 7 × 25 cm). During EPM testing, ambient illumination was maintained at around 15 lux. Mice were placed in the center zone of apparatus at task onset, then allowed to explore freely for 5 minutes. Video recordings were obtained and

analyzed with Ethovision 9.0 (Noldus Information Technologies) to determine the time spent in the open/closed arms and the total number of open arm entries.

Surgical procedure

Mice were anesthetized by injection (i.p., 1.5 mL/kg) of ketamine/xylazine (Ketaset, code EA2489-564; AnaSed, NDC code 59399-111-50), then secured in a stereotaxic frame (Kopf Instruments) for intracranial viral and drug infusions. The insula (from bregma in mm: AP +0.86, ML ±3.59, DV -3.9) was targeted using standard mouse brain atlas coordinates (Paxinos & Franklin, 2007) and substances were microinjected using a 1 µl Neuros Syringe (33-gauge needle, Hamilton) controlled by an infusion pump. For conditional deletion, 300 nL/side of AAV5-CAMKIIα-Cre-eGFP (UNC vector core; 2.3 x 10^{13} vg/mL) or 200 nL/side AAV5-CMV-HI-eGFP-Cre-WPRE-SV4 (Addgene; ≥ 1 x 10^{13} vg/mL) was injected at a rate of at a rate of 100 nL/min into the insula of $Pdyn^{lox/lox}$ and $Oprk1^{lox/lox}$ mice, respectively. After infusion, injectors were left in place for 5 min to allow for viral diffusion. To pharmacologically block KOR, nor-BNI (5 µg/µL, .5 µL/side in PBS) was infused into the insula of C57BL/6J mice over a period of 5 min. In a subset of mice, a GFP-tagged virus (AAV5-CMV-eGFP; 50 µL/side) was added to the nor-BNI mix to assess infusion spread. To minimize postoperative discomfort, meloxicam (5 mg/kg, i.p.; Metacam, NDC code 0010-6013) was administered at the time of surgery.

Histology

Mice were deeply anesthetized with Avertin (250 mg/kg, IP) and transcardially perfused with chilled 0.01 M phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA). Brains were removed and immersed in 4% PFA overnight, then stored in 30% sucrose/PBS before 45 µm coronal sections were taken on a vibratome (Leica VT1000 S). Free-floating sections were processed for immunofluorescence to amplify GFP signal. Briefly, IC-containing sections (from bregma in mm, AP+1.54 to +0.38) were washed in PBS, then blocked and permeabilized in 5% normal donkey serum/0.3% Triton X-100/PBS for 45 min. Tissue was

incubated overnight with gentle agitation at 4°C with a chicken polyclonal anti-GFP antibody (1:2000, Aves Labs) in blocking solution. Sections were rinsed, then blocked for 45 min before 2 h incubation at room temperature in Alexa Fluor 488-conjugated donkey anti-chicken IgG (1:400 in blocking solution, Jackson ImmunoResearch). Sections were rinsed in PBS after the final incubation and mounted with Vectashield Hardset Mounting Medium with DAPI (Vector Labs). Slides were imaged on an Olympus BX43 with attached optiMOS sCMOS camera (QImaging) or Keyence BZ-X800.

Electrophysiology Recordings

Whole-cell patch-clamp recordings were obtained from the insula of $Pdyn^{GFP}$ mice. After rapid decapitation under isoflurane anesthesia, brains were quickly extracted and immersed in a chilled and carbogen (95% O_2/5% CO_2)-saturated sucrose artificial cerebrospinal fluid (aCSF) cutting solution (in mM): 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10 D-glucose and 26 $NaHCO_3$. Coronal slices (300 µM) containing the insula were prepared on a vibratome, then transferred to a holding chamber containing heated oxygenated aCSF (in mM: 124 NaCl, 4.4 KCl, 1 NaH_2PO_4, 1.2 $MgSO_4$, 10 D-glucose, 2 $CaCl_2$, and 26 $NaHCO_3$). After equilibration (≥ 30 min), slices were placed in a submerged recording chamber superfused (2 mL/min) with oxygenated aCSF warmed to approximately 30-35°C. Neurons were visualized under a 40x water immersion objective with video-enhanced differential interference contrast, and a mercury arc lamp-based system was used to visualize fluorescently labeled $pDyn^{GFP}$ neurons. Recording pipettes (2–4 MΩ) were pulled from thin-walled borosilicate glass capillaries. Signals were acquired using an Axon Multiclamp 700B amplifier (Molecular Devices), digitized at 10 kHz, filtered at 3 kHz, and analyzed in pClamp 10.7 or Easy Electrophysiology. Series resistance (R_a) was monitored without compensation and data were discarded from recordings where changes in R_a exceeded 20%.

Potentials were recorded in current-clamp mode with a potassium gluconate-based intracellular solution (in mM): 135 K-gluconate, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.6 EGTA, 4 Na_2ATP, 0.4 Na_2GTP, pH 7.3, 289–292mOsm. To hold cells at a common membrane potential, V_m was adjusted to ~-70 mV by constant current injection. Current-injection evoked action potentials were evaluated by measuring rheobase (minimum current required to evoke an action potential) and number of spikes fired at linearly increasing current steps (50 pA increments, 0 to 650 pA).

Spontaneous synaptic events were recorded in voltage-clamp mode with a cesium methanesulfonate-based intracellular solution (in mM): 135 cesium methanesulfonate, 10 KCl, 1 $MgCl_2$, 10 HEPES, 0.2 EGTA, 4 MgATP, 0.3 Na_2GTP, 20 phosphocreatine, pH 7.3, 285-290 mOsm with 1mg/mL QX-314. The ratio of excitatory to inhibitory (E:I ratio) transmission was measured by isolating glutamate (V_{hold} = -55 mV) and GABA currents (V_{hold} = +10 mV) within individual neurons.

Experimental Design and Statistical Analysis

To assess for sex differences, data were analyzed by two-way ANOVA (sex x group) or three-way (sex x group x time/current) mixed model ANOVA. To simplify presentation, data were collapsed across sex for experiments where analyses yielded no significant interactions by or main effect of sex. One-way ANOVA or t-tests were used to examine the effect of IA on rheobase, sE/IPSC frequency and amplitude, E:I ratio, alcohol/sucrose intake and preference, and time spent in EPM open/closed arms. Data are presented as mean ± SEM and were analyzed using JASP 0.14 (JASP Team) or in Prism 9 (GraphPad Software). Significance threshold was set at $\alpha = 0.05$, and follow-up post-hoc pairwise comparisons were multiplicity adjusted.

Experimental Design and Statistical Analysis

Data were analyzed by mixed model three-way (sex x group x session/time; sex x treatment x time/current) or two-way (sex x group/treatment; group/treatment x session/time/current) ANOVA, where group = GFP/Cre or Vehicle/nor-BNI and treatment =

H2O/IA. Additional analyses were performed using one-way ANOVA or t-tests were used to examine the effects of group on alcohol intake/preference, total fluid intake, sucrose intake, time spent in open/closed arms of EPM or the effects of treatment on RMP, rheobase, sE/IPSC frequency/amplitude, and E/I ratio. Where data violated assumptions of normality or homoscedasticity, a Mann-Whitney U test or Welch's correction was used, respectively, for analysis. Data are presented as mean ± SEM and were analyzed using JASP 0.14 (JASP Team) or in Prism 9 (GraphPad Software, LLC). Significance threshold was set at $\alpha = 0.05$, and post-hoc pairwise comparisons were multiplicity adjusted.

Results

Knockout of Pdyn in the insula decreases alcohol drinking in male and female mice

To define the role of insula Dyn neurons in alcohol intake, we conditionally deleted Pdyn in male and female mice prior to initiation of an 8-week IA drinking procedure. Site-specific knockout of Pdyn was achieved by infusing AAV5 with CAMKIIa-promoter driven expression of a GFP-fused Cre recombinase or a GFP only control into the insula of $Pdyn^{lox/lox}$ mice. Anxiety-like behavior was assessed in the EPM test 24h following the final IA session. To assess the specificity of knockout on alcohol intake, mice were given a 2h sucrose drinking challenge 24h after EPM testing. Pdyn knockout decreased alcohol intake and preference in both males [group x session interaction: $F_{(23,276)} = 1.97$, p = 0.006, intake / $F_{(23,276)} = 2.62$, $p < 0.001$, preference; group main effect: $F_{(1,12)} = 4.62$, $p = 0.053$, intake / $F_{(1,12)} = 6.59$, $p = 0.025$, preference; session main effect: $F_{(23,276)} = 3.89$ $p < 0.001$, intake / $F_{(23,276)} = 3.96$, $p < 0.001$, preference] and females [group x session interaction: $F_{(23,322)} = 1.61$, $p = 0.039$, intake / $F_{(23,322)} = 1.33$, $p = 0.143$, preference; group main effect: $F_{(1,14)} = 11.5$, $p = 0.004$, intake / $F_{(1,14)} = 9.39$, $p = 0.008$, preference; session main effect: $F_{(23,322)} = 9.33$, $p < 0.001$, intake / $F_{(23,322)} = 7.92$, $p < 0.001$, preference]. Knockout of Pdyn in the insula also blocked escalation of alcohol intake, as average intake levels significantly

increased from weeks 1-4 to weeks 5-8 in controls (GFP) [Males: $t_5 = 4.42$, $p = 0.007$; Females: $t_6 = 3.56$, $p = 0.012$] but not in Pdyn knockout (Cre) mice [Males: $p = 0.378$; Females: $p = 0.083$].

Total fluid intake was not affected by Pdyn knockout in male [$p = 0.249$] or female mice [$p = 0.737$]. Interestingly, Pdyn deletion increased sucrose consumption in females [$t_{14} = 3.17$, $p = 0.007$], but this effect was absent in male mice [$p = 0.737$]. Combined, these findings show that Pdyn deletion in the insula selectively reduces alcohol intake in male mice and suggest that insula Pdyn may play a key role in driving escalated alcohol consumption. We next tested the impact of Pdyn deletion on anxiety-like behavior by exposing mice to an EPM test 24h following their final drinking session. We found no effect of Pdyn knockout in either sex on time spent in the open [Males: $p = 0.671$; Females: $p = 0.252$] or closed [Males: $p = 0.188$; Females: $p = 0.843$] arms of the EPM.

Local infusion of nor-BNI decreases early phase alcohol drinking in male, but female mice

Previous studies have found that the KOR antagonist nor-BNI can reduce alcohol consumption when giving systemically (Walker et al., 2011; Anderson et al., 2016). To assess insula KOR involvement in alcohol drinking, we locally infused the long-acting antagonist nor-BNI (Munro et al., 2012) into male and female C57BL/6J mice prior to the start of an 8-week IA to alcohol procedure. This long-acting property of nor-BNI allows for a pharmacological assessment of insula KOR in alcohol consumption over multiple weeks. As above, mice were additionally assessed for anxiety-like behavior (EPM) and sucrose intake at 24h and 48h, respectively, following the final drinking session.

Our findings show that in male mice, insula KOR antagonism by nor-BNI reduced alcohol consumption [group x session: $F_{(23,460)} = 2.08$, $p = 0.003$; main effects of group: $F_{(1,20)} = 8.27$, $p = 0.009$; and session: $F_{(23,460)} = 8.07$, $p < 0.001$] and alcohol preference [group x session: $F_{(23,460)} = 1.45$, $p = 0.082$; main effects of group: $F_{(1,20)} = 6.98$, $p = 0.016$; and session: $F_{(23,460)} = 6.93$, $p < 0.001$]. In female mice, we found no effect of KOR antagonism on intake [group x session: $p =$

0.281; group main effect: $p = 0.647$; session main effect $F_{(23,414)} = 8.78$, $p < 0.001$] or preference [group x session: $p = 0.378$; group main effect: $p = 0.692$; session main effect [$F_{(23,414)} = 16.3$, $p < 0.001$]. The reduction in intake by nor-BNI in males appeared

to occur predominately during the first 9 session or 3 weeks of the IA procedure. Thus, we evaluated this by collapsing alcohol intake over the first 9 sessions of IA and found that nor-BNI decreased average alcohol intake [$t_{22} = 4.56$, $p < 0.001$] and alcohol preference [$t_{20}=3.58$, $p = 0.002$] in male mice but not female mice [intake: $p = 0.578$; preference: $p = 0.245$]. Total fluid intake was not affected by nor-BNI in males [Veh: 5.96 ± 1.7 mL/kg; NBI: 4.82 ± 0.6 mL/kg; $p = 0.865$] or females [Veh: 4.07 ± 0.1 mL/kg; NBI: 4.43 ± 0.3 mL/kg; $p = 0.393$] at this timepoint. Further, nor-BNI did not affect sucrose drinking in male mice [$p = 0.691$], but did lead to a significant increased in sucrose consumption in female mice [$t_{18} = 2.52$, $p = 0.022$]. As a follow-up in female mice, we tested whether nor-BNI would affect the consumption of saccharine, a non-caloric sweet solution. Here, female mice were given access to a 0.15% saccharine solution and intake was measured at the end of a 2-h session. As with sucrose, insula KOR antagonism increased saccharine intake, as female nor-BNI mice consumed significantly more of the sweetened solution than did vehicle controls (Veh: 51.8 ± 7.68 mL/kg; NBI: 91.4 ± 12.3 mL/kg; $U = 17$, $p = 0.011$). Thus, while insula KOR antagonism can selectively decrease alcohol intake in males, it may drive increased consumption of palatable solutions in females. Finally, we tested anxiety-like behavior in the EPM and found no effect of nor-BNI in either sex on time spent in the open arms [Males: Veh, 25.4 ± 7.8 s; NBI, 15.5 ± 5.6 s, $p = 2.80$; Females: Veh, 94.6 ± 17.5 s; NBI, 67.2 ± 12.0 s; $p = 0.213$] or closed arms of the apparatus [Males: Veh, 206 ± 11.7 s; NBI, 223 ± 9.1 s; $p = 0.213$; Females: Veh, 158 ± 15.5 s; NBI, 184 ± 10.7 s; $p = 0.179$].

To test whether the transient reduction in alcohol intake by nor-BNI in male mice was due to a waning effect of the drug, we administered nor-BNI at a later timepoint. In this experiment, male C57BL/6J mice were first exposed to 7 weeks of IA to alcohol before nor-BNI was delivered locally into the insula. After recovery, mice were given 3 additional weeks (8 sessions) of IA

followed by EPM and sucrose testing. When administered after 7 weeks of IA, nor-BNI had no effect on alcohol intake [group x session interaction: $p = 0.594$; group main effect: $p = 0.803$; session main effect: $F_{(8,152)} = 4.86$, $p < 0.001$], alcohol preference [group x session interaction: $p = 0.356$; group main effect: p = 0.564; session main effect: $F_{(8,152)} = 2.41$, $p = 0.018$], or total fluid intake [Veh: 4.37 ± 0.1 mL/kg; NBI: 4.55 ± 0.1 mL/kg; $p = 0.254$] as assessed across the final 3 weeks of IA. Further, there was no effect of nor-BNI administered at this later timepoint on EPM performance or sucrose intake.

Knockout of KOR in the insula does not alter alcohol drinking in male or female mice

Our nor-BNI data suggest that in male mice, local insula KOR can regulate aspects of alcohol consumption. However, KOR can be present at multiple within a given brain region, including presynaptic terminals. Given that nor-BNI can also act presynaptically at KOR expressed on afferent terminals, we wanted to more selectively target locally-expressed KOR in the insula. Using an *Oprk1*$^{lox/lox}$ mouse line, we selectively deleted KOR in the insula by injecting AAV5 encoding for a GFP-tagged Cre recombinase or a GFP only control. Following recovery, mice underwent 8 weeks of IA to alcohol before being tested for anxiety-like behavior (EPM) and sucrose intake. In female mice, knockout of insula KOR had no effect on alcohol intake [group x session interaction: $p = 0.123$; group main effect: $p = 0.734$; session main effect: $F_{(23,667)} = 8.99$, $p < 0.001$] or alcohol preference [group x sex interaction: $p = 0.331$; group main effect, $p = 0.519$; session main effect: $F_{(23,667)} = 9.27$, $p < 0.001$] across the 8 week IA procedure. In male mice, analyses yielded a significant group x session interaction on intake [$F_{(23,575)} = 1.56$, $p = 0.047$] in addition to a main effect of session [$F_{(23,575)} = 2.79$, $p < 0.001$] but not group [$p = 0.097$]. This finding may be due, in part, to the lack of escalation in GFP control mice and the increased intake in KOR knockout mice during the final week of IA (sessions 22-24). However, we found no effect of KOR deletion on alcohol preference in males [group x session interaction: $p = 0.484$; group main effect, $p = 0.162$; session main effect: $F_{(23,575)} = 2.33$, $p < 0.001$]. To further assess intake

and preference, we examined the first and latter halves (weeks 1-4 and 5-8) of the IA procedure. We found that from weeks 1-4 to weeks 5-8, female mice escalated their average alcohol intake [paired t-tests; GFP: $t_{14} = 3.98$, $p < 0.001$; Cre: $t_{15} = 4.42$, $p < 0.001$] whereas male mice did not [GFP: $p = 0.298$; Cre: $p = 0.700$]. In both males and females, KOR knockout did not affect total fluid intake [Males: $p = 0.587$; Females: $p = 0.654$], sucrose intake [Males: $p = 0.810$; Females: $p = 0.594$], or time spent in the open arms [Males: $p = 0.318$; Females: $p = 0.711$] or closed arms of the EPM [Males: $p = 0.177$; Females: $p = 0.654$].

Long-term alcohol drinking exerts a sex-dependent effect on insula neuronal function

Previously, we identified a microcircuit in the medial agranular insular cortex that is modulated by KOR (Pina et al., 2020). Within this microcircuit, Pdyn neurons are densely clustered in layer 2/3 and KOR expression is localized to deep layer 5/6. Functionally, activation of insula KOR by Dyn dampens local inhibitory tone, which leads to a disinhibition of and increase in excitatory synaptic drive in deep layer pyramidal neurons (DLPN). Thus, based on our above results we wanted to determine the effect of long-term alcohol drinking on neuronal function in insula Pdyn and DLPN neurons. To assess the effect of long-term alcohol drinking on Pdyn neuronal function, adult male and female Pdyn^{GFP} mice were exposed to 8 weeks of IA to alcohol before tissue was collected for whole-cell patch clamp electrophysiology. When we examined intrinsic excitability of Pdyn neurons, we found that 8 weeks of IA decreased the excitability of Pdyn neurons in male but not female mice. This was supporting by a significant effect of IA on rheobase (minimum current to elicit firing) in males [$t_{15} = 2.19$, $p = 0.045$] but not females [$p = 0.874$], and by a significant decrease in firing to increasing current steps in males [treatment x current interaction: $F_{(10,180)} = 41.9$, $p < 0.001$; treatment main effect: $F_{(1,18)} = 6.08$, $p = 0.024$; current main effect: $F_{(10,180)} = 4.81$, $p < 0.001$] and not females [treatment x current interaction: $p = 0.999$; treatment main effect: $p = 0.993$; current main effect: $F_{(10,190)} = 18.5$, $p < 0.001$]. Whereas resting membrane potential was

not impacted by IA in males [$p = 0.597$], there was a significant increase in RMP by IA in females [$U = 23.5$, $p = 0.025$].

Next, we evaluated the impact of IA on synaptic transmission in Pdyn neurons. We found no effect of IA or sex on sEPSC frequency [sex x treatment interaction: $p = 0.699$; sex main effect: $p = 0.141$; treatment main effect: $p = 0.502$], suggesting that the frequency of glutamatergic synaptic events is similar in males and female mice and not altered by long-term IA. When we assessed GABAergic transmission, we found that females received less inhibitory input onto insula Pdyn neurons than male mice, as supported by a main effect of sex on sIPSC frequency [$F_{(1,34)} = 4.75$, $p = 0.036$]. As with sEPSCs, we found no effect of IA on sIPSC frequency [sex x treatment interaction: $p = 0.819$; treatment main effect: $p = 0.159$]. Interestingly, when we assessed the ratio of excitatory to inhibitory (E/I) input onto insula Pdyn neurons, we found significant main effects of sex [$F_{(1,37)} = 4.66$, $p = 0.037$] and treatment [$F_{(1,37)} = 6.10$, $p = 0.018$] but no group x sex interaction [$p = 0.776$]. This finding indicates that there is greater excitatory synaptic drive in Pdyn neurons of male mice, and that IA increases E/I in this neuronal population in both males and females. When event amplitude was assessed, we found that sE/IPSC amplitudes were higher in male Pdyn neurons as compared to females, as indicated a significant main effects of sex [sEPSC: $F_{(1,34)} = 18.5$, $p < 0.001$; sIPSC: $F_{(1,34)} = 12.1$, $p < 0.001$]. Whereas there was no effect of IA on sEPSC amplitude in male and female mice [sex x treatment: $p = 0.802$; treatment main effect: $p = 0.222$], sIPSC amplitude was decreased in IA exposed animals, as revealed by a significant main effect of treatment [$F_{(1,34)} = 4.23$, $p = 0.047$] but no interaction of sex x treatment [$p = 0.330$].

We subsequently examined the effect of long-term IA on excitability and synaptic transmission in insula DLPNs of male and female mice. Intrinsic excitability of insula DLPNs was reduced in IA exposed mice compared to water controls, as indicated by a deceased in RMP and an increase in rheobase in IA mice. These findings were supported by a main effect of treatment [RMP: $F_{(1,36)} = 9.71$, $p = 0.004$; rheobase: $F_{(1,36)} = 9.03$, $p = 0.005$] but not sex [RMP: $p = 0.628$;

rheobase: p = 0.112] and no sex x treatment interaction [RMP: p = 0.353; rheobase: 0.792]. When analyzed separately, we found that IA significantly decreased RMP and increased rheobase in males [RMP: $t_{9.98}$ = 2.46, p = 0.034; rheobase: t_{18} = 3.09, p = 0.006] but not females [RMP: p = 0.110; rheobase: p = 0.082]. Additionally, IA decreased the number of action potentials fired at increasing current steps in male mice [treatment x current interaction: $F_{(10, 190)}$ = 2.92, p = 0.002; treatment main effect: $F_{(1,19)}$ = 10.4, p = 0.005; current main effect: $F_{(10,190)}$ = 92.0, p < 0.001], but not in female mice [treatment x current interaction: p = 0.052; treatment main effect: p = 0.557; current main effect: $F_{(10,170)}$ = 62.3, p < 0.001]. Synaptic transmission was next assessed in DLPNs of IA-exposed and water control male and female mice. In these experiments we found that long-term IA produced a sex-dependent increase in excitatory synaptic transmission. This was revealed by a significant sex x treatment interaction on sEPSC frequency [$F_{(1,39)}$ = 5.63, p = 0.023] as well as a significant main effect of sex [$F_{(1,39)}$ = 10.2, p = 0.003] but not group [p = 0.159]. Post-hoc analyses revealed that IA increased DLPN sEPSC frequency in male but not female mice [p = 0.046], which subsequently resulted in a significant increase in sEPSC frequency in IA-exposed males compared to females [p < 0.001]. Conversely, there was no effect of long-term alcohol intake on inhibitory synaptic transmission in insula DLPN neurons, as sIPSC frequency did not differ between IA mice and water controls. This finding was demonstrated by the absence of both a sex x treatment interaction [p = 0.270] and main effect of treatment [p = 0.983]. Analyses yielded a main effect of sex on sIPSC frequency [$F_{(1,39)}$ = 32.3, p < 0.001] and post-hoc comparisons revealed that H2O and IA exposed males exhibited higher sIPSC frequency as compared to females of the same treatment group [H2O: p = 0.003; IA: p < 0.001]. We next assessed E/I ratio in insula DLPNs and found that IA significantly increase synaptic drive in this neuronal population. This was demonstrated by a significant main effect of treatment [$F_{(1,41)}$ = 4.58, p = 0.038] but not sex [p = 0.748] and no sex x treatment interaction [p = 0.180]. Follow-up analyses showed that there was a significant difference in E/I ratio between IA and water exposed males [p = 0.028] but not females [p = 0.526], indicating that excitatory synaptic drive was

increased in male mice only. Moreover, we found sex-related differences in event amplitude, as sEPSCs and sIPSCs were higher in female mice. This was illustrated by a significant main effect of sex on sE/IPSC amplitude [sEPSC: $F_{(1,39)} = 22.6$, $p < 0.001$; sIPSC: $F_{(1,39)} = 32.1$, $p < 0.001$] followed by post-hoc analyses showing significant difference between male and female mice

Discussion

In this study we used converging pharmacological and genetic approaches to understand how KOR signaling in the insula can contribute to excessive alcohol consumption in male and female mice. In addition, we use slice electrophysiology to probe physiological changes in insula neuronal populations, focusing on DYN expressing neurons and Layer 5 pyramidal neurons. Across these studies we identify numerous sex-differences at the behavioral and physiological level. This adds to a growing literature demonstrating sex-differences in how KOR systems can modulate behavior and neuronal function.

Divergence between Pharmacology and Genetic Approaches

In this study, there were several key differences between the pharmacological approach (blocking KOR locally) and the genetic approach (deletion of KOR/DYN) in the insula to probe impact on drinking. In the males, we saw an early impact on alcohol consumption that diminished over time. We hypothesized that could either be due to the waning effects of nor-BNI, or a shift to a different state insensitive to local KOR antagonism. To test this, we infused nor-BNI after another set of male mice had escalated their consumption, in keeping with the second possibility, we found no effect on consumption. This suggested that in males only, local KOR antagonism in the insula could reduce early alcohol consumption, but following escalation, it was no longer sensitive. This result was surprising, as in many other studies, KOR antagonists effects emerge as animals become dependent or escalate their consumption (Walker et al., 2011; Erikson et al., 2018). However, it is notable, that KOR antagonists can impact drinking in more acute models of

escalated consumption, such as Drinking in the Dark (DID) (Anderson et al., 2019; Haun et al., 2020). The antagonist approach does have limitations, including difficulty to accurately quantify spread and potential off-target actions of the compound.

To address these limitations, we used a genetic approach, similar to our previous work in the central nucleus of the amygdala (CeA) (Bloodgood et al., 2021). In contrast to local inhibition, we found that deletion of KOR had no effect on any measures of alcohol intake. This divergence is likely due the dissociation between the receptors that local antagonist can block compared to what deletion removes. It is within the realm of possibility that the insular cortex receives inputs that have KOR located on the terminals, these receptors would be blocked by local pharmacology but not by deletion from insula neurons. Another possibility is that deletion of KOR from insula neurons also leads to removal or KOR in insula output regions, where KOR signaling may play a different role on alcohol consumption.

We also examined how deletion of DYN from the insula could impact alcohol drinking and found that it led to a robust reduction of alcohol consumption in both males and females. There were some slight differences between the sexes. Notably, in females there was a reduction, both early and after escalation, whereas in the males the reduction was only apparent in later escalated consumption phases. Deletion of DYN in females also reduced sucrose intake. Taken together, our findings suggests that there is an overlying effective on appetitive behavior in females that is additive with the emerging difference in alcohol consumption. There is a large difference between this DYN manipulation and the KOR manipulations, both genetic and pharmacological. One possible reason for this may be that when we delete Dyn, we are altering both local and distal neuropeptide release. There are a number of insula targets that could contribute this effect, such as the CeA, which has been implicated in KOR regulation of alcohol consumption.

Notably, none of the manipulations we performed that altered alcohol consumption altered avoidance behavior, assessed via the elevated plus maze. This suggests that the role of KOR/DYN system may be distinct from regulation of avoidance behavior. The insula has also

been related to affective behavior, or more specifically struggling behavior during restraint stress, as shown in a recent study (WINDER). We did not explore these behaviors, and it would be an interesting future direction, especially to see how long-term alcohol drinking could alter this behavior. In addition, work from the Hopf and Lasek groups have found that manipulations in the insula are related primarily to punished alcohol responding. We did not explore that phenotype in our model, and it represents another path for exploration.

Alcohol consumption has complex effects in distinct insula cell types

Given our results with the DYN deletion, we wanted to evaluate the effect that long-term alcohol consumption had on properties of insula Pdyn neurons. We found that with the excitability of these neurons, there were divergent effects depending on sex. In males, we found a reduction in current-evoked firing and an increase in rheobase, consistent with reduced excitability. In female mice, we found that IA increased the resting membrane potential in Pdyn neurons only. There was a main effect of alcohol drinking on the E/I ratio across both sexes, however there was reduced GABAergic tone in insula Pdyn neurons from females, suggesting possible differential interneuron function. Taken together, the results are suggestive of a synaptic scaling process occurring in insula Pdyn neurons in males. This is especially interesting considering recent results suggesting synaptic scaling plays a role in learning in the closely related gustatory cortex (Wu et al., 2021). The increase in RMP and synaptic balance in insula Pdyn neuron from female mice could be linked to the greater effect of DYN deletion in females, however our data does not directly address this possibility. It is noteworthy that these findings, in particular the sex differences, are similar to our recent study exploring the impact of binge-like alcohol drinking on DYN neurons in the CeA (Bloodgood et al., 2021), and in keeping with recent data from the Herman lab demonstrating sex-specific effects of alcohol in the CeA (Agoglia et al., 2020). This raises the important issue that sex is a critical factor to consider when evaluating KOR ligands in preclinical models, as has been noted by Chertoff and Mavrikaki (Chartoff and Mavrikaki, 2015).

We also evaluated the impact of long-term alcohol consumption on Layer 5 pyramidal neurons in the IC, as outputs from the insula have been implicated in a range of important behaviors (Gogolla, 2017), and found to be altered following alcohol exposure (McGinnis et al., 2020). A similar pattern emerged with these layer 5 neurons showing reductions in excitability, but an increase in E/I ratio from males, suggestive of a scaling like phenomena, with no differences in the properties of layer 5 insula from female mice. This again suggest a sex-dependent plasticity following alcohol drinking, but it is unclear how this relates to the behaviors we have observed. Work from the Besheer lab (Jaramillo et al., 2018b, 2018a, 2018c) and the Hopf lab (Seif et al., 2013) has found that the insula to nucleus accumbens pathway is important for alcohol self-administration and punished alcohol drinking, suggesting one possible mechanism for how these cellular changes could promote increased alcohol consumption. The Hopf lab also found a role for insula outputs to brainstem in punished alcohol drinking but not alcohol only drinking (De Oliveira Sergio et al., 2021), suggesting that while this path is important, it may not be related to the drinking phenotypes seen. However, there is also intriguing work that insula outputs to the BLA can play a critical role in learning (Yiannakas et al., 2021). This is especially exciting when taken with recent data supporting the role of the BLA in alcohol reinforcement (Faccidomo et al., 2021). Future studies should focus on the investigating plasticity in these distinct outputs, as it can provide important insight into how alcohol can impact insula circuits.

Taken together, our data suggest that KOR/DYN signaling in the insula can regulate alcohol consumption and there are key sex differences in the mechanism. Pharmacology suggests that in males, there is an early KOR signal in the insula that plays a role in induction of escalated alcohol drinking, but does not impact maintenance of escalated alcohol drinking. In contrast, dynorphin deletion can reduce alcohol consumption during the later phases of escalation, suggesting a shift to KOR sensitivity in downstream structures, potentially the CeA. In females, dynorphin deletion appears to robustly impact drinking both early and late, but this may be due to general effects on appetitive response. There are similar sex-dependent changes on

neuronal function, however our data does not specifically identify how they are related to escalated consumption. These data reinforce the importance of studying sex difference in alcohol related behavior, and specifically the mechanisms by which KOR can reduce alcohol consumption. As a potential target for treatment of AUD, it is important to identify these mechanisms and determine how they relate to sex differences in clinical populations.

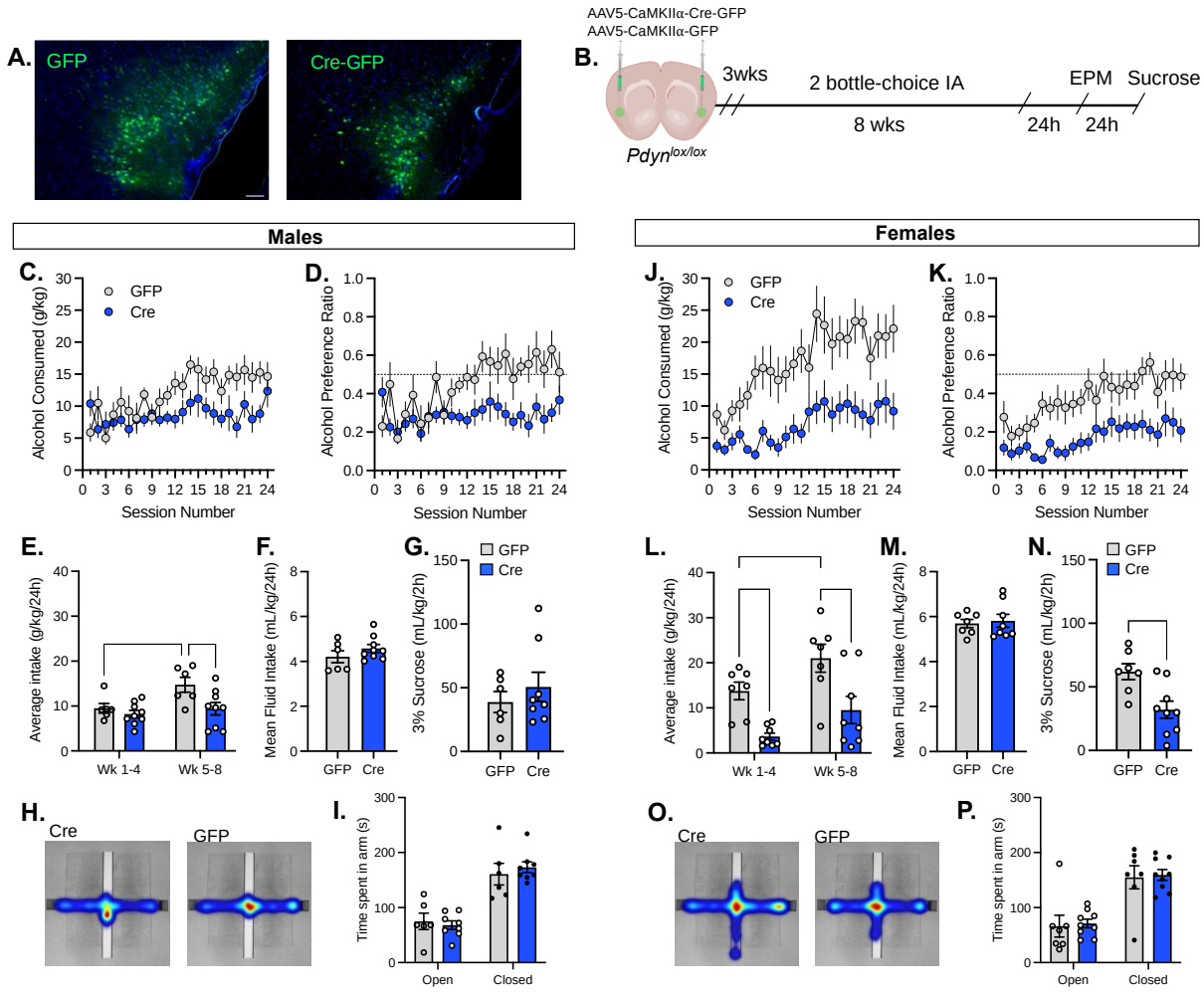

Figure 1. Genetic deletion of insula Pdyn blocks intermittency-induced escalation of alcohol intake. (A) Representative images showing expression following injection of AAV-CAMKIIa-GFP control vector (left) and AAV-CAMKIIa-Cre-GFP (right) in the mouse insula. (B) Experimental timeline: Following AAV injections and a 3 week delay for expression, male and female mice were run through an 8-week 2-bottle choice intermittent access to alcohol (IA) procedure. 24 hours after the final alcohol exposure, mice were tested for anxiety-like behavior in the elevated plus maze (EPM), then given a 2-hour sucrose challenge 24 thereafter. (C-N) Deletion of insula Pdyn significantly reduced alcohol consumption and preference in both male (C-D) and female (J-K) mice. This effect was most prominent during the final 4 weeks of drinking (highlighted blue in C-D and J-K), during which control mice consumed significantly more alcohol as compared to the first 4 weeks of IA (E, L). Cre mice did not exhibit this escalated pattern of intake and showed lower average intake levels compared to GFP controls, with males consuming significantly less alcohol at weeks 5-8 and females at weeks 1-4 and 5-8. In male mice, the effect of insula Pdyn deletion was specific to alcohol, as it did not affect mean fluid intake (F) or intake of a 3% sucrose solution (G). While insula Pdyn deletion did not impact mean fluid intake in female mice (M), it decreased sucrose consumption (N). (O-P) There was no effect of insula Pdyn deletion on anxiety-like behavior as measured in the EPM test. In both male and female mice, Cre and GFP groups spent a similar amount of time in the open and closed arms of the EPM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

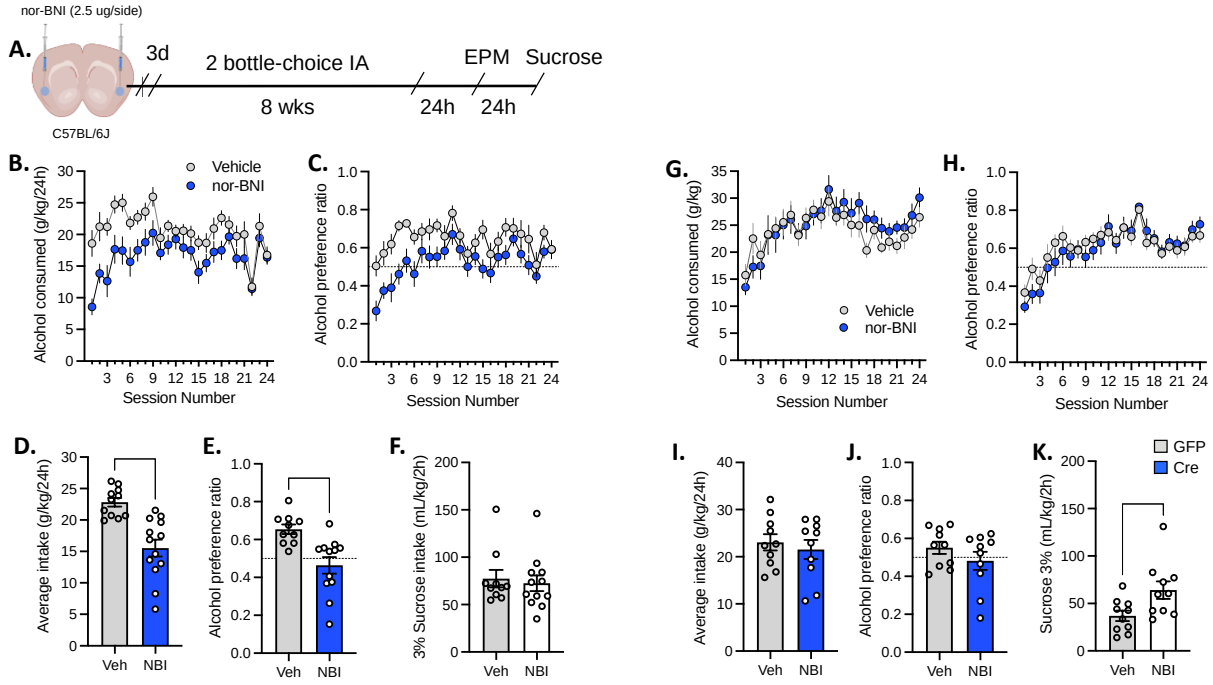

Figure 2. Pharmacological blockade of insula KOR by nor-BNI decreases alcohol intake in early phases of drinking in male mice. (A) Experimental timeline: The KOR antagonist nor-BNI (2.5 ug/side) or PBS (vehicle) was microinjected into the insula about 3 days before male and female C57BL/6J mice were exposed to an 8-week 2-bottle choice intermittent access to alcohol (IA) procedure. 24 hours after their final alcohol exposure, mice were tested for anxiety-like behavior in the elevated plus maze (EPM), then given a 2-hour sucrose challenge 24 later. In male (B-C) but not female (G-H) mice, nor-BNI produced a transient decrease in alcohol consumption (B) and preference (C) during the first 3 weeks or 9 drinking sessions (highlighted blue). In males, average alcohol intake (D) and preference (E) levels during the first 9 sessions of IA were significantly lower in nor-BNI (NBI) mice compared to vehicle (Veh) controls. There was no effect of NBI on sucrose intake (F) in males, suggesting KOR antagonism selectively reduced the consumption of alcohol. In female mice, alcohol consumption (G) and preference (H) did not significantly differ between NBI and Veh groups across the 8 week IA procedure. In females, NBII did not impact average alcohol intake (I) and preference (J) on the first 9 drinking sessions, but did increased sucrose intake (K) compared to Veh. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

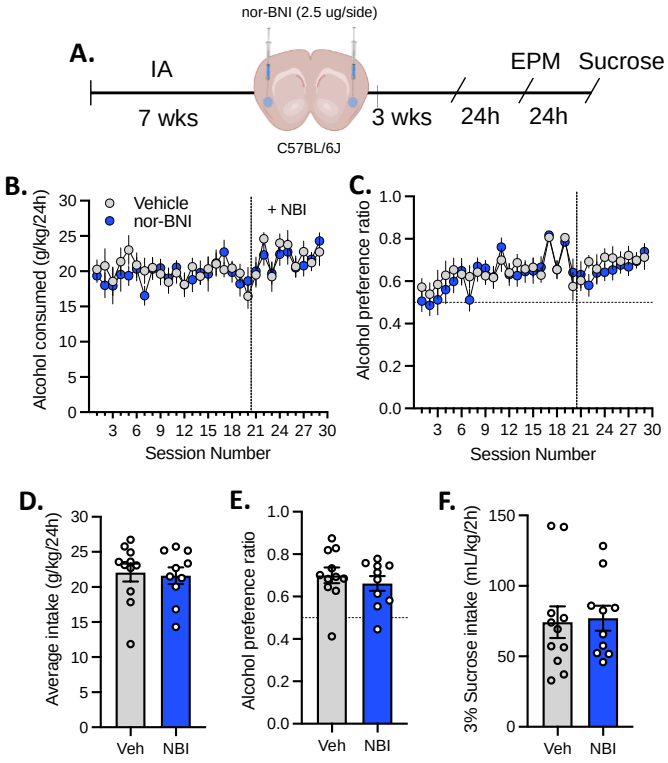

Figure 3. Pharmacological blockade of insula KOR by nor-BNI does not affect alcohol drinking in male mice during later stages of intake. A) Experimental timeline: To test the effect of nor-BNI (NBI) on the later phase of alcohol drinking, male mice were first exposed to 7 weeks of IA before NBI or PBS vehicle (Veh) was injected into the insula. Following recovery, mice were given an additional 3 weeks of IA exposure, and 24h following the final IA session, mice were tested in the EPM, then given a 2-h sucrose challenge 24h later. When injected after 7 week of IA, NBI did not affect intake of (B) or preference for (C) alcohol. There was no difference between groups in average alcohol intake (D) and preference (E) levels during the 8 sessions following injections, and NBI did not affect sucrose intake (F).

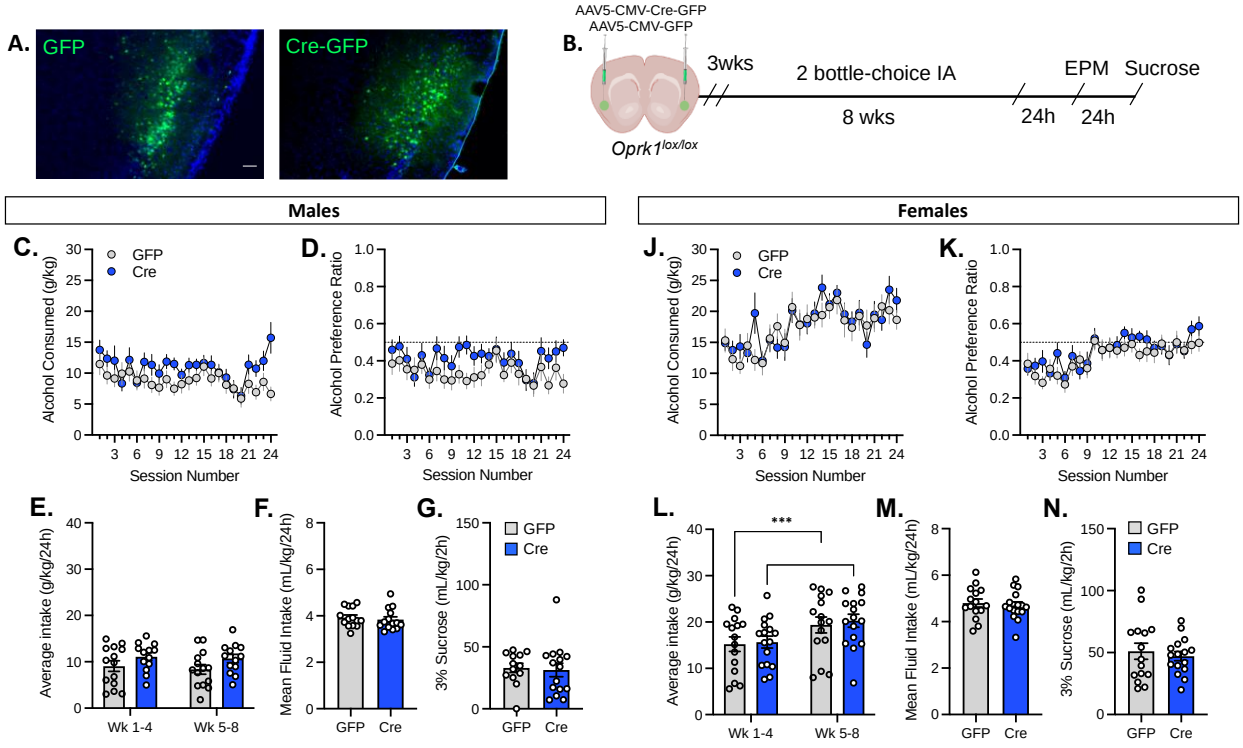

Figure 4. Genetic deletion of insula KOR does not impact alcohol intake. (A) Representative images from mice injected with AAV5-CMV-GFP (control vector, right) or AAV5-CMV-Cre-GFP (left) in the insula. (B) Experimental timeline: 3 weeks following AAV injections, male and female mice were run through an 8-week 2-bottle choice intermittent access to alcohol (IA) procedure. 24 hours after the final alcohol exposure, mice were tested for anxiety-like behavior in the elevated plus maze (EPM), then given a 2-hour sucrose challenge 24 later. (C-N) Compared to GFP mice, Cre-mediated deletion of insula KOR did not affect alcohol consumption or preference in male (C-D) or female (J-K) mice. In males, there was no difference in alcohol intake (E), mean fluid intake (F), and sucrose intake (G) between groups. Female mice showed escalated alcohol intake between weeks 1-4 and 5-8 (L). Insula KOR deletion did not impact their alcohol intake (L), mean fluid intake (M), or sucrose intake (N). *** $p < 0.001$.

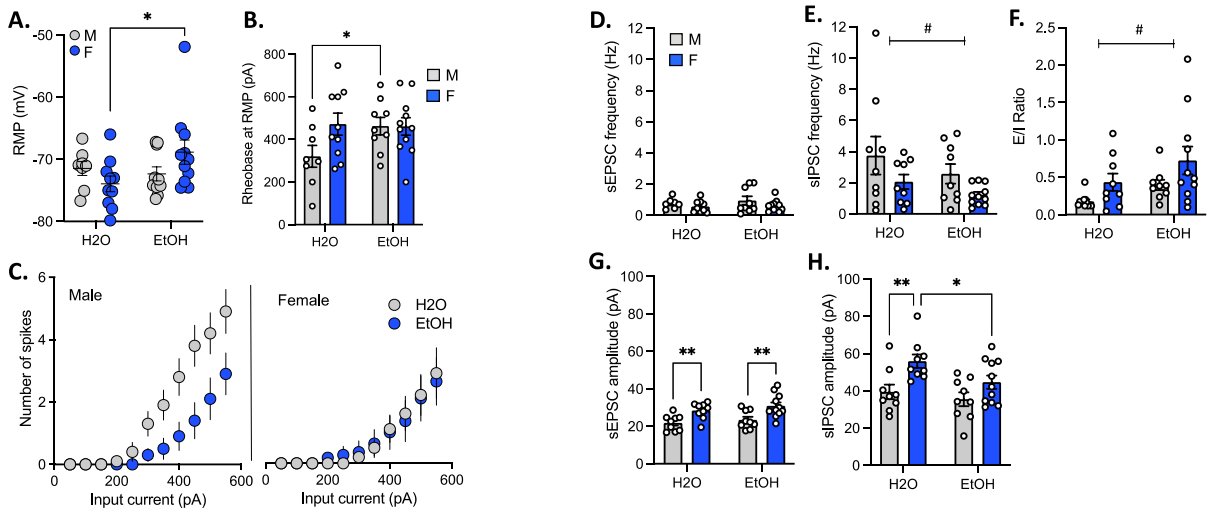

Figure 5. Long-term alcohol intake induces plasticity in insula dynorphin neurons. Whole-cell patch clamp recordings were obtained from Pdyn neurons in the insula of male and female PdynGFP mice following 8 weeks of intermittent alcohol (EtOH) access. (A-C) Intrinsic excitability was altered in Pdyn mice by alcohol drinking. Resting membrane potential (RMP) was increased in EtOH-exposed female mice as compared to water controls. In male mice, EtOH decreased the excitability of Pdyn neurons as indicated by (B) increased rheobase (minimum current needed to elicit firing) and (C) decrease firing to increasing current steps. (D-H) EtOH exposure altered synaptic transmission in Pdyn neurons. (E) Female mice exhibited lower sIPSC frequency than male mice and (F) EtOH-exposure increased the ratio of excitatory to inhibitory input onto Pdyn neurons. (G-H) There were sex-dependent differences in sE/IPSC amplitude in Pdyn neurons, as (G) sEPSC and (H) sIPSC amplitudes were higher in females than males. In female mice, (H) sIPSC amplitude was lower in EtOH-exposed mice as compared to water controls. * $p < 0.05$, ** $p < 0.01$, # $p < 0.05$ main effect of sex (E) or treatment (F).

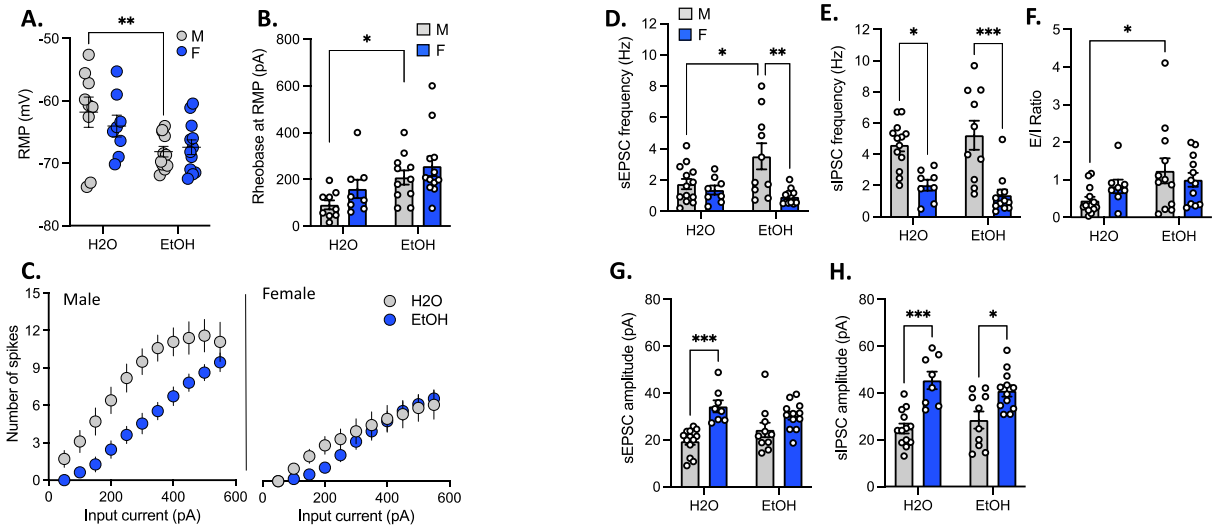

Figure 6. Long-term alcohol intake induces plasticity in deep layer pyramidal neurons (DLPNs) of the insula. Whole-cell patch clamp recordings were obtained from insula DLPN neurons in the insula of male and female mice following 8 weeks of intermittent alcohol (EtOH) access. (A-C) In male mice, EtOH decreased the excitability of DLPNs. Compared to H2O controls, EtOH mice showed (A) decreased resting membrane potential (RMP), (B) increased rheobase (minimum current needed to elicit firing), and (C) decreased firing to increasing current steps. (D-H) EtOH exposure altered synaptic transmission in DLPNs of male mice. (D) EtOH increased sEPSC frequency in male mice only, as sEPSC frequency was higher in male EtOH-exposed mice as compared to male H2O controls and female EtOH mice. (E) There was no effect of EtOH on sIPSC frequency. However, in males, sIPSC frequency was higher than in females. (F) EtOH increased the ratio of excitatory to inhibitory transmission in DLPNs in male mice only. (G-H) In male mice, sE/IPSC amplitudes were lower than female mice. (G) In H2O mice, males had a lower sEPSC amplitude than females. (H) In males, sIPSC amplitudes were lower as compared to females in H2O and EtOH groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$