Sexual dimorphism of insular cortex function in persistent alcohol drinking despite aversion in mice

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ABSTRACT:

BACKGROUND: One major hallmark of alcohol use disorder (AUD) is the persistence of alcohol drinking despite negative consequences. Among the indicators of AUD vulnerability, binge drinking is a strong risk factor. Although the lifetime prevalence of binge and AUD has been historically higher in men than women, this gap dramatically narrowed in the last decade. Additionally, sex differences in AUD and binge drinking have been shown in clinical and preclinical studies, respectively. The insular cortex plays an important role in AUD, and the anterior (aIC) and posterior (pIC) divisions have dimorphic functions. However, the contributions of the aIC and pIC sections in alcohol binge drinking and alcohol persistent drinking despite aversion, as well as the sexual dimorphism of these contributions, remained to be uncovered.

METHODS: First, by combining the drinking in the dark model with chemogenetics, we studied the causal role of aIC and pIC excitatory neurons in binge and persistent ethanol drinking in C57BL6/J male (n=49) and female (n=49) mice. Second, using calcium fiber photometry, we investigated pIC neuronal activity in both sexes (male n=14, female n=11) during both binge and persistent ethanol drinking.

RESULTS: We identified a higher binge and persistent ethanol consumption in females compared to males. Chemogenetic inhibition of aIC glutamatergic neurons reduced bitter solutions intake independently of the solvent (ethanol or water), in both sexes. In contrast, inhibition of pIC glutamatergic neurons exclusively reduced persistent ethanol drinking in female mice. Finally, using fiber photometry recordings, we uncovered that pIC glutamatergic neuron activity was selectivity increased during ethanol persistent drinking in female mice.

CONCLUSIONS: These findings suggest a sex-dependent function of the pIC in persistent ethanol drinking, providing a starting point in our understanding of the insular cortex function in the neurobiology of AUD in both sexes.

INTRODUCTION

Alcohol use disorder (AUD) is a chronic relapsing disorder defined by the persistence of excessive alcohol consumption despite physical and psychological negative consequences (1), which are a major impediment to AUD treatment (2,3). AUD is a major public health burden as 6% of the world population is affected by alcohol morbidity or mortality (4). Among the indicators of vulnerability to AUD, binge drinking, an episodic and excessive pattern of alcohol consumption leading to the intoxication threshold of 80 mg/dL, has been identified as a strong risk factor (3,5–7). Indeed, a longitudinal study showed that 43% of adolescents (13 to 18 years old) that binge-drink alcohol developed AUD at the age of 21, whereas only 7% of non-bingers developed AUD at the same age (8). In preclinical studies, binge-drinking is modeled using the drinking in the dark (DID) procedure, where mice have repeated access to alcohol for a limited time during the dark phase of the circadian rhythm (active period) which allows to reach blood ethanol concentration (BEC) of 80 mg/dL as observed in humans (8–10). Interestingly, this model can be adapted to study the persistence of alcohol drinking despite aversive outcomes, for example by adulterating ethanol with quinine, a bitter and aversive substance to mice (11–14).

Historically, men have been more likely than women to drink alcohol and exhibit pathological drinking behaviors. In 2020, AUD lifetime prevalence was still higher in men than women, reaching respectively 36% and 23% (15). However, a recent longitudinal study showed that the increase rate of AUD over a decade was drastically higher in women than men, with respective rates of 84% and 35% (16). These epidemiological data highlight that the sex difference of AUD prevalence is narrowing. Furthermore, women transition faster to AUD after regular or chronic alcohol consumption (17,18) and are subsequently more likely to develop alcohol-related diseases (e.g. cardiovascular and hepatic diseases) (19–21). Interestingly, preclinical studies revealed sex differences in alcohol intake, with female rodents drinking more than males across different models of intake, including binge drinking and drinking despite taste aversion (12,22–

29). This higher propensity to alcohol drinking in females is proposed to be, at least in part, due to their lowest sensitivity to aversive properties of alcohol, as female rodents are more resistant to ethanol conditioned taste aversion (30,31).

The insular cortex (or insula), is strongly involved in drug addiction (32–34), including AUD (35–37). Indeed, human functional imaging studies have demonstrated that alcohol cues trigger greater activity responses in the insula, in alcohol dependent subjects (38,39). Furthermore, insula white matter volume is linked to binge drinking frequency in adolescents (40). Indeed, a higher white matter volume in the left insula was associated with stronger drinking motivations, and therefore a greater frequency of binge drinking. Anatomically, the insula is subdivided into the anterior (aIC) and posterior (pIC) sections, which are proposed to have antagonistic functions in a wide range of behaviors (41–43). In AUD patients, structural studies report a reduction of the aIC volume and gray matter density compared to healthy controls, which is correlated with higher compulsive drinking measures (44–47). In addition, aIC functional connectivity with several brain regions (e.g. hippocampus, medial orbitofrontal regions) is higher in patients with AUD compared to social drinkers (48) and healthy controls (49). Intriguingly, less information is known about the role of pIC in AUD. Indeed, only two recent studies reported a decrease of pIC gray matter volumes in AUD patients (49,50) and a higher resting state of the pIC relative to healthy controls (49), suggesting that the pIC plays a role in AUD as well. Preclinical studies confirmed the role of both aIC and pIC in ethanol drinking behaviors. Indeed, chemogenetic activation or inhibition of aIC neurons respectively decrease and increase alcohol intake in several behavioral paradigms such as self-administration and intermittent 2-bottle choice (51–53). In contrast, pharmacological inactivation of pIC neurons decreases alcohol self-administration (54), suggesting divergent functions of aIC and pIC in alcohol drinking. However, how sex as a biological variable influence those divergent contributions remained untested.

Recent findings point towards structural sexual dimorphism of the insula, with a larger volume in men than women in physiological conditions, whereas in AUD long-term abstinent patients this ratio is inverted (47). In support of these clinical findings, preclinical studies highlighted sex differences of insula functions in alcohol drinking behaviors. For example, in alcohol binge drinking mice, optogenetic stimulation of aIC terminals in the dorso-lateral striatum induced larger excitatory post-synaptic currents and smaller AMPA/NMDA current ratio recorded in medium spiny neurons compared to water drinking mice (55). Interestingly, this synaptic plasticity was observed in males but not female mice (55). Additionally, in mice, short-term ethanol exposure increases the excitability of pIC projection neurons targeting the bed nucleus of the stria terminalis neurons in females, but not males (56). Although the literature pinpoints the insular cortex as a key player in alcohol-related behaviors, the sexual dimorphism of aIC and pIC function in binge and/or persistent alcohol intake despite quinine aversion remains to be uncovered.

Using an adapted DID model, we repeatedly demonstrated a higher binge and persistent ethanol consumption despite aversion in female compared to male mice. Combining the DID protocol with chemogenetics in a within-subject experimental design, we showed that inhibition of aIC glutamatergic neurons reduced bitter solutions intake independently of the solvent (e.g. ethanol or water), in both sexes. In contrast, pIC glutamatergic neuron inhibition exclusively reduced persistent ethanol drinking in female mice. Then, using *in vivo* calcium fiber photometry recordings, we identified that pIC excitatory neurons are activated during drinking of all liquids tested, when we did not consider sex as a variable. However, when comparing males and females, we uncovered that pIC glutamatergic neuron activity was selectivity increased in female mice during ethanol persistent drinking.

Altogether, we identified a sex-dependent function of the pIC in persistent ethanol drinking, providing a starting point in our understanding of the insular cortex function in the neurobiology of AUD in both sexes.

METHODS AND MATERIALS

For additional details on the methods, see the Supplement.

Stereotactic surgeries

Chemogenetic viral injection. The adeno-associated viruses encoding the inhibitory hM4Di receptor coupled to mCherry under the control of CaMKII promoter (AAV_9-CaMKIIa-hM4D(Gi)-mCherry, Addgene), to target glutamatergic neurons, or the control viral vector (AAV9/2-mCaMKII-mCherry-WPRE, ETH Zürich) were injected bilaterally (200-250 nL, 1nL/sec) in the aIC (antero-posterior +1.7mm; medial-lateral ±3.1mm; dorso-ventral -3.5mm from the bregma) or in the pIC (antero-posterior -0.35mm; medial-lateral ±4.0mm; dorso-ventral -4.2mm from the bregma).

Calcium fiber photometry viral injection and fiber implantation. A viral vector coding for the calcium sensor GCaMP6f (AAV_9-CaMKII-GCaMP6f-WPRE-SV40, Addgene) was injected unilaterally (250 nl, 1nl/sec) in the right pIC. An optic fiber (400µm diameter, 0.48 NA, >90% efficiency) inserted in a ceramic ferrule was implanted 50µm above the virus injection site.

Drinking in the dark procedure (DID)

The DID protocol is based on the circadian rhythm where animals have access to different liquids (e.g. ethanol) during the nocturnal period (8,9). Two and a half hours after the onset of the dark phase, the water bottle in the home cage was replaced by different liquids for 2 hours per day for 4 consecutive days, followed by 3 days with water access only. This 7-day cycle is repeated for 4 weeks (**Figure 2C**), starting with tap water (cycle 0), then ethanol (20% v/v in tap water, cycles 1 and 2) to study binge drinking behavior, and ethanol adulterated with quinine (500µM of quinine, cycle 3) to study persistent drinking behavior despite aversion. Finally, water or water adulterated with quinine (500µM quinine) consumption was measured during a single 2-hour session and served as control. Daily liquid consumption was obtained by weighting the bottle before and after the 2-hour session, and mice were weighed the first day of each cycle.

Blood ethanol concentration measurement. To measure blood ethanol concentrations (BEC), blood sampling was performed by incision of the lateral tail vein 30 minutes or 2 hours after the onset of the ethanol session. The samples were immediately centrifuged at 10,000 rpm for 10 minutes at 4°C. The BEC in mg/dL was obtained from the serum using the Analox GL5 analyzer. We excluded 3 males and 3 females from the BEC analysis after 2-hour ethanol intake because the blood sample did not contain enough serum.

Chemogenetic inhibition of aIC or pIC excitatory neurons during binge and persistent ethanol drinking

The same DID procedure as described above was used. Additionally, a subgroup of animals underwent three more days of water+quinine followed by a last cycle of ethanol+quinine to control the resumption of persistent ethanol drinking. The day before the chemogenetic manipulation an intraperitoneal (i.p.) injection of vehicle (NaCl, 0.1 mL/kg) was performed 30 minutes before the drinking session for habituation. The causal role of aIC or pIC glutamatergic neurons on drinking was tested after injection of CNO (i.p. 3 mg/kg in NaCl, Tocris) or vehicle 30 minutes before the last session of binge (day 18), persistent ethanol drinking (day 25) and a single session of water or water+quinine (day 29). To note, mice tested for chemogenetic inhibition of pIC on water+quinine drinking (day 29) were CNO naive.

Coding properties of pIC excitatory neurons during binge and persistent ethanol drinking

The same DID procedure as described before was used, except that no initial water cycle was performed. To study the coding properties of pIC neurons during binge and persistent ethanol drinking, the calcium signal and the drinking behavior (e.g. licking) has to be synchronized. Thus, after two ethanol binge cycles in their home cage, mice (n=14 males and n=11 females) underwent three cycles of ethanol drinking (cycle 3 to 5) in multifunctional boxes equipped with lickometers (**Figure S2A**). Then, after one cycle of ethanol+quinine drinking in the home cages

(cycle 6), mice underwent a cycle of ethanol+quinine drinking in the multifunctional boxes (cycle 7). Finally, a water and water+quinine photometry recording session was performed in these boxes as a control. To note 3 male and 2 female ethanol naive mice were included in water recording analysis and 4 male and 2 female ethanol naive mice were included in water+quinine recording analysis.

Fiber photometry recordings. The recordings were performed with Neurophotometrics fiber photometry system (FP3002 V2), on the last day of the last ethanol and ethanol+quinine cycles, and during a single water and water+quinine session (**Figure S2A**). The recordings were performed using a 4-branch patch cord (400µm diameter, NA=0.48, 1 branch per animal). Then, the drinking sprout was filled with the appropriate solution, and the recording lasted 1 hour as previously done (33). Mice were kept in the boxes for an additional hour for a total of a 2-hour session. The drinking sprout was automatically refilled to give *ad-libitum* access to the mice. The optimal wavelength for GCaMP6f excitation is 470 nm, while the isosbestic wavelength is 415 nm. To minimize the photobleaching effect of the recording, the light intensity at the tip of the patch cord was adjusted between 80 and 100 µW for both channels.

Calcium fiber photometry data analysis. Photometry recordings were analyzed using custom Python scripts which can be downloaded at: https://github.com/praneethnamburi/photometry_collab_ab. The timestamps for lick events were extracted and classified in bouts. A bout event is defined as a lick or a group of licks with an inter lick interval (ILI) less than or equal to 10 seconds, for the peri-event analysis. The first 10 minutes of signals were discarded. Then, GCaMP and isosbestic signals were de-trended using the airPLS algorithm (57), and the detrended isosbestic signal was regressed from the detrended GCaMP signal. The resulting signal was bandpass filtered with cutoff frequencies of 0.2 and 6 Hz using a finite impulse response filter implemented in the scipy package. Finally, a time window of -10 to +10 s was defined for the peri-event analysis of the resulting signal aligned to the first lick

of each bout. Signal from each window was z-normalized by subtracting the mean of the baseline and dividing the result by the standard deviation of the baseline, where the baseline signal was defined as the signal from 10 s to 8 s before a lick bout onset. Z-normalized calcium signals were averaged for each male and female mouse during the reference (-8 to -5 sec) and licking window (0 to +3 sec) to describe the signal changes.

RESULTS

Sexual dimorphism of ethanol binge drinking: validation of the drinking in the dark model

After 2 ethanol cycles (**Figure 1A**), the average of ethanol consumption over 2-hour access was higher in females than males (**Figure 1B,** unpaired Student t-test, t=3.864, p=0.0007) whereas the BEC levels were similar between sexes (**Figure 1C**), with 23% of males and 15% of females reaching the binge intoxication threshold of 80 mg/dL. The literature suggests that mice drink the majority of the total ethanol intake at the beginning of the DID sessions (10). Thus, we measured the proportion of ethanol consumed during the first 30-minutes of the 2-hour session and we showed that males consumed 67% and females 84% of the total ethanol intake during this period (**Figure 1D**). Moreover, in an additional session, ethanol intake and BEC were measured after 30 minutes of ethanol access and revealed a higher ethanol intake (**Figure 1E**, unpaired Student t-test, t=3.679, p=0.0009) and BEC (**Figure 1F**, unpaired Mann-Whitney, U=71, p=0.0318) in females than males with a respective proportion of 75% and 44% of mice reaching the intoxication threshold of 80 mg/dL which validates the model.

Chemogenetic inhibition of aIC and pIC glutamatergic neurons during binge and persistent ethanol drinking

At the behavioral level, for all liquids during the sessions without chemogenetic manipulation, animals injected into aIC or pIC showed similar results, so we grouped them together (**Figure 3A-H**). Across the sessions, both male and female mice had similar levels of water intake as well as

average water intake (**Figure 3A, B**). In contrast, female mice consumed a larger amount of ethanol across the sessions (**Figure 3C**, 2-way RM-ANOVA, sex factor $F_{1,97}$=94.91, p<0.0001 and session factor $F_{5.192,\ 503.7}$=4.629, p=0.0003 without interaction $F_{6,\ 582}$=0.5145, p=0.7976) and on average than males (**Figure 3D**, unpaired Student t-test, t=9.742, p<0.0001) although the absolute volume of ethanol solution was similar between sexes (**Figure S1A**). Similarly, when ethanol was adulterated with quinine, female mice had a higher consumption than males across the sessions (**Figure 3E**, 2-way RM-ANOVA, sex factor $F_{1,\ 96}$=94.71, p<0.0001 and session factor $F_{1.880,\ 180.5}$=4.931, p=0.0095 without interaction $F_{2,\ 192}$=1.233, p=0.2938), on average (**Figure 3F**, unpaired Student t-test, t=9.732, p<0.0001) as well as on absolute volume (**Figure S1B**, unpaired Student t-test, t=4.472, p<0.0001). Both ethanol and ethanol adulterated with quinine drinking were independent of the hormonal cycle in female mice (**Figure S1C, D**). To test the strength of persistent ethanol drinking despite quinine aversion, a subgroup of mice had access to water+quinine for 3 sessions, followed by 4 sessions of ethanol+quinine access. Both males and females show an increase of ethanol+quinine intake after exposure to 3 days of quinine diluted in water (**Figure 3G**, 2-way RM-ANOVA, sex factor $F_{1,\ 35}$=11.00, p=0.0021, session factor $F_{6,\ 210}$=17.35, p<0.0001, with an interaction $F_{6,\ 210}$=3.316, p=0.0039). Interestingly, in both sexes, the average of quinine solution consumed was larger when quinine was mixed to ethanol, than when quinine was diluted in water (**Figure 3H**, 2-way RM-ANOVA, sex factor $F_{1,\ 46}$=17.32, p=0.0001, liquid factor $F_{1,\ 46}$=52.47, p<0.0001, with a sex x liquid interaction $F_{1,\ 46}$=8.905, p=0.0045). These results validate the aversiveness of quinine and that ethanol reintroduction to quinine is sufficient to resume ethanol persistent drinking in both sexes.

To study the causal role of aIC and pIC neurons in binge and persistent ethanol drinking, we used a chemogenetic approach (**Figure 2A-C**), that we validated using *in-vivo* electrophysiology (**Figure S1E, F**, paired Wilcoxon test, p=0.023), to inhibit aIC or pIC glutamatergic neurons by expressing hM4Di, under the CaMKIIα promoter, before the mice underwent the behavioral

protocol. Then, we investigated the effect of chemogenetic inhibition of aIC or pIC excitatory neurons on binge and persistent ethanol intake. Chemogenetic inhibition of aIC or pIC glutamatergic neurons (**Figure S1M, N**) do not influence ethanol drinking in both sexes (**Figure 4A**, 2-way ANOVA, sex factor $F_{1, 42}$=33.65, p<0.0001, virus factor $F_{1, 42}$=0.9755, p=0.3290, with no sex x virus interaction $F_{1, 42}$=1.340, p=0.2535, **Figure 4F**, 2-way ANOVA, sex factor $F_{1, 26}$=27.71, p<0.0001, virus factor $F_{1, 26}$=0.0001289, p=0.9910, without sex x virus interaction $F_{1, 26}$=0.2937, p=0.5925). In contrast, the inhibition of aIC excitatory neurons reduced ethanol+quinine drinking independently of the sex (**Figure 4B**, 2-way ANOVA, sex factor $F_{1, 41}$=18.71, p<0.0001, virus factor $F_{1, 41}$=8.624, p=0.0054, with no sex x virus interaction $F_{1, 41}$=2.201, p=0.1456), whereas the inhibition of pIC excitatory neurons reduced ethanol+quinine intake exclusively in female mice (**Figure 4G**, 2-way ANOVA, sex factor $F_{1, 26}$=42.01, p<0.0001, virus factor $F_{1, 26}$=14.04, p=0.0009, with a sex x virus interaction $F_{1, 26}$=7.055, p=0.0133). To control whether aIC and pIC glutamatergic neurons inhibition was specific to ethanol persistent drinking, we tested the effect of the same chemogenetic manipulation on water and water+quinine intake. The inhibition of aIC or pIC glutamatergic neurons did not change water consumption nor in males neither in females (**Figure 4C, H**). In contrast, inhibition of aIC neurons decreased water+quinine intake independently of the sex (**Figure 4D**, 2-way ANOVA, sex factor $F_{1, 19}$=1.481, p=0.2386, virus factor $F_{1, 19}$=10.80, p=0.0039, with no sex x virus interaction $F_{1, 19}$=1.296, p=0.2691), while, we observed a strong trend of pIC inhibition to reduce water+quinine intake mainly driven by the male group (**Figure 4L**, 2-way ANOVA, sex factor $F_{1, 19}$=2.677, p=0.1183, virus factor $F_{1, 19}$=4.230, p=0.0537, with no sex x virus interaction $F_{1, 19}$=2.999, p=0.0995). Finally, to rule out any behavioral confound due to CNO treatment, the locomotion was measured in control mice that received CNO or vehicle injection, and in mice expressing the inhibitory viral vector in aIC or pIC that received CNO injection. Locomotion was similar between the three groups (**Figure 4E, J**). Finally, CNO injection in control animals did not alter ethanol,

ethanol+quinine and water intake compared to control animals injected with vehicle (**Figure S1G-L**).

Together, our results demonstrate a divergent function of aIC and pIC glutamatergic neurons on drinking behaviors with the aIC neurons supporting bitter solution drinking in both sexes and the pIC neurons underpinning persistent ethanol drinking only in females.

Coding properties of pIC glutamatergic neurons during binge and persistent ethanol drinking

Using fiber photometry with the genetically encoded calcium sensor GCaMP6f (**Figure 5A, B, S2F**), we recorded pIC glutamatergic neurons activity during licking behaviors over 1 hour (**Figure 5C**, **S2**). Behaviorally, the average of ethanol intake across all drinking sessions, excluding the recording session, was similar between males and females (**Figure 5D**), whereas the average of ethanol+quinine intake was higher in females (**Figure 5E,** unpaired Student t-test, t=4.023, p=0.0024). Both sexes did the same average number of bouts for ethanol, ethanol+quinine, water and water+quinine during the recording sessions (**Figure S2B-E**). At the neural activity level, calcium signal in pIC neurons was increased during ethanol licking compared to the reference independently of the sex (**Figure 5F, G**, 2-way RM-ANOVA, sex factor $F_{1, 10}$=1.593, p=0.2355, period factor $F_{1, 10}$=7.708, p=0.0196, with no sex x period interaction $F_{1, 10}$=0.3726, p=0.5552). Furthermore, in females, ethanol+quinine licking increased the calcium signal in pIC neurons compared to the reference. Moreover, pIC neurons activity during ethanol+quinine licking was higher in female compared to male mice (**Figure 5H, I**, 2-way RM-ANOVA, sex factor $F_{1, 10}$=8.368, p=0.0160, period factor $F_{1, 10}$=12.98, p=0.0048, with a sex x period interaction $F_{1, 10}$=5.026, p=0.0489). To control the specificity of the increase of pIC neuronal activity in female mice in response to persistent ethanol drinking, we performed fiber photometry recording during water and water+quinine licking. Independently of the sex, the calcium signal in pIC neurons increased during water licking compared to the reference (**Figure**

5J, K, 2-way RM-ANOVA, sex factor $F_{1, 12}$=1.081, p=0.3189, period factor $F_{1, 12}$=12.51, p=0.0041, without interaction $F_{1, 12}$=3.316, p=0.0936). Similarly, independently of the sex, pIC neurons exhibited an increase of calcium signal during water+quinine licking compared to the reference (**Figure 5L, M,** 2-way RM-ANOVA, sex factor $F_{1, 15}$=1.708, p=0.2109, period factor $F_{1, 15}$=8.119, p=0.0122, without interaction $F_{1, 15}$=0.4080, p=0.5326)**.**

DISCUSSION

The goal of our study was to elucidate whether the aIC and pIC have sexually dimorphic function on ethanol binge drinking and persistent ethanol drinking despite bitter aversion in mice. First, we observed higher binge and persistent ethanol intake in females compared to males (**Figure 3C-F**). Second, we demonstrated that, independently of sex, chemogenetic inhibition of aIC glutamatergic neurons reduced the intake of quinine adulterated solutions (e.g. ethanol and water, **Figure 4B, D**). In contrast, chemogenetic inhibition of pIC glutamatergic neurons reduced exclusively persistent ethanol drinking in females but not males (**Figure 4G**). Finally, pIC neuronal activity increases during ethanol, water and water+quinine drinking, independently of sex, (**Figure 5F, G, J-M**). However, pIC neuronal activity responses to persistent ethanol intake, specifically in females but not in males (**Figure 5H, I**). All together, these findings suggest a causal role of aIC glutamatergic neurons in bitter liquid drinking, and a sex-dependent function of pIC excitatory neurons in persistent ethanol drinking.

Sex-dependent behavior on binge and persistent ethanol drinking

Our results repeatedly showed a higher binge and persistent ethanol intake per body mass in females compared to males, independently of the estrous cycle, which is consistent and extend previous literature highlighting sex differences in drug addiction (58,59). Indeed, multiple preclinical studies also reported a higher binge (12,26,56) and persistent (12,27,60) ethanol intake per body mass in females, than in males. Importantly, to rule out any confound on sex-dependent aversion resistance for quinine, we adulterated the ethanol solution with 500 µM quinine, a

concentration for which both male and female mice have similar aversive threshold (61). Therefore, the sex differences observed in the consumption of ethanol adulterated with quinine is independent of the aversion sensitivity but driven toward the ethanol intake.

Bitter taste processing in aIC excitatory neurons

We demonstrated that inhibition of aIC glutamatergic neurons reduced the intake of solutions adulterated with quinine (e.g. ethanol or water) but did not alter binge ethanol intake. Our results support and extend Haaranen and al. study, which demonstrated that chemogenetic inhibition of aIC neurons had no effect on alcohol intake in alcohol-preferring male rats in a two-bottle choice model (53). However, it contrasts with previous literature demonstrating that chemogenetic manipulation of aIC neurons modulates alcohol binge drinking, although the results are divergent. Indeed, while silencing aIC neurons increased operant responses for alcohol (51), it decreased alcohol intake in a two-bottle choice paradigm (62). Altogether, these results suggest that aIC function in ethanol binge drinking might be dependent on the animal model used. Moreover, while we specifically manipulated aIC glutamatergic neurons, the studies cited previously manipulated all aIC mature neurons independently of their phenotype which could explain some discrepancy in the results observed.

On the other hand, we demonstrated that aIC excitatory neurons play a role in bitter solution intake as shown by the reduction of ethanol and water adulterated with quinine consumption following aIC glutamatergic inhibition. These results extend previous findings demonstrating a reduction of ethanol+quinine intake in male mice compared to controls after inhibition of aIC neurons, as well as its projections to locus coeruleus or nucleus accumbens core (62,63). In addition, Chen and Lasek reported an increase expression of the neuronal activity marker *cFos* in the aIC after ethanol+quinine intake in male mice compared to water, ethanol and quinine drinking (64). Overall, these observations confirm the implication of aIC in quinine-adulterated ethanol drinking in males, and our study extends this finding to female mice. In regard to the role of aIC in water+quinine intake, the literature is more divergent. On one hand, inhibition or lesion

of the aIC respectively had no effect (65,66) or decreased (67) sensitivity to quinine, leading to an increase of the intake. On the other hand, activation of aIC neurons and its projection to basolateral amygdala also decreased quinine sensitivity (41,68). These findings contrast with our results where aIC excitatory inhibition reduced quinine intake. In our study, we chose not to water deprive our animals in order to observe behavioral responses as close as possible to physiological conditions. In contrast, in previously cited studies, animals were water deprived before quinine exposure which could have induced a physiological stress altering aIC response to quinine.

Overall, these results suggest a potential role of aIC in bitter solution intake, however further investigations are necessary to sharpen our understanding of aIC neurons and its projections in aversive solutions consumption in males and females.

Uncovering sex-dependent pIC function in persistent ethanol intake

We demonstrated that inhibition of pIC glutamatergic neurons reduces persistent ethanol drinking only in females without altering ethanol, water, or water adulterated with quinine drinking. To our knowledge, these results are the first demonstration of a sex-dependent function of pIC neurons on persistent ethanol drinking. Using the two-bottle choice paradigm, a recent study showed a higher expression of the neuronal activity marker *cFos* in the pIC of males compared to females after ethanol+quinine intake (69). Importantly, in this study ethanol was adulterated with 100 µM of quinine. Previous reports suggests that females are not sensitive to ethanol adulterated with quinine at this concentration whereas males are (27) which could explain the discrepancy with our results where we used a concentration of quinine at 500 µM. Furthermore, using an operant alcohol drinking model in male rats, pharmacological inactivation of pIC neurons reduces alcohol intake (54) while in our study, chemogenetic inhibition of pIC excitatory neurons did not change ethanol drinking nor in males neither in females. Several methodological aspects can, at least in part, support this discrepancy. First, operant and voluntary alcohol drinking could recruit different

neuronal circuits, second, the methods used for the neuronal inhibition (e.g. pharmacology vs chemogenetics) and the animal model used (e.g. rat vs mouse) were different.

To rule out the possibility that pIC glutamatergic inhibition was due to non-selective effects of CNO, we performed multiple control experiments. Indeed, it has been shown that CNO can bind to non-DREADD targets, and does not cross the blood brain barrier. However, clozapine, a CNO metabolite, is reaching the brain and can also have multiple pharmacological targets which can induce several physiological and behavioral effects (70,71). In our control experiments, we found that CNO injection in mice expressing the control viral vector in pIC, had no effects on either liquid consumption (e.g. ethanol, ethanol+quinine or water) or locomotion. Furthermore, CNO injection in mice injecting with the viral vector carrying the gene coding for hM4Di also had no effect on locomotion. These results indicate first, that CNO does not have non-specific effects neither on drinking nor on locomotion and secondly, that the inhibition of pIC excitatory neurons did not affect locomotion. Overall, these results confirm that the behavioral effect of CNO on ethanol+quinine drinking is due to selective inhibition of pIC excitatory neurons.

Functional sex differences in pIC coding properties of persistent ethanol drinking

The chemogenetic manipulations demonstrated the necessity of pIC excitatory neurons to change persistent ethanol drinking in females, but these artificial manipulations do not reflect how pIC excitatory neurons encode this behavior. Thus, we performed *in vivo* fiber photometry recordings of pIC calcium signal and identified an increased activity of pIC glutamatergic neurons during ethanol licking, as well as water and water+quinine licking, independently of the sex. These results consolidate recent findings demonstrating that pIC excitatory neurons respond to water intake (72) which suggests that these neurons encode drinking behaviors in both males and females. Furthermore, pIC neuronal activity increased during water+quinine drinking, which is consistent with previous studies reporting that pIC neurons encode bitter taste, independently of the sex (43,73). In contrast, we decipher a sex-dependent pIC neuronal coding property in persistent

ethanol drinking with an increase of pIC excitatory neurons activity exclusively in females during ethanol adulterated with quinine drinking. Our results strengthen a recent study demonstrating that short ethanol exposure increases intrinsic excitability of pIC-BNST projection in female mice only (56) highlighting pIC glutamatergic neurons and their projections as one of the key regions supporting sex-dependent functions in alcohol-related behaviors. One limitation of our study is the manipulation and the recording of pIC excitatory neurons regardless of their outputs. The pIC is a highly wired region sending dense projections to several regions (43,74), including the central amygdala, a brain region highly involved in alcohol-related behaviors and supporting sex-differences in alcohol intake (75–77). Therefore, future investigations will be crucial to understand the sex-dependent function of pIC neurons in alcohol-related behaviors at the circuit level.

Conclusions

To conclude, our study demonstrated that aIC and pIC have different roles in drinking behaviors. While aIC excitatory neuron inhibition decreases bitter-tastant solutions intake independently of mice sex, pIC glutamatergic neurons inhibition exclusively reduces persistent ethanol intake despite aversion in female mice. In line with this result, pIC glutamatergic neurons activity increased specifically in females during ethanol consumption despite bitter aversion. Our results provide a starting point to further characterize the role of pIC circuits in sex-dependent addictive-related behaviors.

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AUTHORSHIP CONTRIBUTIONS

CF, CN and AB designed the study. CF, CGM, YC, CN and AB carried out the experiments and performed data analysis. PN wrote the custom Python code for fiber photometry analysis. CF, CN and AB wrote the manuscript. All authors critically reviewed the content and approved the final version before submission.

DISCLOSURE

The authors declare that they do not have any competing interests or conflicts of interest (financial or non-financial) related to the material presented in this manuscript.

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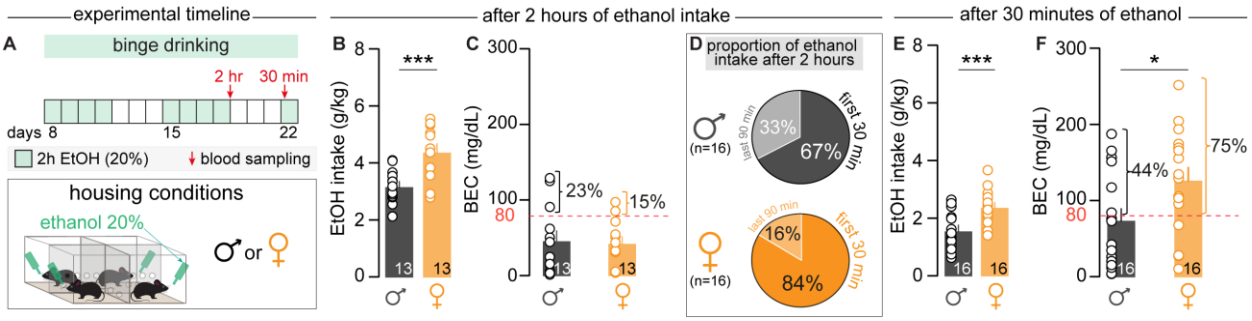


Figure 1. Validation of the drinking in the dark model. **(A)** Experimental timeline of binge ethanol exposure according to the drinking in the dark procedure. Blood was sampled after 2 hours of session at day 18 and after 30 minutes of session at day 22. **(B, C)** Average of ethanol intake (**B**) and blood ethanol concentration (**C**) after a 2 hours of ethanol binge drinking (day 18) in male and female mice. The red dashed line represents the intoxication threshold of 80 mg/dL of ethanol in blood. **(D)** Proportion of ethanol intake during the first 30 minutes and last 90 minutes of a 2-hour session. **(E, F)** Average of ethanol intake (**E**) and blood ethanol concentration (**F**) after 30 minutes of session (day 22) in male and female mice. Data are shown as mean ± SEM. *p<0.05 ***p<0.001.

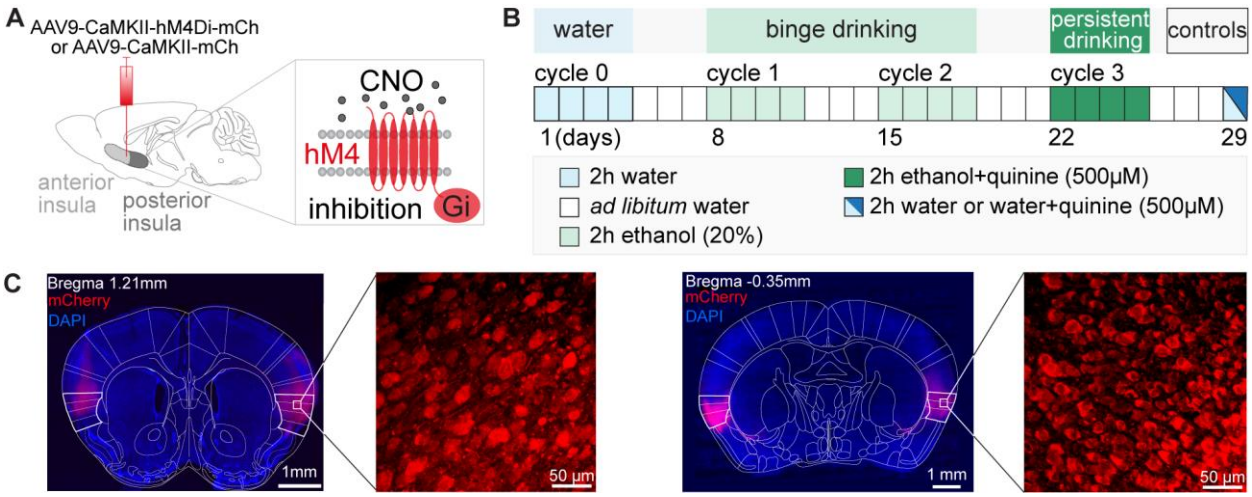


Figure 2. Experimental approach for chemogenetic manipulation of anterior (aIC) and posterior (pIC) insular cortex neurons during drinking in the dark procedure. **(A)** Experimental design to inhibit aIC and pIC excitatory neurons. A viral vector carrying the gene coding for the inhibitory receptor hM4Di fused to the fluorescent reporter mCherry, or the control virus, containing only the gene for mCherry, was bilaterally injected in the aIC or pIC. **(B)** Behavioral timeline during the drinking in the dark procedure. **(C)** Representative images of the chemogenetic viral vector expression in the aIC (left) and the pIC (right) neurons. The mouse brain atlas delineation has been overlaid to the image, with the borders of the aIC and pIC in bold.

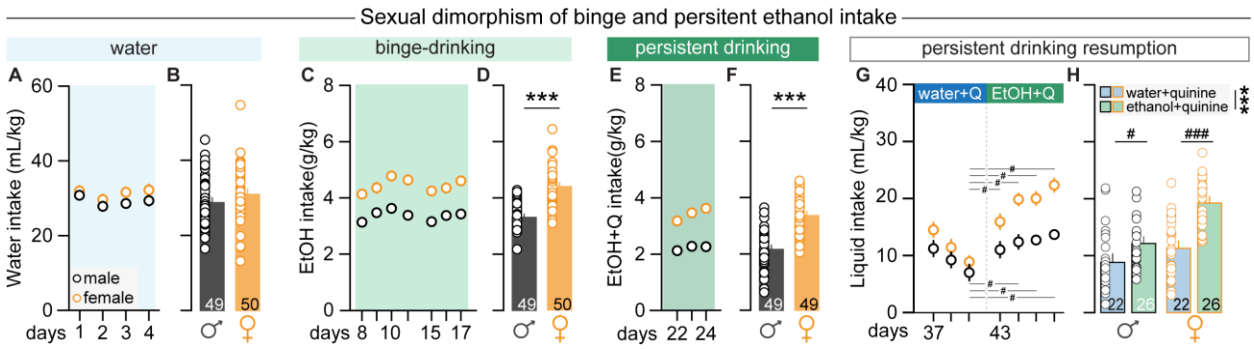


Figure 3. Sexual dimorphism of binge and persistent ethanol drinking. **(A, B)** Kinetic (**A**) and average (**B**) of water intake during cycle 0 in male and female mice. **(C, D)** Kinetic (**C**) and average (**D**) of ethanol intake (binge drinking) during cycle 1 and 2 in male and female mice. **(E, F)** Kinetic (**E**) and average (**F**) of ethanol+quinine intake (persistent drinking) during cycle 3 in male and female mice. **(G)** Kinetic of water+quinine (water+Q) and ethanol+quinine (EtOH+Q) intake in males and females. # $p<0.05$ compared to day 39. **(H)** Comparison of water and ethanol adulterated with quinine intake during the last session exposure in male and female mice. Data are shown as mean ± SEM. ***$p<0.001$ represent significant t-test and main effect of 2-way ANOVA. ##$p<0.01$ ###$p<0.001$ represent significant Bonferroni post-hoc test.

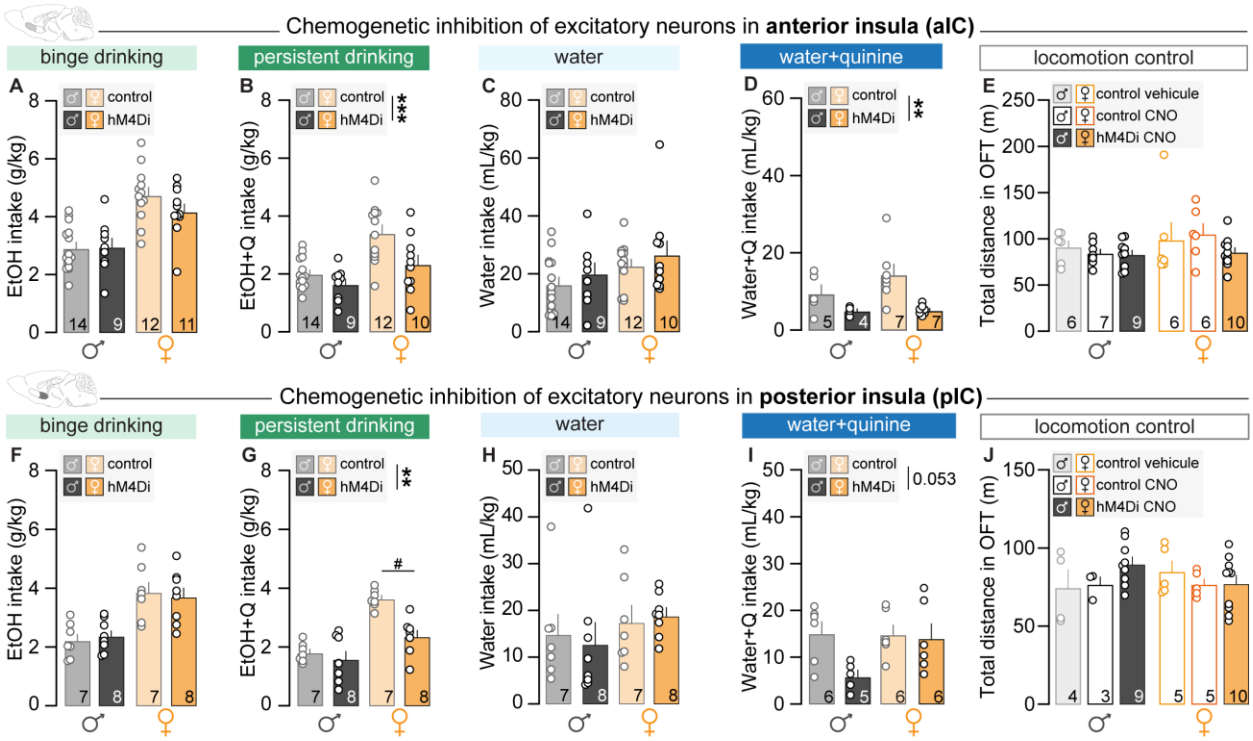


Figure 4. Chemogenetic inhibition of anterior (aIC) or posterior (pIC) insular cortex excitatory neurons during binge and persistent ethanol intake. **(A)** Average of ethanol intake during chemogenetic manipulation of the aIC glutamateric neurons at day 18 in males and females. **(B)** Average of ethanol+quinine intake during chemogenetic manipulation of the aIC at day 25, in males and females. **(C)** Average of water intake during chemogenetic manipulation of the aIC at day 29, in males and females. **(D)** Average of water+quinine intake during chemogenetic manipulation of the aIC at day 29, in male and female mice. **(E)** Average of total distance traveled in an open field over 15 minutes. **(F)** Average of ethanol intake during chemogenetic manipulation of the pIC neurons at day 18, in males and females. **(G)** Average of ethanol+quinine intake during chemogenetic manipulation of the pIC neurons at day 25, in males and females. **(H)** Average of water intake during chemogenetic manipulation of the pIC neurons at day 29, in males and females. **(I)** Average of water+quinine intake during chemogenetic manipulation of the pIC neurons day 29, in an independent group of male and female mice. **(J)** Average of total distance traveled in an open field over 15 minutes. Data are shown as mean ± SEM. **p<0.01 ***p<0.001

represent significant main effect of 2-way ANOVA. #p<0.05 represents significant Bonferroni post-hoc test.

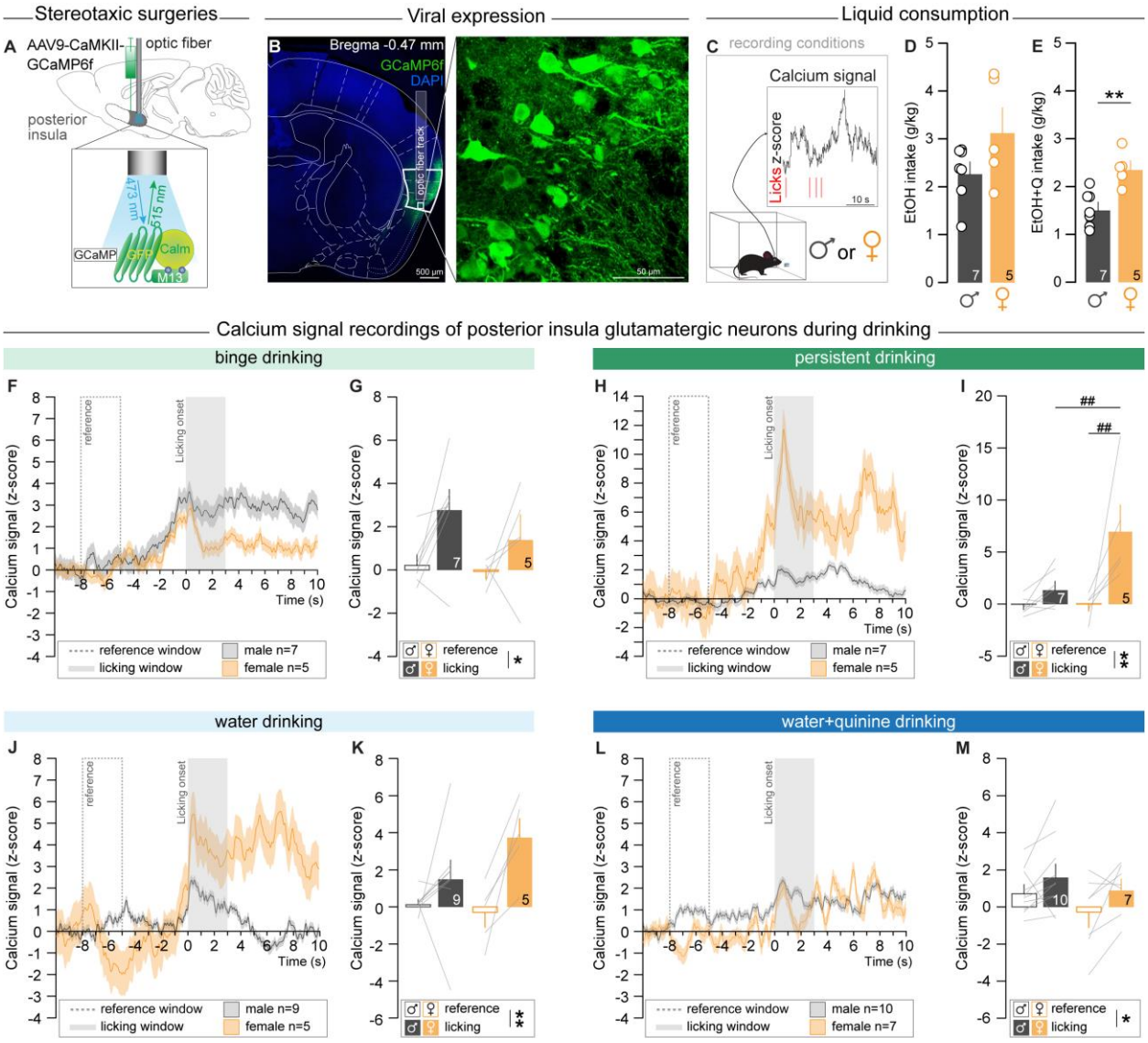
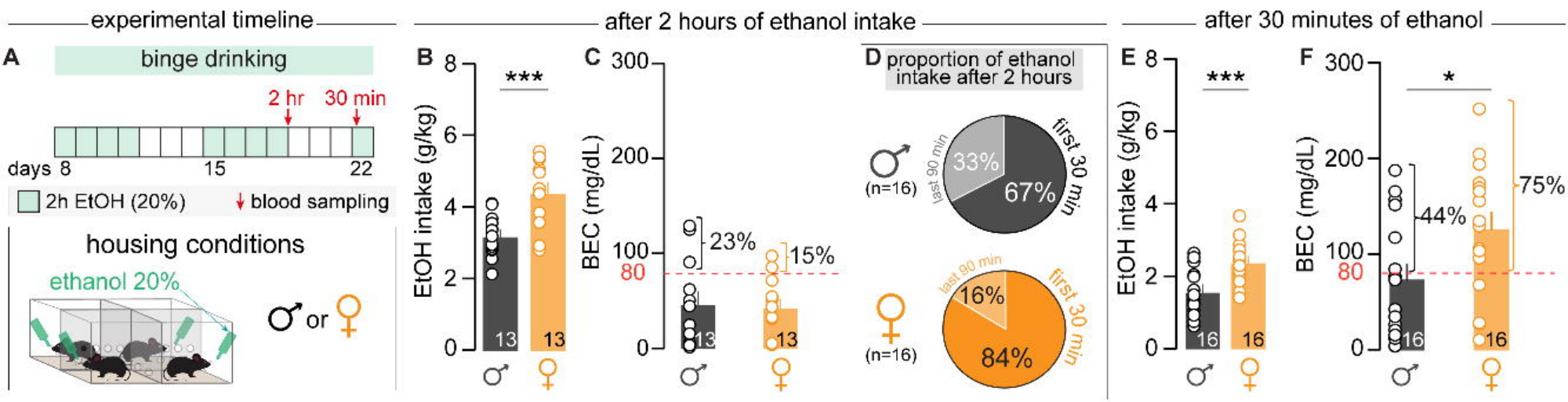
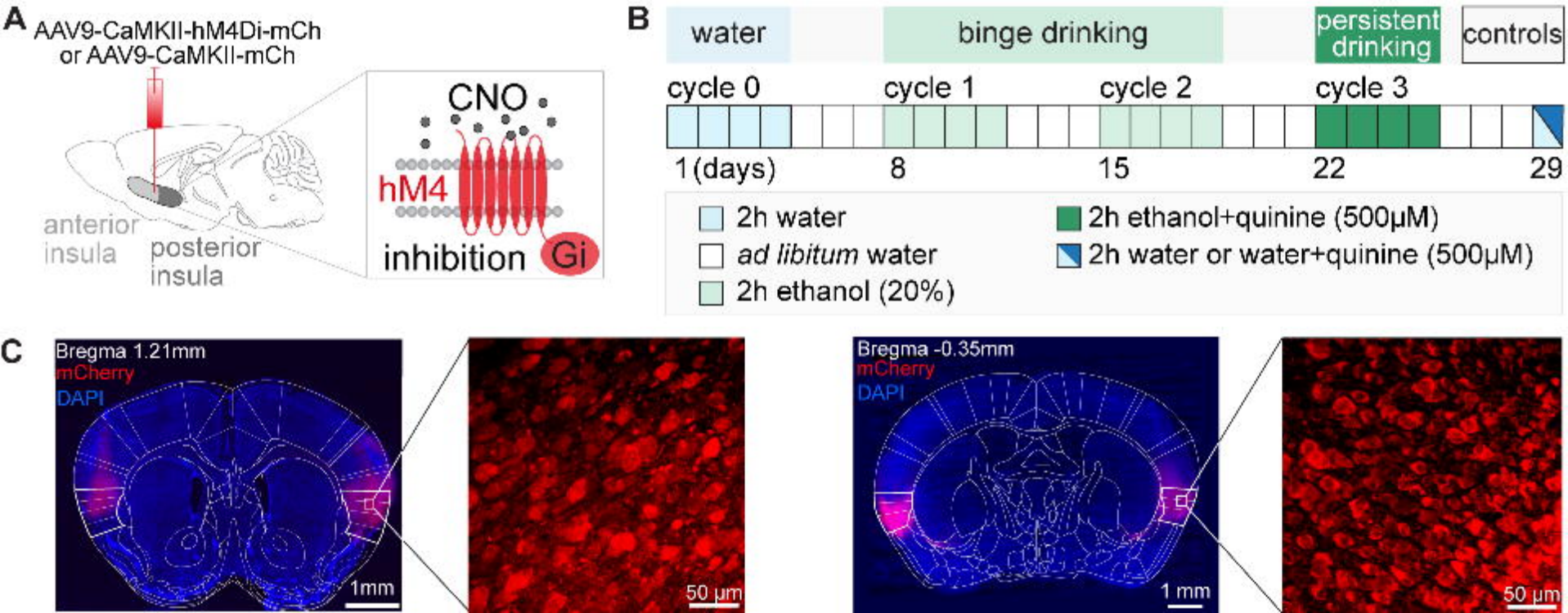
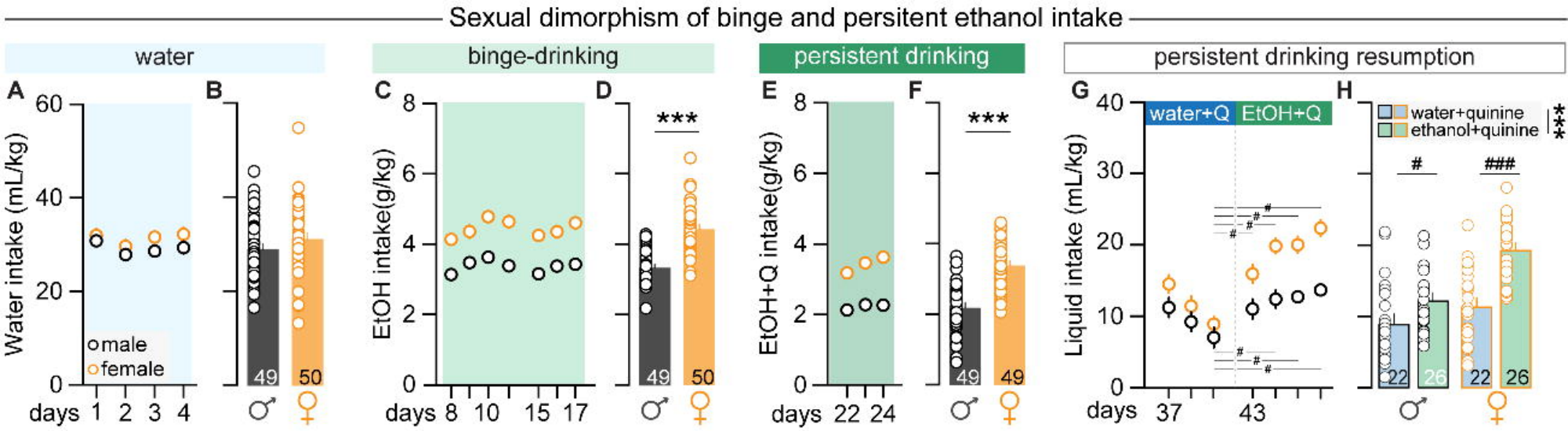


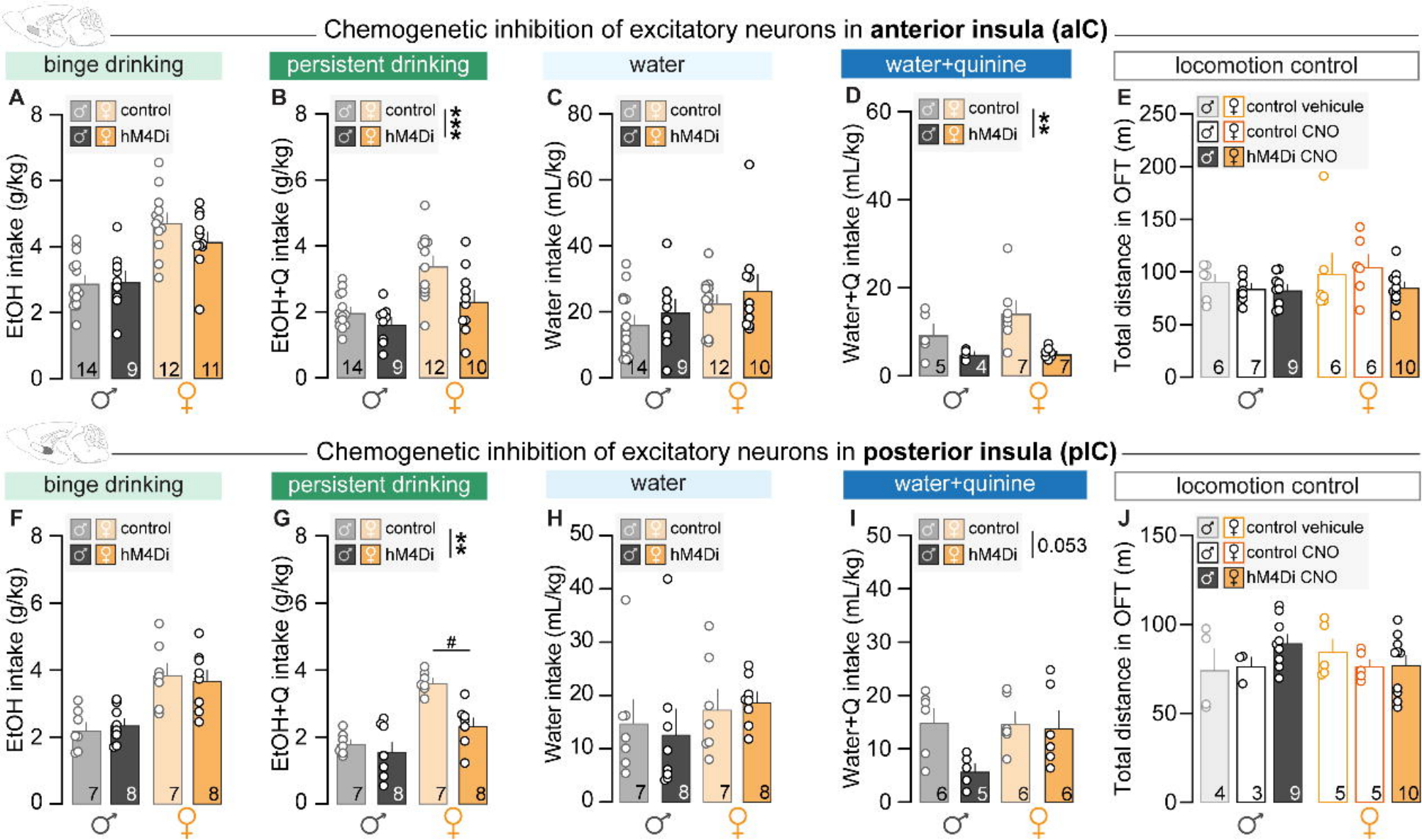
Figure 5. Coding properties of posterior insular cortex (pIC) excitatory neurons during binge and persistent ethanol drinking. **(A)** Viral strategy to record calcium changes in pIC excitatory neurons. A viral vector carrying the gene coding for the calcium sensor GCaMP6f was injected unilaterally in the pIC. **(B)** Representative image of GCaMP6f expression in pIC excitatory neurons. The mouse brain atlas delineation has been overlaid to the image, with the borders of the pIC in bold. **(C)** Housing conditions during photometry recordings to synchronize calcium signal and licking

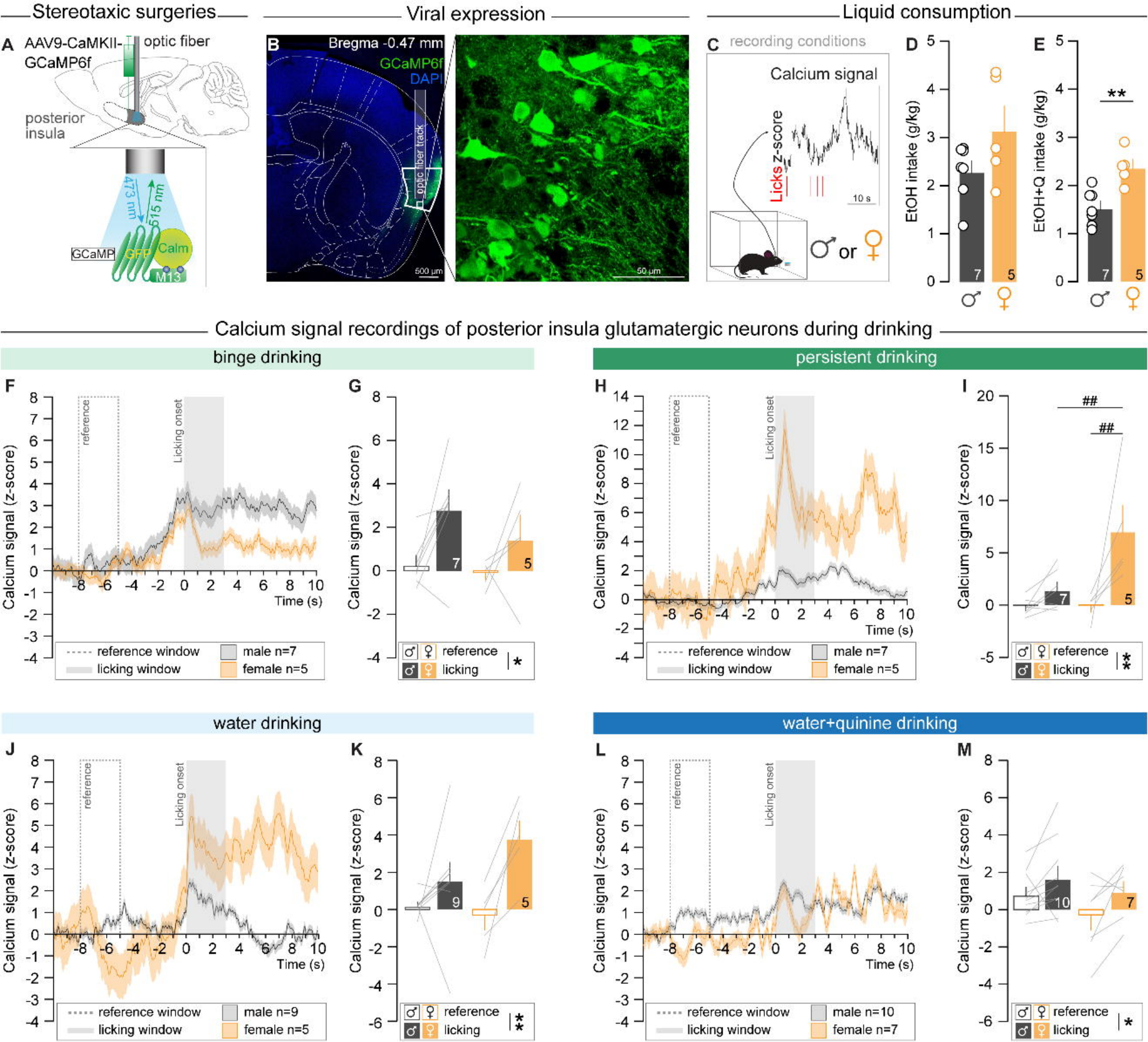
behavior. **(D)** Average of ethanol intake during all binge-drinking sessions except the recording day (day 32) in male and female mice. **(E)** Average of ethanol+quinine intake during all persistent drinking sessions except the recording day (day 46) in male and female mice. **(F)** Peri-ethanol licking time course of the calcium signal in pIC glutamatergic neurons in males and females. **(G)** Average of calcium signal in pIC glutamatergic neurons during reference and ethanol licking, in both sexes. **(H)** Peri-ethanol+quinine licking time course of the calcium signal in pIC glutamatergic neurons in male and female mice. **(I)** Average calcium signal in pIC glutamatergic neurons during reference and ethanol+quinine licking, in both sexes. **(J)** Peri-water licking time course of the calcium signal recorded from pIC glutamatergic neurons in males and females. **(K)** Average of calcium signal from pIC glutamatergic neurons during reference and water licking in both sexes. **(L)** Peri-water+quinine licking time course of the calcium signal from pIC glutamatergic neurons, in male and female mice. **(M)** Average of calcium signal from pIC glutamatergic neurons during reference and water+quinine licking, in both sexes. Data are shown as mean ± SEM. *$p<0.05$ **$p<0.01$ represent significant t-test and main effect of 2-way ANOVA. ##$p<0.01$ represents significant Bonferroni post-hoc test.


experimental timeline
A
binge drinking
2 hr
30 min
days 8
15
22
2h EtOH (20%)
blood sampling
housing conditions
ethanol 20%
or
after 2 hours of ethanol intake
B
EtOH intake (g/kg)
***
13
13
C
BEC (mg/dL)
80
23%
15%
13
13
D proportion of ethanol intake after 2 hours
(n=16)
33%
67%
last 90 min
first 30 min
(n=16)
16%
84%
last 90 min
first 30 min
after 30 minutes of ethanol
E
EtOH intake (g/kg)
***
16
16
F
BEC (mg/dL)
80
*
44%
75%
16
16


A
AAV9-CaMKII-hM4Di-mCh
or AAV9-CaMKII-mCh
anterior insula
posterior insula
CNO
hM4
inhibition
Gi
B
water
binge drinking
persistent drinking
controls
cycle 0
cycle 1
cycle 2
cycle 3
1 (days)
8
15
22
29
2h water
ad libitum water
2h ethanol (20%)
2h ethanol+quinine (500μM)
2h water or water+quinine (500μM)
C
Bregma 1.21mm
mCherry
DAPI
1mm
50 μm
Bregma -0.35mm
mCherry
DAPI
1 mm
50 μm


Sexual dimorphism of binge and persitent ethanol intake
water
binge-drinking
persistent drinking
persistent drinking resumption
A
B
C
D
E
F
G
H
Water intake (mL/kg)
EtOH intake(g/kg)
EtOH+Q intake(g/kg)
Liquid intake (mL/kg)
male
female
days 1 2 3 4
days 8 10 15 17
days 22 24
days 37 43
water+Q
EtOH+Q
water+quinine
ethanol+quinine
***
#
###
49
50
22
26


Chemogenetic inhibition of excitatory neurons in anterior insula (aIC)
binge drinking
persistent drinking
water
water+quinine
locomotion control
A
B
C
D
E
control
hM4Di
control vehicule
control CNO
hM4Di CNO
EtOH intake (g/kg)
EtOH+Q intake (g/kg)
Water intake (mL/kg)
Water+Q intake (mL/kg)
Total distance in OFT (m)
**
0.053
Chemogenetic inhibition of excitatory neurons in posterior insula (pIC)
F
G
H
I
J
#


Stereotaxic surgeries
Viral expression
Liquid consumption
A
AAV9-CaMKII-GCaMP6f
optic fiber
posterior insula
473 nm
515 nm
GCaMP
GFP
Calm
M13
B
Bregma -0.47 mm
GCaMP6f
DAPI
optic fiber track
500 µm
50 µm
C
recording conditions
Calcium signal
Licks
z-score
10 s
or
D
EtOH intake (g/kg)
E
EtOH+Q intake (g/kg)
**
Calcium signal recordings of posterior insula glutamatergic neurons during drinking
binge drinking
persistent drinking
water drinking
water+quinine drinking
F
G
H
I
J
K
L
M
reference
Licking onset
Calcium signal (z-score)
Time (s)
reference window
licking window
male n=7
female n=5
male n=9
male n=10
female n=7
reference
licking
##