Generation and Validation of Pdyn^{Lox} Conditional Knockout Mice

The 5' arm of the targeting construct (3.8 kb) was PCR amplified from a C57Bl/6 BAC clone using Q5 DNA polymerase with *Pac*1 site at the 5' end and *Xba*1 site at the 3' end (Supplementary Figure 2a). A synthetic loxP site was inserted into the *BamH*1 site 5' of the first coding exon. The 3' arm of the targeting construct (3.05 kb) was PCR amplified with *Sal*1 site at the 5' end and *Not*1 site at the 3' end. The two arms were cloned into the polylinkers of a targeting vector with a loxP site, a frt-flanked SV40-neomycin resistance gene for positive selection, *Pgk*-DTa and HSV-TK genes for negative selection. The targeting construct was electroporated into G4 ES cells and correct targeting was established by Southern blot of DNA digested with *Bcl*1 using a 32P-labelled probe located beyond the 3' arm of the targeting construct. Seven of 60 clones were correctly targeted and all of them retained the distal loxP site assessed using PCR primers (5' GACTCACTTGTTTGCTGGAGAG and 5' CAGAGTACGTGGATTGTCACAG) flanking the distal loxP site. Several clones were injected into blastocysts of C57Bl/6J mice and then transferred to pseudo-pregnant females. One of the clones that gave high percentage of chimeric mice, was bred with mice expressing FLP recombinase to remove the NeoR gene. The mice were then continuously backcrossed to C57BL/6 mice. Routine genotyping was performed using the primers indicated above.

Histology

Following the completion of behavioral experiments, mice were deeply anesthetized with an overdose of tribromoethanol (250 mg/kg, intraperitoneal) and transcardially perfused with 0.01 M phosphate buffered saline (4°C, pH 7.4) followed by 4% paraformaldehyde in PBS. Brains were post-fixed for 24 hours in 4% PFA and then cryoprotected in 30% sucrose in PBS. Coronal sections containing the CeA were cut at a thickness of 45 μm using a Vibratome (Leica VT1000S, Buffalo

Grove, IL, USA) and stored in a 50% glycerol/PBS solution. Slides were prepared with a Vectashield anti-fade mounting medium containing DAPI (Vector Labs, Burlingame, CA, USA).

In Situ Hybridization

Mice were anesthetized with isoflurane and rapidly decapitated. Brains were dissected and flash frozen on dry ice for 15 min and stored at -80°C until sectioned for in situ hybridization (ISH). Brain slices (16 µm) containing the CeA were obtained on a Leica CM 3050S cryostat (Leica Biosystems, Nussloch, Germany) at -20°C and were mounted directly onto microscope slides. Slides were stored at -80°C until tissue was processed for ISH. RNAscope ISH was conducted using the Multiplex Fluorescence Assay following the manufacturer's protocol (Advanced Cell Diagnostics, Newark, CA, USA). The probes used to assess gene expression were purchased from Advanced Cell Diagnostics and are as follows: PDYN-C1 (Accession number NM_018863.3), OPRK1-C2 (Accession Number NM_001204371.1), and EGFP-C2 (Accession Number U55763.1). Slides were coverslipped with ProLong Gold Mounting Medium containing DAPI (Fisher Scientific, Pittsburgh, PA, USA) and stored at 4°C until they were imaged.

Microscopy

Correct placements for viral infusions were verified using a wide-field epifluorescent microscope (BX-43, Olympus, Waltham, MA, USA) using a stereotaxic atlas (Franklin & Paxinos, 2008) and mice with viral expression outside the CeA were removed from the analysis for behavioral cohorts. Images for the quantification of fluorescent in situ hybridization were collected on a Zeiss 800 Laser Scanning Confocal Microscope (20x objective, NA=0.8) (Carl Zeiss, Jena, Germany). Regions of Interest (ROIs) containing the Central Lateral (CeL) and Central Medial (CeM) were annotated using Zeiss Zen 2 Blue Edition software. Manual quantification of PDYN positive and KOR positive cells was performed using manual counts with the cell counter plugin in FIJI (Curtis

Rueden, LOCI, University of Wisconsin-Madison, Madison, WI, USA). The definition of a cell was operationally defined as described previously (Bloodgood, Sugam, Holmes, & Kash, 2018). Briefly, a cell was considered PDYN+ or KOR+ if it contained at least one punctum on top of or immediately adjacent to the DAPI-defined nucleus. The DAPI channel was omitted from the representative image to more clearly show the distribution of PDYN and KOR mRNA. For the quantification of KOR knockout-mediated reduction of alcohol drinking, counts of GFP+ cells in the CeA were tabulated using a semi-automated pipeline in the Zeiss Zen Blue Advanced processing module. Briefly, a threshold for fluorescence that adequately segmented cells was empirically determined within each experiment cohort, and GFP+ nuclei with a sufficient spatial extent above the intensity threshold were counted as cells. One watershed correction was applied to each image to separate adjacent nuclei. For an example of the automated cell detection, see supplementary figure 1a.

qPCR on Tissue Punches

Mice were anesthetized with isoflurane and rapidly decapitated. The brain was dissected out and 1-mm coronal sections were made using a brain block. Slices were flash frozen on dry ice and tissue punches containing bilateral samples from the CeA were taken and stored at -80°C until future use. Total RNA from tissue punches was extracted using Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA, USA). Reverse transcription and qPCR were performed using Superscript II and Applied Biosystems Taqman Assay on a StepOnePlus Real Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The following TaqMan assay probes were purchased from Invitrogen (USA): ACTB Mm00607939_s1, *Oprk1* Mm01230885_m1, *Pdyn* Mm00457573_m1. Three technical replicates were averaged together to compute mean cycle threshold (Ct) values for the housekeeping gene and experimental genes of interest. The Ct value

for β-actin was then subtracted from the Ct values for *Oprk1* and *Pdyn* to compute normalized ΔCt values for each sample.

Randomization and blinding

All surgical and behavioral manipulations performed on each animal were determined randomly. For determining assignment to knockout or control conditions, equal numbers of $Pdyn^{lox/lox}$ or $Oprk1^{lox/lox}$ littermates were assigned to the two experimental conditions. All randomization was performed by an experimenter, and no explicit randomization algorithm was used. For electrophysiology experiments, ethanol and water drinkers were counterbalanced so that recordings were performed from one animal from each treatment condition on any recording day. For manual counts of PDYN+ and KOR+ neurons in the CeA, the experimenter was blinded to the treatment condition (ethanol or water). The experimenter was not blinded to the treatment condition for behavior and electrophysiology experiments.

Alcohol and Tastant Drinking Procedures

All alcohol and tastant drinking experiments were conducted with mice single housed in a reverse light cycle space (lights off at 7am, lights on at 7pm). For the duration of all experiments, mice were maintained on an Isopro RMH 3000 (LabDiet, St. Louis, MO, USA) diet, as this been shown to result in the highest levels of ethanol consumption (Marshall et al., 2015). All animals were transferred to the reverse light space to acclimate to the chow and light cycle for at least one week prior to the start of experiments.

Drinking in the Dark (DID) was carried out as described (Lowery-Gionta et al., 2012; Pleil et al., 2015; Rhodes, Best, Belknap, Finn, & Crabbe, 2005). Briefly, on days 1–3 beginning 3 hr into the dark cycle, water bottles were removed from all cages and replaced with a bottle containing 20% (v/v) ethanol solution. Mice had 2 hr of access to ethanol, after which the

ethanol bottles were removed from cages and water bottles were replaced. The same procedure was followed on day 4 except that ethanol access was extended to 4 hr. Bottle weights were recorded after 2 and 4 hr of access to ethanol on day 4. Additionally, a bottle was placed on an empty cage during all experiment days in order to record the amount of volume loss due to ethanol drip alone. This value was generally less than 0.1 mL/2 hr and was subtracted from the raw daily intake. The difference in start and end bottles weights minus the drip value was corrected for the density and concentration of ethanol and divided by the mouse's body weight to obtain normalized intake g/Kg. Days 1-4 comprised one cycle of DID which was repeated for a total of 4 weeks, a point at which we have previously observed dynorphin and KOR modulation of binge-like drinking in the CeA (Anderson et al., 2018).

Following completion of DID experiments, cage tops were replaced with lids designed for 2-bottle drinking. Following 3 days of acclimation to bottles containing only water, animals began Intermittent Access to Ethanol (IA) as described previously (Hwa et al., 2011). Briefly, on Monday, Wednesday, and Friday mornings 3 hr into the dark cycle, one of the water bottles was replaced with 20% ethanol (w/v). Animals then had access to ethanol for 24 hr at which point the difference in weights of the water and ethanol bottles was measured. Drip values for water and ethanol bottles were calculated separately and used to calculate normalized consumption using the method described above. The side of the ethanol bottle (left or right) was counterbalanced across session to prevent the development of a side preference. Animals underwent IA for 2 wk to assess the effects of the genetic knockout on total fluid consumption and alcohol preference.

Ethanol-naïve animals underwent drinking of aversive or palatable solutions using the same procedures as the drinkers. However, the animals always had access to one bottle containing water and the other containing the tastant solution of interest. The concentrations of

quinine (0.03, 0.06, and 0.09 mM), saccharin (0.33% and 0.66%), and sucrose (1%) were chosen based on values previously used to characterize global PDYN and KOR knockout mice (Blednov, Walker, Martinez, & Harris, 2006; Kovacs et al., 2005). The bottles for the tastant solution were changed 3 hr into the dark cycle and the animals had access to each solution for 24 hr thereafter. The weight of the tastant bottles and the drip bottles were recorded the following day and replaced on the opposite side as the previous day. The difference in consumption was averaged across the two days to obtain a single value for the preference of each solution. The bottle choice assay began with the lowest concentration of tastant in the series (0.03 mM quinine or 0.33% saccharin), which was then increased over the course of the next 6 days. This was then followed by 2 days of washout during which only water was available. We then began the preference test with the lowest concentration of the next series of solutions. The order of presentation of each solution type (palatable or aversive) was counterbalanced across cohorts within an experiment.

Elevated Plus Maze

The Elevated Plus Maze (EPM) (Med Associates, St. Albans, VT) was made of white-and-black plastic and consisted of two open arms (75 × 7 cm) and two closed arms (75 × 7 × 25 cm) adjoined by a central area (7 × 7 × 25 cm). The arms were arranged in a plus configuration with arms of the same type (open or closed) opposite of each other. The maze was elevated 75 cm with light levels maintained at 15 lux throughout the experiment. Mice were placed in the center of the EPM and allowed to explore freely. The EPM was cleaned with 70% ethanol between each trial. Movements were video recorded and analyzed using Ethovision 9.0 (Noldus Information Technologies). The primary measures of reduced anxiety-like behavior were time spent in the open arm and number of entries into the open arm. One mouse in the PDYN

knockout naïve male mice was excluded from the analysis as the subject exhibited only one entry into the closed arm. EPM testing was conducted 8 hrs after the last DID binge session (approximately 10pm or 3 hr in the light cycle). Testing in ethanol naïve animals was performed at the same time of day with respect to the light/dark cycle.

Slice Electrophysiology

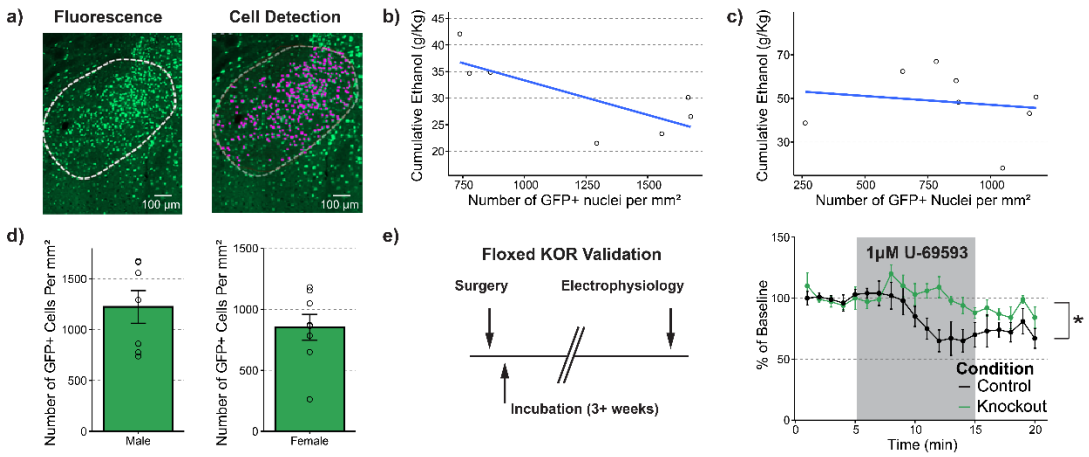
To ensure that only animals in the ethanol group consumed a sufficient amount of alcohol only mice that consumed greater than cumulative 20 g/Kg across the three cycles of DID (This corresponded to on average >4 g/Kg on binge test days). We performed whole-cell electrophysiology experiments similar to those published previously (Crowley et al., 2016; Lowery-Gionta et al., 2012). Briefly, 300-µm coronal slices containing the CeA were prepared on a vibratome (Leica VT1200, Leica, Wetzlar, Germany) from mice rapidly decapitated under isoflurane. The brains were removed and placed in ice-cold modified high sucrose artificial cerebrospinal fluid (aCSF) containing the following (in mM): 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10.0 glucose, and 26.0 $NaHCO_3$. Slices were then transferred to normal aCSF maintained at approximately 30°C (Warner Instruments, Hamden, Connecticut) containing the following (in mM): 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 NaH_2PO_4, 10.0 glucose, and 26.0 $NaHCO_3$. Slices were placed in a holding chamber where they were allowed to rest for at least one hour. Slices were continuously bubbled with a 95% O_2 / 5% CO_2 mixture throughout slicing and experiments. Thin-walled borosilicate glass capillary recording electrodes (3–6 MΩ) were pulled on a Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA).

Following rupture of the cell membrane, cells were allowed to rest and equilibrate to the intracellular recording solutions (below). Recordings were made from cells in both the lateral subdivision (CeL) and medial subdivision (CeM) and were pooled within each experimental group. Signals were acquired via a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, California), digitized at 10 kHz and filtered at 3 kHz. Current clamp experiments were analyzed using Clampfit 10.2 software (Molecular Devices) and spontaneous synaptic transmission experiments were analyzed using Mini Analysis version 6.0.7 (Synaptosoft, Decatur, GA). Access resistance was monitored continuously throughout the experiment, and when it deviated by more than 20% the experiment was discarded. No more than two cells per animal were included in each experiment. For current clamp experiments, cells were recorded using a potassium-gluconate based internal recording solution containing the following (in mM): 135 K-gluc, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.6 EGTA, 4 Na_2ATP, 0.4 Na_2GTP. Experiments were conducted both at resting membrane potential (RMP) and -70mV.

Lidocaine N-ethyl bromide (1 mg/mL) was included in the intracellular recording solution to prevent postsynaptic sodium spikes for all voltage-clamp experiments. For voltage-clamp experiments requiring the simultaneous recording of excitatory and inhibitory events within the same neuron, a cesium-methanesulfonate based intracellular recording solution containing the following (in mM) was used: 135 Cs-meth, 10 KCl, 1 $MgCl_2$, 0.2 EGTA, 4 MgATP, 0.3 GTP, 20 phosphocreatine. Excitatory events were recorded at -55mV and inhibitory events were recorded at +10mV. E/I synaptic drive ratio was calculated using the following formula: (EPSC frequency X amplitude)/ (IPSC frequency X amplitude).

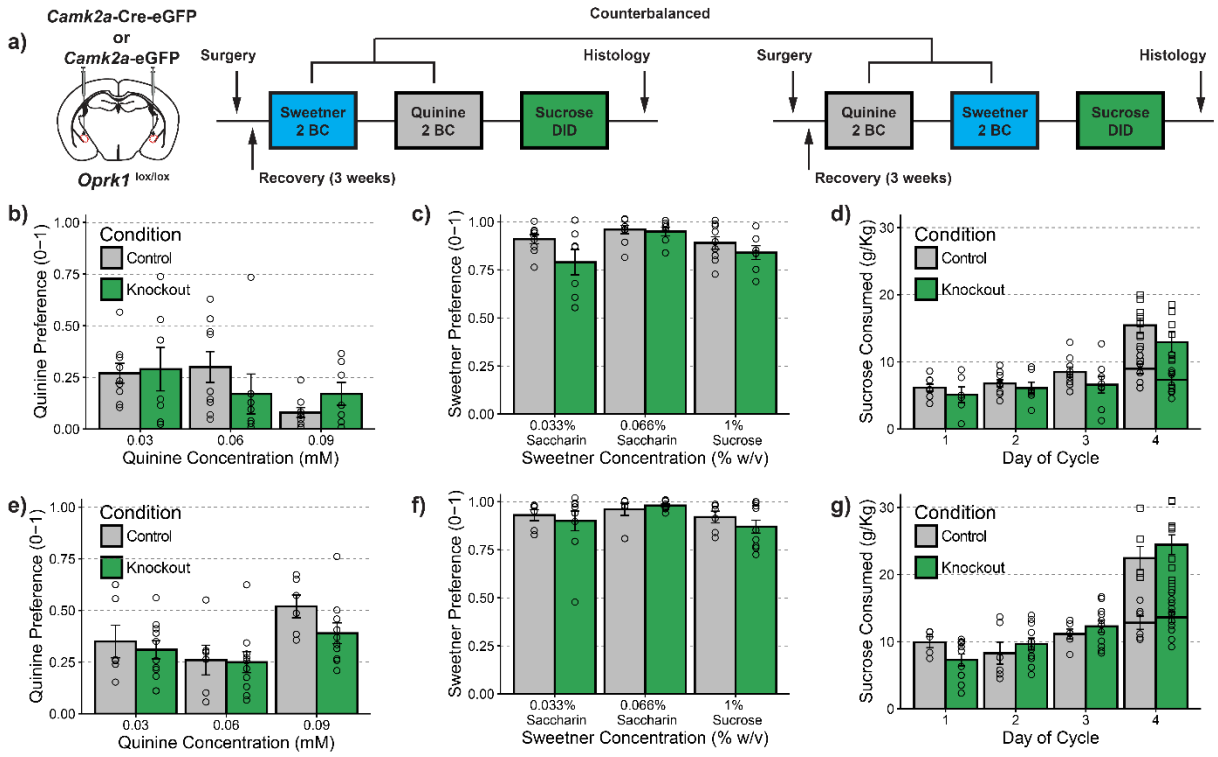
For experiments examining the effects of KOR modulation of GABAergic transmission, a high chloride Cs-based internal solution was used (in mM): 117 Cs-Gluc, 20 HEPES, 0.4

EGTA, 5 TEA, 2 $MgCl_2$, 4 Na_2ATP, 0.4 Na_2GTP. Electrode stimulation-evoked GABAergic inhibitory postsynaptic currents (eIPSCs) were pharmacologically isolated using 3mM kynurenic acid to block α-amino-3-hydroxy-5-methyl-4- isoxazole-propionic acid and N-methyl-D-aspartate receptor-dependent postsynaptic currents. Evoked IPSCs were induced via twisted bipolar nichrome wire placed dorsal to the recording electrode, 100 μm to 500 μm dorsal from the recorded neuron. Electric output was set to stimulate at 0.1 Hz, between 2 V and 16 V with a 100 microsecond to 150 microsecond duration. Following 5 minutes of a stable baseline, 1 μM U-69593 was applied for 10 minutes followed by 5 minutes of washout. Evoked IPSC experiments were analyzed by measuring the average peak amplitude of the synaptic response per minute, which was normalized to the baseline period 5 minutes immediately preceding application of the drug.



Supplementary Figure 1- Correlation of KOR Knockout with Alcohol Drinking and Validation of KOR-Flox Line

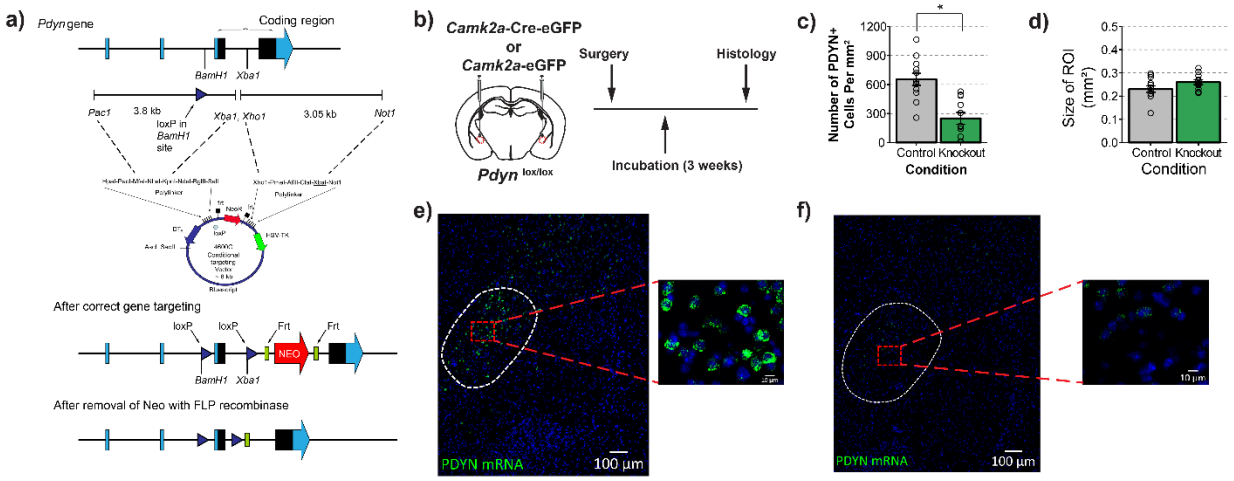
a) Representative image of GFP expression in the CeA and automated cell detection threshold b) Degree of KOR knockout as measured by number of GFP+ nuclei per mm^2 was associated with a significant reduction in alcohol drinking in male mice r^2=0.571, p=0.048 c) Degree of KOR knockout was not correlated with ethanol consumption in female mice r^2=0.024, p=0.712 d) Average number of GFP+ nuclei in the CeA of male and female KOR knockout mice e) CeA KOR knockout abolished U69-mediated inhibition of eIPSCs, main effect of condition F(1,12)=5.448, p=0.038, time by condition interaction F(3,36)=3.945, p=0.016. r^2 values reflect Pearson's correlation coefficient.



Supplementary Figure 2- CeA KOR knockout does not alter the preference of palatable or aversive tastants.

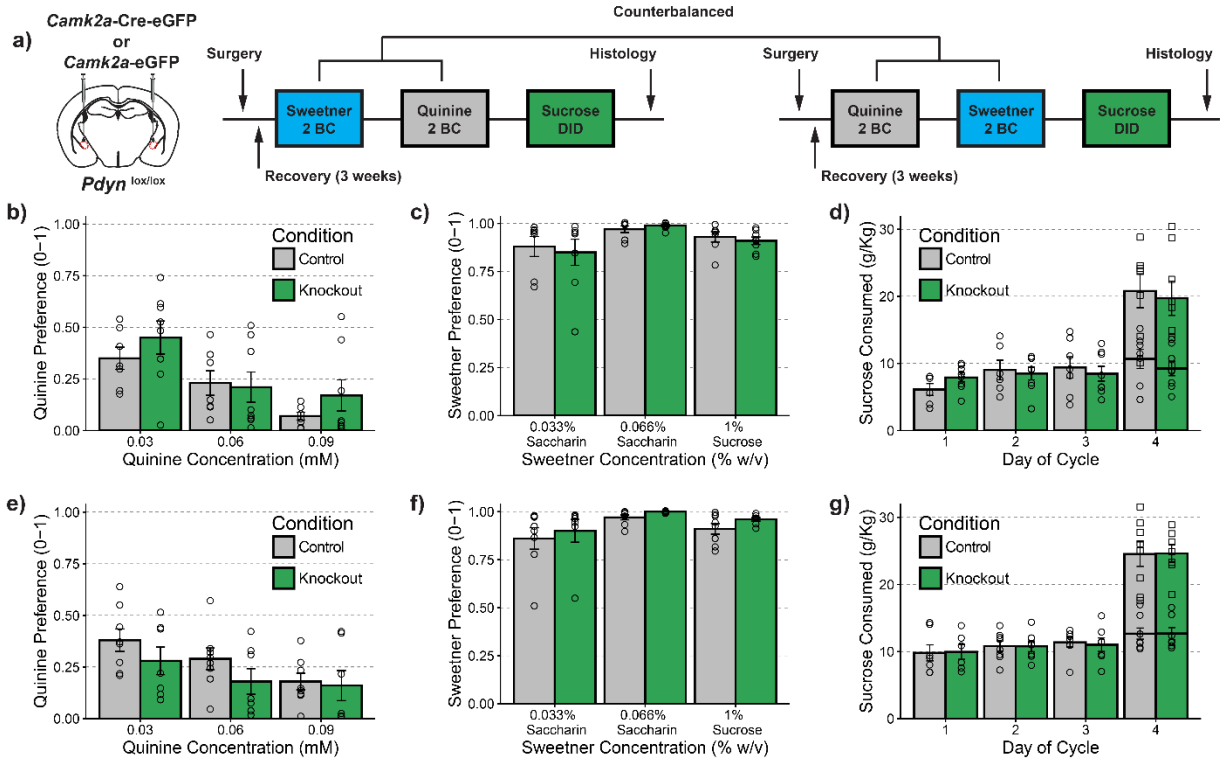
(a) Timeline for tastant experiments. Ethanol-naïve animals underwent two-bottle choice (2BC) for series of palatable (saccharin and sucrose) or aversive (quinine) tastants. The presentation of the first series (palatable or aversive) was counterbalanced across multiple cohorts. Following completion of the two-bottle choice experiments, animals then had binge access to 10% sucrose during same access schedule as DID. (b-d) Experiments in male animals. (b) CeA KOR knockout did not alter preference for quinine at the range of concentrations tested, main effect of genotype; $F(1,14)=0.005$, $p=0.945$. (c) KOR knockout did not alter preference for saccharin or sucrose, main effect of genotype; $F(1,14)=3.015$, $p=0.104$. (d) KOR knockout did not affect binge consumption of sucrose at any of the 2-hr time points; main effect of genotype: $F(1,12)=1.435$, $p=0.254$ or 4-hr time point on the final day $t(13.168)$, $p=0.193$. (e-g) Experiments

in female animals. (e) CeA KOR knockout did not alter preference for quinine at the range of concentrations tested, main effect of genotype: $F(1,14)=2.069$, $p=0.172$. (f) KOR knockout did not alter preference for saccharin or sucrose, main effect of genotype: $F(1,14)=0.175$, $p=0.682$. (g) KOR knockout did not affect binge consumption of sucrose at any of the 2-hr time points, main effect of genotype: $F(1,14)=0.007$, $p=0.979$, or 4-hr time point on the final day $t(11.655)$, $p=0.397$. (d,g) round dots indicate values at 2-hr time points and square dots indicate values at 4-hr time points (b-d) n=9 control males and n=7 knockout males, (e-g) n=7 control females and n=10 knockout females



Supplementary Figure 3- Validation of PDYN-Flox Line

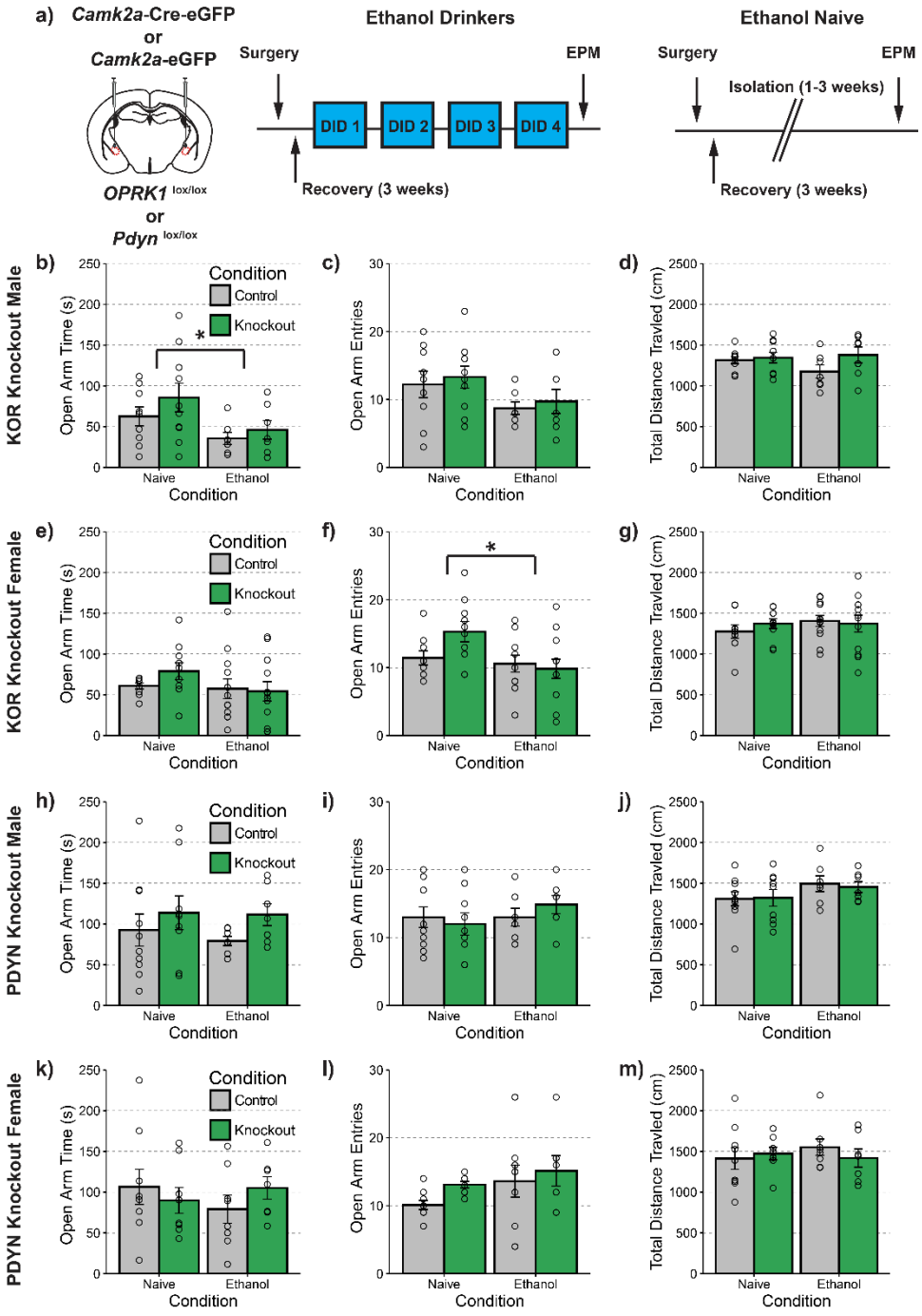
(a) Cloning vector used to target *Pdyn* gene. (b) Experimental timeline for virus injection and histology. (c) Injection of an adeno associated virus containing Cre recombinase results in a significant reduction in the number of PDYN+ cells in the CeA $t(21)= 4.663$, $p<0.001$. (d) The size of the ROI drawn was no different between groups $t(19.953)=1.660$, $p=0.113$. (e) Representative image of *Pdyn* mRNA expression in a control mouse and (f) in a PDYN-knockout mouse. n=12 images per group from N=4 mice per condition.



Supplementary Figure 4- PDYN knockout in CeA does not alter the preference of palatable or aversive tastants.

(a) Timeline for tastant experiments. Ethanol naïve animals underwent two-bottle choice (2BC) for series of palatable (saccharin and sucrose) or aversive (quinine) tastants. The presentation of the first series (palatable or aversive) was counterbalanced across multiple cohorts. Following completion of the two-bottle choice experiments, animals then had binge access to 10% sucrose during same access schedule as DID. (b-d) Experiments in male animals. (b) CeA PDYN knockout did not alter preference for quinine at the range of concentrations tested; main effect of genotype: $F(1,13)=0.752$, $p=0.402$. (c) PDYN knockout did not alter preference for saccharin or sucrose, main effect of genotype: $F(1,13)=0.093$, $p=0.765$. (d) PDYN knockout did not affect binge consumption of sucrose at any of the 2-hr time points; main effect of genotype: $F(1,11)=0.322$, $p=0.582$ or 4-hr time point on the final day $t(12.963)=0.306$, $p=0.764$. (e-g)

Experiments in female mice. (e) CeA PDYN knockout did not alter preference for quinine at the range of concentrations tested, main effect of genotype: $F(1,13)=1.119$, $p=0.309$. (f) PDYN knockout did not alter preference for saccharin or sucrose, main effect of genotype: $F(1,13)=1.331$, $p=0.269$. (g) PDYN knockout did not affect binge consumption of sucrose at any of the 2-hr time points, main effect of genotype: $F(1,12)=1.184$, $p=0.298$ or 4-hr time point on the final day $t(13.18)=0.325$, $p=0.750$. (d,g) round dots indicate values at 2-hr time points and square dots indicate values at 4-hr time points (b-d) n=7 control males and n=8 knockout males (e-g) n=8 control females and n=7 knockout females.



Supplementary Figure 5- Neither KOR nor PDYN Knockout Protect Against Ethanol-Induced Increases In Anxiety-Like Behavior

(a) Experimental timeline. (b) There was a significant main effect of ethanol drinking $F(1,29)=5.739$, $p=0.023$ but not gene knockout $F(1,29)=1.651$, $p=0.209$ on open arm time. (c)

There was a significant main effect of ethanol drinking $F(1,29)=4.372$, $p=0.045$ but not gene knockout $F(1,29)=0.388$, $p=0.538$ on open arm entries. (d) There was no effect of ethanol drinking $F(1,29)=0.522$, $p=0.476$ or gene knockout $F(1,29)=2.18$, $p=0.151$ on total distance traveled in the elevated plus maze. (e) There was no significant effect of ethanol drinking $F(1,39)= 1.765$, $p=0.192$ or gene knockout $F(1,39)=0.332$, $p=0.568$ on open arm time. (f) There was a significant main effect of ethanol drinking $F(1,39)=5.718$, $p=0.022$ but not gene knockout on open arm entries. (g) There was no effect of ethanol drinking $F(1,39)=0.582$, $p=0.450$ or gene knockout $F(1,39)=0.081$, $p=0.777$ on total distance traveled. (h) There was no effect of ethanol drinking $F(1,29)=0.197$, $p=0.660$ or gene knockout $F(1,29)=2.149$, $p=0.153$ on open-arm time. (i) There was no effect of ethanol drinking $F(1,29)=0.827$, $p=0.371$ or gene knockout $F(1,29)=0.020$, $p=0.889$ on open-arm entries. (j) There was no significant main effect of ethanol treatment $F(1,29)=3.009$, $p=0.093$ or gene knockout $F(1,29)=0.011$, $p=0.915$ on total distance traveled. (k) There was no effect of ethanol drinking $F(1,26)=0.213$, $p=0.648$, or gene knockout $F(1,26)=0.009$, $p=0.922$ on open-arm time. (l) There was no effect of ethanol drinking $F(1,26)=2.374$, $p=0.135$ or gene knockout $F(1,26)=1.564$, $p=0.222$ on open-arm entries. (m) There was no effect of ethanol drinking $F(1, 26)=0.198$, $p=0.660$ or gene knockout $F(1, 26)=0.048$, $p=0.829$ on total distance traveled. (b-d) n=8 ethanol control males, n=7 ethanol knockout males, n=9 naïve control males, n=7 naïve knockout males; (e-g) n=9 ethanol control females, n=10 knockout females, n=7 naïve control females, n=10 knockout females; (h-j) n=7 ethanol control males, n=7 ethanol knockout males, n=10 naïve control males, n=9 naïve knockout males; (k-m) n=8 ethanol control females, n=7 knockout females, n=9 naïve control females, n=8 naïve knockout females.

Supplementary Table 1- Tests of Homogeneity of Variance

Panel	Test Statistic
Figure 1d	Levene's Test F=0.110, p=0.744
Figure 1e	Levene's Test F=1.408, p=0.255
Figure 2e	Mauchly's Test W=0.507, p=0.202
Figure 2f	Levene's Test F=2.7378, p=0.124
Figure 2g	Mauchly's Test W=0.091, p=0.049
Figure 2h	Mauchly's Test W=0.068, p=0.021
Figure 2i	Mauchly's Test W=0.065, p=0.001
Figure 2j	Mauchly's Test W=8.967e-06, p=8.225e-18
Figure 2l	Mauchly's Test W=0.475, p=0.039
Figure 2m	Levene's Test F=0.015, p=0.903
Figure 2n	Mauchly's Test W=0.195, p=0.040
Figure 2o	Mauchly's Test W=0.226, p=0.073
Figure 2p	Mauchly's Test W=0.018, p=1.303e-07
Figure 2q	Mauchly's Test W=2.108e-06, p=9.142e-34
Figure 3e	Mauchly's Test W=0.507, p=0.202
Figure 3f	Levene's Test F= 4.221, p=0.064
Figure 3g	Mauchly's Test W=0.176, p=0.343
Figure 3h	Mauchly's Test W=0.057, p=0.030
Figure 3i	Mauchly's Test W=0.091, p=0.093
Figure 3j	Mauchly's Test W=2.684e-06, p=1.281e-17
Figure 3l	Mauchly's Test W=0.475, p=0.039
Figure 3m	Levene's Test F=0.079, p=0.783
Figure 3n	Mauchly's Test W=0.200, p=0.044
Figure 3o	Mauchly's Test W=0.340, p=0.302
Figure 3p	Mauchly's Test W=0.186, p=0.032
Figure 3q	Mauchly's Test W=0.001, p=4.415e-21
Figure 4b	Mauchly's Test W=0.710, p=0.504
Figure 4c	Mauchly's Test W=0.059, p=0.015
Figure 4e	Levene's Test F=0.054, p=0.818 Levene's Test F=0.305, p=0.588
Figure 4f	Levene's Test F=0.010, p=0.919 Levene's Test F=0.400, p=0.536
Figure 4g	Levene's Test F=0.003, p=0.953
Figure 4i	Levene's Test F=1.146, p=0.304 Levene's Test F=0.142, p=0.712
Figure 4j	Levene's Test F=1.358, p=0.265 Levene's Test F=0.191, p=0.669
Figure 4k	Levene's Test F=0.004, p=0.953
Figure 5a	Levene's Test F=0.483, p=0.497
Figure 5b	Levene's Test F=0.003, p=0.958
Figure 5c	Levene's Test F=1.152, p=0.298
Figure 5e	Mauchly's Test W= 4.988e-09, p=5.501e-24

Figure 5f	Levene's Test F=0.364, p=0.555
Figure 5g	Levene's Test F=0.209, p=0.655
Figure 5h	Levene's Test F=0.894, p=0.361
Figure 5j	Mauchly's Test W= 5.936e-12, p=1.377e-38

Supplementary Table 2- Kinetics for spontaneous Excitatory Post-synaptic Currents (sEPSCs)

	Water		Ethanol	
	Rise (ms) ± SEM	Decay (ms) ± SEM	Rise (ms) ± SEM	Decay (ms) ± SEM
Male	1.91 ± 0.14	3.79 ± 0.16	2.01 ± 0.11	5.17 ± 0.89
Female	1.90 ± 0.20	3.62 ± 0.44	2.14 ± 0.22	4.74 ± 0.72

Supplementary Table 3- Kinetics for spontaneous Inhibitory Post-synaptic Currents (sIPSCs)

	Water		Ethanol	
	Rise (ms) ± SEM	Decay (ms) ± SEM	Rise (ms) ± SEM	Decay (ms) ± SEM
Male	3.79 ± 0.28	13.01 ± 1.03	4.17 ± 0.53	10.55 ± 1.41
Female	3.75 ± 0.26	10.61 ± 1.38	4.05 ± 0.37	12.20 ± 1.54

Supplementary Table 4- Cell Properties in Synaptic-Transmission Experiments

	Water		Ethanol	
	Capacitance (pF) ± SEM	Resistance (MΩ) ± SEM	Capacitance (pF) ± SEM	Resistance (MΩ) ± SEM
Male	77.11 ± 7.58	313.96 ± 73.19	85.32 ± 6.45	259.26 ± 35.44
Female	81.26 ± 6.68	388.67 ± 79.45	82.38 ± 6.52	210.51 ± 19.44

Supplementary Table 5- Cell Properties in Excitability Experiments

	Water		Ethanol	
	Capacitance (pF) ± SEM	Resistance (MΩ) ± SEM	Capacitance (pF) ± SEM	Resistance (MΩ) ± SEM
Male	74.33 ± 9.00	139.44 ± 24.24	65.81 ± 8.24	156.27 ± 16.32
Female	68.93 ± 7.39	222.63 ± 36.61	61.71 ± 7.29	163.58 ± 46.36