Title: Sex-specific prefrontal cortex dysfunction underlying opioid-induced cognitive impairment

Authors: Eden M. Anderson, Annabel Engelhardt, Elissa Porath, and Matthew C. Hearing*

Author Contributions: EMA and MCH designed, discussed, and planned all experiments. EMA, EP, AE, and MCH performed experiments and analyzed data. EMA and MCH wrote the manuscript.

Department of Biomedical Sciences, Marquette University, Milwaukee, WI, 53233

Corresponding Author:

Matthew C Hearing
matthew.hearing@marquette.edu
560 16th Street
Milwaukee, WI 53233

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ABSTRACT

Deficits in cognitive control, whether intrinsic or arising from drug use increases relapse vulnerability and risk for out of control drug use. In susceptible populations, women tend to transition to addiction faster and experience greater difficulties remaining abstinent[1,2] however, what drives this is unknown. Even when taken as prescribed, opioid-based pain therapies carry a risk of misuse and addiction. Thus, identifying adaptations responsible for reduced cognitive control and how intrinsic factors like biological sex influence risk for opioid abuse provides an opportunity to enhance relapse mitigating therapies and reduce susceptibility to opioid use disorders. Decreases in intrinsic excitability (hypoexcitability) of frontal cortex pyramidal neurons has been linked with loss of cognitive control in a variety of mental disorders, including addiction, however underlying mechanisms of this cortical dysfunction are poorly characterized. We show that self-administration of the opioid, remifentanil, causes a long-lasting decrease in *ex vivo* excitability but augments firing capacity of pyramidal neurons in the prelimbic cortex. This phenomenon occurs faster in females and aligns with a shift in synaptic drive towards inhibition and a significant impairment in cognitive flexibility. Further, compensating for this hypoactive state using chemogenetics protected against inflexibility that results from opiate self-administration. These data define cellular and synaptic mechanisms by which opioids and perhaps other drugs of abuse impair prefrontal function and cognitive control and have implications for sex differences in opioid addiction vulnerability.

INTRODUCTION AND RESULTS

Compared to men, women report more lifetime use of prescription opioids[3], exhibit increased vulnerability to out of control use, and greater rates of heroin relapse[4,5], however the mechanisms underlying these sex differences remain unclear. To investigate potential sex differences in neurobiological dysfunctions, we focused on the prelimbic region (PrL) of the

medial PFC (mPFC) as this region in rodents, and a homologous region in humans, is known to regulate opioid-seeking and inhibitory response control[6,7], and encode cognitive flexibility in rodents and humans[8-10]. Impairments in cognitive control and compulsive drug use in cocaine and heroin addicts is thought to reflect reductions in PFC pyramidal neuron (PN) activity[8,9,11], however the impact that opioids have on PFC activation, the mechanisms underlying these changes, and resultant effects on cognitive function remains poorly defined. We hypothesized that opioids produce time-dependent shifts in PN excitability – culminating in basal state hypoactivation that impairs cognitive control, and that the more rapid transition to uncontrollable drug taking in women reflects emergence of hypoexcitability of PrL PN on a shorter time-scale. To identify the temporal and sex-specific nature of opioid-induced mPFC dysfunction, male and female mice underwent 10-16 (short-term) or 25-30 days (long-term) of remifentanil (Ultiva®) self-administration followed by 14-21 or 40-45 days of forced abstinence (Fig. 1a). Remifentanil males and females had similar active and inactive lever pressing across the first 10 and 25 days in short- and long-term self-administration, respectively (Fig. 1b, 1e). Cumulative earned infusions was greater in long-term remifentanil mice compared to short-term, with females showing greater cumulative intake versus males with more prolonged self-administration (Fig. 1c, 1f).

Remifentanil Effects on mPFC Layer 5/6 Pyramidal Neuron Intrinsic Excitability

Functional integrity of mPFC information processing and PN output is dependent on extrasynaptic factors that dictate neuronal firing threshold and how cells respond to synaptic input[12-15]. Thus, we used *ex vivo* electrophysiology to examine these properties in PrL PN following remifentanil self-administration. Intrinsic excitability of deep-layer (L5/6) PrL PN was assessed by measuring current required to evoke an initial action potential (rheobase) in response to depolarizing current steps (1 s, -80-380 pA, 20 pA steps). Following 14-21 days abstinence, PN in short-term remifentanil males showed a reduction in threshold to fire an action potential (hyperexcitability), whereas short-term females exhibited an increased threshold

to fire (hypoexcitability; Fig. 1d). Following prolonged abstinence (45 days), hypoexcitability in females but not hyperexcitability in males was still present (Supplemental 1). Following 14-21 days abstinence from long-term self-administration, PrL PN in female and males exhibited an equivalent hypoexcitability compared to controls (Fig. 1g). These data indicate that long-term opioid self-administration produces a hypoactive state in PrL PN that emerges more rapidly in females that is independent of drug intake and more enduring than hyperexcitability in males. Further, as PN in females show increased excitability under basal conditions akin to short-term remifentanil males, hyperexcitability in males may reflect permissive plasticity in the transition to hypoactive states and heightened susceptibility in females.

Clinical studies in opiate-dependent individuals indicates that the human equivalent of the mPFC exhibits a dichotomous reduction in basal metabolic activity but heightened activity during cue-induced craving[6,8,9,16]. Further, PFC dysfunction and impaired cognition in numerous pathological states has been linked to reduced or increased/irregular spike firing activity[17-20] -- highlighting the importance of spike firing in cortical processing and opiate addiction pathology. Following short-term remifentanil self-administration, PN in males exhibited a leftward shift (increase) in firing frequency in response to depolarizing current steps (Fig. 1h) that was no longer present at 45 days (Supplemental 1), whereas PN in females exhibited increased firing capacity at depolarized currents (Fig. 1i) that persisted up to 45 days (Supplemental 1). Following more prolonged self-administration, PN in both male and female remifentanil mice exhibited divergent shifts in firing capacity at low versus high currents (Fig. 1j, 1k). These data indicate that PrL PN are less likely to fire but exhibit increased firing capacity once activated – potentially explaining the dichotomy in PFC activation observed in humans in basal versus craving states. Notably, no significant effects on excitability were observed in infralimbic cortex PN following short- or long-term self-administration (Supplemental 2) suggesting adaptations may be time-, cell-, or pathway-specific[21-23].

Remifentanil Effects on PrL Pyramidal Neuron Inhibitory and Excitatory Signaling

Activation and firing patterns of PN are heavily influenced by synaptic and perisynaptic excitatory and inhibitory signaling at the soma and dendrites[13,14]. Cocaine-induced hyperexcitablity of PrL PN and their increased responsivity to cocaine and related stimuli[24] has been linked to adaptations in multiple ion conductances in PN[25-28], including weakening of $GABA_BR$-dependent activation of G protein inwardly-rectifying K+ channels ($GABA_BR$-GIRK)[29]. Following short-term self-administration, hyperexcitability in males but not hypoexcitablity in females, aligned with reduced somatodendritic currents evoked by the $GABA_BR$ agonist, baclofen ($I_{Baclofen}$; 200 µM; Fig. 2a, 2b), which similar to rheobase and spike firing, was not present at 45 days abstinence (Supplemental 1).

Previous studies have implicated mPFC excitatory:inhibitory synaptic and structural modifications in opioid sensitivity and reinstatement[30-33], no known studies have directly measured synaptic adaptations following abstinence from opioid self-administration selectively in PrL PN. Given the lack of change in $I_{Baclofen}$ in females, we next examined modifications in excitatory and inhibitory synaptic transmission by measuring AMPA receptor (AMPAR)-specific miniature excitatory postsynaptic currents (mEPSCs) and $GABA_AR$-specific inhibitory postsynaptic currents (mIPSCs). PN from short-term remifentanil females showed reductions in mEPSC frequency (Fig. 2d-e) and increased frequency of mIPSCs compared to controls (Fig. 2f-g), with no effects on mEPSC or IPSC amplitude. Saline males exhibited a lower baseline of mEPSC frequency versus females; with only a trend towards reduced mIPSC frequency in remifentanil versus saline males (Fig. 2f-g). These data indicate that mechanisms underlying hyperexcitabilty and hypoexcitability vary, with hypoexcitability aligning with a shift towards synaptic inhibition, and that sex differences in basal excitatory transmission may also confer abuse susceptibility.

PrL dysfunction has been linked to impairments in flexible behavior, affect dysregulation, and increased risk for developing compulsive/impulsive behavior - all of which are observed in

opioid dependent subjects and aligns with poor (relapse) treatment outcome in cocaine addicts[8,9,30,34-36], however little is known regarding the influence of biological sex on these risk factors or mechanisms underlying this dysfunction. We first examined whether hypo- (or hyper-) active PN states equate to changes in anxiety- and depressive-like behaviors. Short-term self-administration, a time point in which we see differences in male and female plasticity, did not alter anxiety- or depressive-like coping behaviors as measured in the elevated plus maze or forced swim test (Supplemental 3). As the PrL is critical for cognitive flexibility[10,37], we next used an attentional set-shifting model to measure deficits in cognitive flexibility -- a task that resembles the Wisconsin Card Sorting Task in the sense that the stimuli are easy to detect, but rules are implicit and learned while the task is performed (Fig. 3a). Similar to previous lesion studies of the PrL[38], learning of the visual cue discrimination task was unaltered by short-term remifentanil in males or females (Fig. 3b). Alternatively, remifentanil in females increased the number of trials and errors to reach criteria during an extradimensional (cue-to-response) set-shift test compared to saline females and remifentanil males (Fig. 3c). Deficits cannot be attributed to differences in acquisition of lever training or omission of response as no differences in these measures was observed (Supplemental 4). Further, performance in reversal learning – a task more reliant on the orbitofrontal region[39] -- was unaffected by remifentanil in males and females (Supplemental 5). A critical step in future studies will be determining what role, if any, gonadal hormones have on these remifentanil-induced deficits, as recent work has demonstrated a role of estrous stage on cue/drug associations[40], heroin self-administration[41], and unconstrained remifentanil demand[42].

Impact of Remifentanil on Affective Behavior and Cognitive Flexibility

We propose that if PrL PN hypoexcitability underlies reductions in cognitive flexibility following repeated drug use, compensating this should restore flexibility. We used an *in vivo* chemogenetic approach to express the excitatory AAV8-hm3d-Gq designer receptor

exclusively activated by designer drugs (DREADD) in CaMKII-expressing cells (i.e., PN) in female mice undergoing remifentanil self-administration (Fig. 3d). *Ex vivo* analysis of CNO effects on PN rheobase showed that CNO bath application (5µM) lead to spontaneous firing in 4 out of 5 cells. Interestingly, CNO treatment also significantly reduced rheobase in non-virus expressing and GFP-control expressing PN, albeit to a lesser extent (Fig. 3e). No differences across treatment groups was observed in lever training or trials to criteria during the visual cue test (Supplemental 6). Injection of the DREADD agonist, clozapine-N-oxide (CNO; 5 mg/kg), into mice 30 minutes prior to extradimensional set-shift testing significantly reduced trials and errors to reach criteria compared to remifentanil exposed females and akin to performance in saline controls (Fig. 3f-g). Although not significant, CNO administration in remifentanil females expressing a GFP-control virus produced a trend towards better performance. These data, along with the effect of CNO on GFP-control neurons, suggests that partial recovery of set-shifting may reflect back metabolism of CNO to clozapine[43] which improves set-shifting performance in rats[44]. Regardless, this is the first evidence that opioid-induced hypofrontality underlies cognitive inflexibility and a potential means by which women transition to uncontrolled drug-seeking on a more rapid time scale, highlighting this as a critical preventative therapeutic target.

DISCUSSION/CONCLUSION

The current results demonstrate that low dose self-administration of a clinically used short-acting opioid (i.e. remifentanil) is sufficient to promote an enduring hypoexcitable state akin to that in cocaine and heroin addicted individuals[8,9] which emerges on a shorter time-scale in females and directly contributes to deficits in flexible decision-making[8,11,45]. A similar hypoexcitable state was observed following eight weeks of cocaine self-administration in males that underlies compulsive drug-seeking[11], thus opioids may promote a faster transition towards reduced cognitive control. Our data highlight important considerations for clinical use and highlight a sex

difference in risk for developing early deficits in cognitive control that likely increases risk of opioid misuse and addiction on a shorter timescale than previously thought. Although the mechanisms responsible for this more rapid shift require further exploration, we find that PN in females are intrinsically more excitable compared to males. As a similar hyperexcitable state was observed following short-term opioid exposure in males, it is possible that this adaptation represents a permissive functional plasticity that serves to lower the rheobase in females under naïve conditions[46]. Together, the current results fill critical gaps in understanding the influence of self-administered opioid use on mPFC states and how these contribute to cognitive flexibility in both *males* and *females*, which has implications for sex-specific targets to mitigate opioid abuse.

METHODS

***Subjects*.** Adult male and female wild-type C57BL/6 mice (postnatal day 65 ± 0.77 at self-administration onset) were bred in-house or commercially purchased (Jackson Laboratory) and maintained in a temperature and humidity-controlled room. Animal use was approved by the Institutional Animal Care and Use Committee at Marquette University.

Catheter surgery. Mice were implanted with an intravenous standard mouse jugular vein catheter (Access Technologies; 2/3Fr. x 6cm silicone, Cat No. AT-MJVC-2914A) connected to a backmount (PlasticsOne; 22GA, Part No. 8I31300BM01) under general isofluorane (1-3%) anesthesia. Following catheter surgery, mice were single-housed and allowed to recover for at least 5 days prior to beginning self-administration. During this time, mice were acclimated to handling and catheter manipulation. For all self-administration sessions, catheters were flushed with 0.05 mL (i.v.) of heparinized (20 IU/ml; Hospira, Inc.) bacteriostatic 0.9% saline (Hospira, Inc.) containing gentamicin sulphate (Sparhawk Laboratories, Inc.; 0.25 mg/ml) immediately before and containing enrofloxacin (Norbrook Laboratories; 4.994mg/mL)

immediately after each session. Catheter patency was checked using 0.05mL (i.v.) Brevital Sodium (5mg/mL).

Opioid self-administration. To facilitate acquisition of opioid self-administration, mice were initially food-restricted to maintain a weight of 85-90% their original body weight and were habituated overnight to 50% liquid Ensure (diluted with water). The current study utilizes the highly μOR-specific and potent synthetic opioid, remifentanil HCl (Ultiva®; Mylan Institutional, LLC; purchased from Froedtert Hospital Pharmacy, Milwaukee, WI), whereby pressing the left lever resulted in delivery of both liquid Ensure (50%; Abbott Laboratories) and intravenous infusion of either saline or 5μg/kg/infusion based on previous dosing in mice and rats[47-49]. Conversely, pressing the right lever resulted in no consequence.

In most instances, mice were trained to press a lever on an increasing fixed ratio schedule using a liquid dipper system (Med Associates, Inc.). Training involved reaching a criterion of 25 (fixed ratio 1), 50 (fixed ratio 2), and 100 (fixed ratio 3) dipper/infusions on a given day and an increasing fixed ratio schedule was enforced, with reaching criteria necessary before progressing (i.e. Paired). Following completion of FR3 paired with 100 dipper/infusions, mice were given 1-3 days of abstinence followed by one self-administration session for the remifentanil or saline alone and then food was returned *ad libitum.* In a subset of mice, acquisition involved responding on a fixed ratio 1 schedule for ensure alone for one day, then responding on a fixed ratio 1 schedule for remifentanil or saline alone for the remainder of self-administration (i.e. Unpaired). Importantly, there were no differences in PrL rheobase of 14 day saline male mice that received Paired versus Unpaired training ($t_{(21)}$= 0.41, p=0.69; Supplemental 7). Following acquisition, male and female mice received remifentanil or saline on a fixed ratio 1 schedule (+cue-light, time out 20 s) for 3 hours per day for 10-14 days or 26-30 days (with a minimum of 10 infusions for each self-administration day as a criteria) for initial electrophysiology recordings. Mice undergoing set-shifting underwent 2 hours of administration

per day, however no difference in cumulative intake was observed in males or females compared to 3 hour counterparts (Supplemental 8).

In vivo activation of designer receptor exclusively activated by designer drugs. Two to three days following completion of self-administration, a subset of mice received a bilateral intracranial infusion of AAV8-CamKII-hm3d(Gq)-mcherry (addgene: 50476-AAV8) or AAV8-CamKII-GFP (UNC) into the PrL (coordinates from bregma: AP: +1.8, ML: ± 0.4; DV: -2.3). Mice were allowed to recover for five days then were food deprived to 85-90% of their free feeding weight to be ran in the attention set-shifting task, as described below. Mice with viral infusions were given a saline intraperitoneal injection 30 minutes prior to testing in the visual cue test. On the day of the extradimensional shift, mice were given 5mg/kg clozapine-n-oxide (CNO; Tocris Biosciences, Cat. No: 4936).

Elevated plus maze. The morning following the last self-administration sessions, a subset of mice were tested for anxiety-like behaviors using the elevated plus maze as previously described (Anderson et al., 2019) (EPM; San Diego Instruments). Behavior was recorded using AnyMaze (Stoelting Co.) tracking software. Percent time in the open arms was calculated as total time in the open arms divided by total time in the open and closed arms.

Forced swim test. A subset of mice were tested for immobility in a forced swim test approximately 16 days following the last day of self-administration, as previously described[50]. Briefly, mice were placed in an inescapable 800mL glass jar that was filled with 25°C water and were habituated for two minutes. The test consisted of four minutes during which the time immobile was measured using AnyMaze tracking software (Stoelting Co.). This test was performed following testing with the set-shifting task to avoid confounds of acute stress on subsequent behavior.

Attention set-shifting task. Cognitive flexibility was measured using an operant based attentional set-shifting task that was modified based off of methods previously described[51]. Three to seven days following the last self-administration session, mice were food-deprived 85-90% of their free feeding weight. Training and testing was done in the operant chamber that was used for self-administration (Med Associates, Inc). During *food training*, a fixed ratio 1 schedule was used, whereby a right lever response (opposite lever to the reinforced lever during self-administration) resulted in delivery of a 50% liquid Ensure reward. Food training sessions were 30 minutes in length and required mice to earn 50 rewards to progress to lever training. During *lever training*, levers were extended pseudorandomly with no more than two consecutive extensions of each lever. Mice were required to press the lever within 10 seconds of extension to receive a 50% liquid Ensure reward, absence of which was deemed as an omission but was not counted as a trial. Lever training consisted of a total of 90 trials (45 of each the left and right lever), with each followed by a 20 second time out. Mice had to reach criteria of five or fewer omissions on two consecutive days. Once lever training criteria was reached, *lever bias* was assessed through 7 trials, during which both the left and the right lever were reinforced.

The following day, *visual cue testing* was conducted until 150 trials or a streak of 10 consecutive correct responses, whichever occurred first. The correct response was the lever below the illuminated cue light and resulted in delivery of the ensure reward and a time out. Omissions were counted as stated above. If this criterion was not reached on the first day of testing, a second or third day of testing was conducted. The day following visual cue testing, *extradimensional shift testing* was conducted and the reinforced lever was always the lever opposite of the bias, however during this test the cue light was presented in a manner/order similar to that during the visual cue test. Following criteria of the extradimensional shift, reversal testing was conducted during which the reinforced lever was always that of the bias,

however the cue light was presented as above. The criterion for both the extradimensional shift and reversal test was the same as the visual cue test. Once all attentional set-shifting tests were complete, food was provided *ad libitum* for the remainder of the experiments.

Slice electrophysiology. Acute slice electrophysiology was performed either 14-21 or 40-45 days following the self-administration session. Mice were mildly anesthetized with isoflurane (Henry Schein), decapitated, and the brain removed and put in ice-cold sucrose solution oxygenated using 95% O_2 5% CO_2. A vibratome (Leica VT1000S) was used to obtain coronal slices (300µm) containing the mPFC. Slices were immediately incubated at 31°C for 10 minutes in a solution 119mM NaCl, 2.5mM KCl, 1mM NaH_2PO_4, 26.2mM $NaHCO_3$, 11mM glucose, 0.4mM ascorbic acid, 4mM $MgCl_2$, and 1mM $CaCl_2$. Slices were then removed, allowed to cool to room temperature and incubated further for a minimum of 35 min.

Whole-cell recordings were performed as previously described [29,50]. Briefly, slices were gravity perfused with oxygenated ACSF at a temperature of 29°C-33°C using at a flow rate of ~2-2.5 ml/min. Sutter Integrated Patch Amplifier (IPA) with Igor Pro (Wave Metrics, Inc.) was used for the data acquisition software. Recordings were filtered at 2kHz and sampled at 5kHz for current-clamp recordings and voltage-clamp recordings assessing baclofen-evoked currents. Miniature postsynaptic current recordings were filtered at 2kHz and sampled at 20kHz.

Layer 5/6 PN were identified based on pyramidal-shaped soma, long apical dendrite extending superficially, capacitance (PrL >100pf; IL >75pF), resting membrane potential (<-55mV), and lack of spontaneous activity[29,50,52-54]. For all recordings, adequate whole-cell access (Ra<40 MΩ) was maintained. Borosilicate glass pipettes were filled with 140mM K-Gluconate, 5.0mM HEPES, 1.1mM EGTA, 2.0mM $MgCl_2$, 2.0mM Na_2-ATP, 0.3mM Na-GTP, and 5.0mM phosphocreatine (pH 7.3, 290mOsm) for rheobase, action potential firing, and baclofen recordings. For miniature excitatory and inhibitory postsynaptic currents, borosilicate glass pipettes were filled with 120mM CsMeSO4, 15mM CsCl, 10mM TEA-Cl, 8mM NaCl, 10mM

HEPES, 5mM EGTA, 0.1mM spermine, 5mM QX-314, 4mM ATP-Mg, and 0.3mM GTP-Na and 0.7mM lidocaine was added to the recording solution. To assess the influence of CNO bath application on rheobase, rheobase was measured as above followed by application of 5µM CNO and rheobase was tested 5-10 minutes following application.

Histological assessment of virus expression. Accuracy and selectivity of virus targeting was assessed on in slices prior to electrophysiology studies or post-hoc in fixed tissue. Viral placement was either verified by fixing brains in 4% paraformaldehyde for 24-48 hours then placed in phosphate buffered saline until sliced using a vibratome during which 100µm were collected, mounted onto a slide using ProLong Gold Antifade Mountant (Thermo Fisher Scientific) and imaged using a Nikon inverted fluorescent microscope with a 4x objective. Virus placement was conducted for a subset of mice during electrophysiology recordings to assess the influence of CNO on rheobase. Only mice with virus expressed bilaterally in the PrL were used in analyses. Animals with inaccurate targeting of virus or excessive virus expression outside the PrL were removed. Histological analysis was performed blind to conditions.

Data analysis. Statistical significance for electrophysiological measurements pertaining to rheobase, mEPSC/mIPSC frequency/amplitude, $I_{baclofen}$) as well as behavior tests involving EPM, FST, and ASST were determined using 2 (male, female) x 2 (saline, remifentanil) analysis of variance. For current-spike analysis (Fig. 1, Supplemental 1-2), two-way (frequency x current) repeated measure ANOVA was used. For subsequent analysis of DREADD rescue, a one-way ANOVA RM for within-cell analysis was used. Here, data from saline and remifentanil mice were combined with additional animals and groups (GFP control, GqDREADD). When interactions were found, main effects were not reported. Student-Newman-Keuls post-hoc comparisons were conducted when necessary. mIPSCs/mEPSCs were analyzed using MiniAnalysis software using a 5pA detection threshold (Synaptosoft). Data was analyzed using Sigma Plot (Systat Software, Inc) and graphed using Prism 7.0

(GraphPad). The threshold for statistical significance was $p<0.05$. Statistical outliers were defined as being at least 2 standard deviations away from the mean and were removed from analyses. A total of 6 cells were deemed as outliers for electrophysiology recordings (n=3 for mPSCs; n=3 for rheobase), with no more than one per experimental group removed. For behavior studies, no statistical outliers were removed. For electrophysiology studies, in most instances, no more than 3 recordings were used for a given metric (e.g., mEPSCs) from the same animal to reduce data bias. Exceptions were made when multiple recordings were acquired by different electrophysiologists on a given day for a total of n=4/animal.

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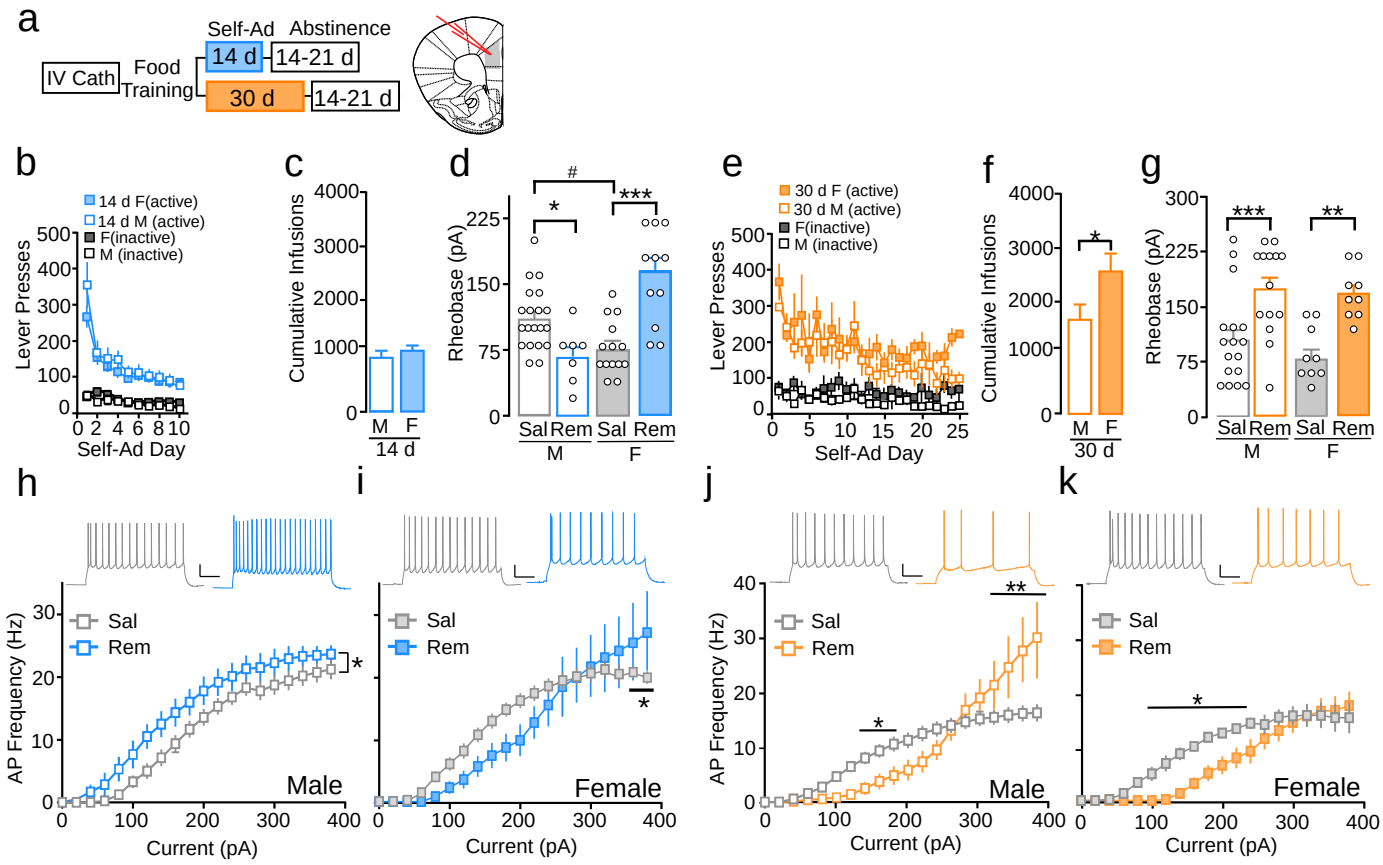


Figure 1. Bi**directional effect of remifentanil self-administration on PrL L5/6 PN in males and females.** a, Self-administration and abstinence timeline and location of current-injection whole-cell recordings in the prelimbic cortex (PrL). b, For 10-16 day groups, no sex by day interaction or main effect of sex was observed for active [interaction: F(9, 279)= 1.26, p=0.26; sex: F(1, 31)= 0.76, p=0.39)] or inactive lever presses [interaction: F(9, 279)= 0.90, p=0.52; sex: F(1, 31)= 2.39, p=0.13]. c, Cumulative remifentanil infusions did not differ in males (M) and females (F) self-administering for 10-16 days (t(31)= -1.27, p=0.21). d, Mean action potential (AP) threshold (rheobase) in PrL L5/6 PN following 14-21 d abstinence from 10-16 d self-administration showed an interaction of sex by treatment (F(1, 50)= 32.11, p<0.001). Rheobase was lower in females compared to males under saline conditions (SAL) (N/n= F: 9/15, M:12/16, #p=0.03). Remifentanil (REM) increased rheobase in females (N/n=4/12), ***p<0.001) but reduced it in males (N/n=7/10, *p=0.01) compared to respective controls. e, For 25-30 day groups, no sex by day interaction or main effect of sex was observed for active [interaction: F(24, 168)= 1.03, p=0.43; sex: F(1, 7)= 0.85, p=0.39] or inactive lever presses [interaction: F(24, 168)= 1.35, p=0.14; sex: F(1, 7)= 3.62, p=0.10]. f, Cumulative remifentanil infusions were elevated in females (F) compared to males (M) for 25-60 days (t(7)=2.37, *p=0.05). g, Following ~30 days of remifentanil, PrL L5/6 PYR exhibited higher rheobase in males (N/n=6/14) and females (N/n=4/9) compared to respective saline controls (Male: N/n=8/17, Female: N/n=4/10; F(1,45)= 22.39, p<0.001). h-i, Current-spike analysis showed an increase in mean firing frequency in remifentanil (N/n=5/6) versus saline (N/n=11/16) males (h; treatment: F(1,20)=4.28, p=0.05) but an interaction of treatment by current in females (i; F(19,416)=1.97, p<0.01; N/n= Sal:9/14, Rem:4/10). j, Current-spike analysis showed firing frequency was reduced at lower currents but increased at more depolarized potentials in remifentanil (N/n=6/12) versus saline (N/n=8/16) males (interaction: F(19,551)=4.74,*p<0.05, ***p<0.001). k, Remifentanil females (N/n=4/9) had reduced firing frequency at lower currents but similar firing frequency at more depolarized potentials versus saline females (N/n=5/9) (Two-way RM: F(19,304)= 2.95, p<0.001). All data are presented as the mean ± SEM.

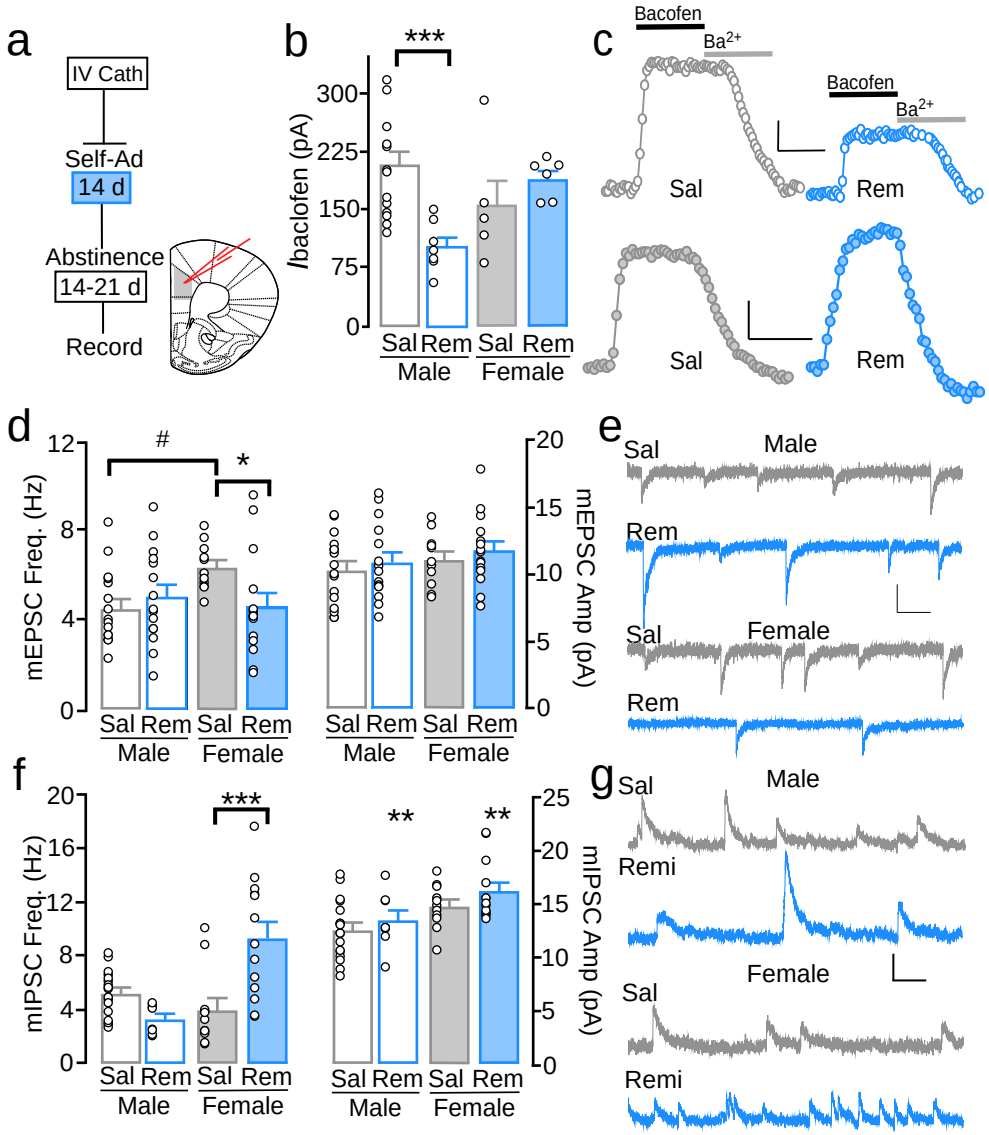


Figure 2. **Remifentanil effects on PrL L5/6 PN excitatory and inhibitory plasticity in males and females. a, Self-administration and abstinence timepoints**. b, Mean baclofen-evoked currents (IBaclofen) from male (unfilled) and female (filled) PrL L5/6 PN 14-21 days after 10-14 day remifentanil (blue) or saline (gray). A significant sex by treatment interaction was observed following short-term abstinence ($F_{(1,28)}$=9.63, p<0.01). Currents were reduced in remifentanil males (N/n=6/8) versus saline (N/n=11/13, ***p<0.001). Saline females (N/n=5/5) showed similar IBaclofen compared to remifentanil (N/n=3/6, p=0.35). c, Representative IBaclofen traces following 14-21 d abstinence. d, Mean frequency (left) and amplitude (right) of miniature excitatory postsynaptic currents (mEPSCs) following short-term abstinence. An interaction was observed for mEPSC frequency ($F_{(1,53)}$=5.06, p=0.03). Saline females (N/n=6/8) exhibited elevated frequency compared to saline males (N/n=8/16; #p=0.016) and remifentanil females (N/n=8/15, *p=0.023), with no change in remifentanil versus saline males (N/n=8/15, p=0.45). No main effects or interaction was observed for mEPSC amplitude (sex: $F_{(1,53)}$=1.48, p=0.23; treatment: F=0.79, p=0.38; interaction: F= 0.01, p=0.94). e, Representative mEPSCs (recorded at -72 mV) from PrL L5/6 PN. f, Mean frequency and amplitude of miniature inhibitory PSCs (mIPSCs) following short-term abstinence. A significant interaction was observed for mIPSC frequency ($F_{(1,45)}$=17.85, p<0.001). Frequency was increased in remifentanil (N/n=10/13) versus saline (N/n=6/11) females (***p<0.001), with no differences in remifentanil (N/n=5/8) versus saline (N=8/17, p=0.13) males. A significant effect of sex, but not treatment or interaction was observed for mIPSC amplitude (sex: $F_{(1,45)}$=10.19, **p=0.003; treatment: F=2.18, p=0.15; interaction: F=0.11, p=0.74). g, Representative mIPSCs (recorded at 0 mV) from PrL L5/6 PN.

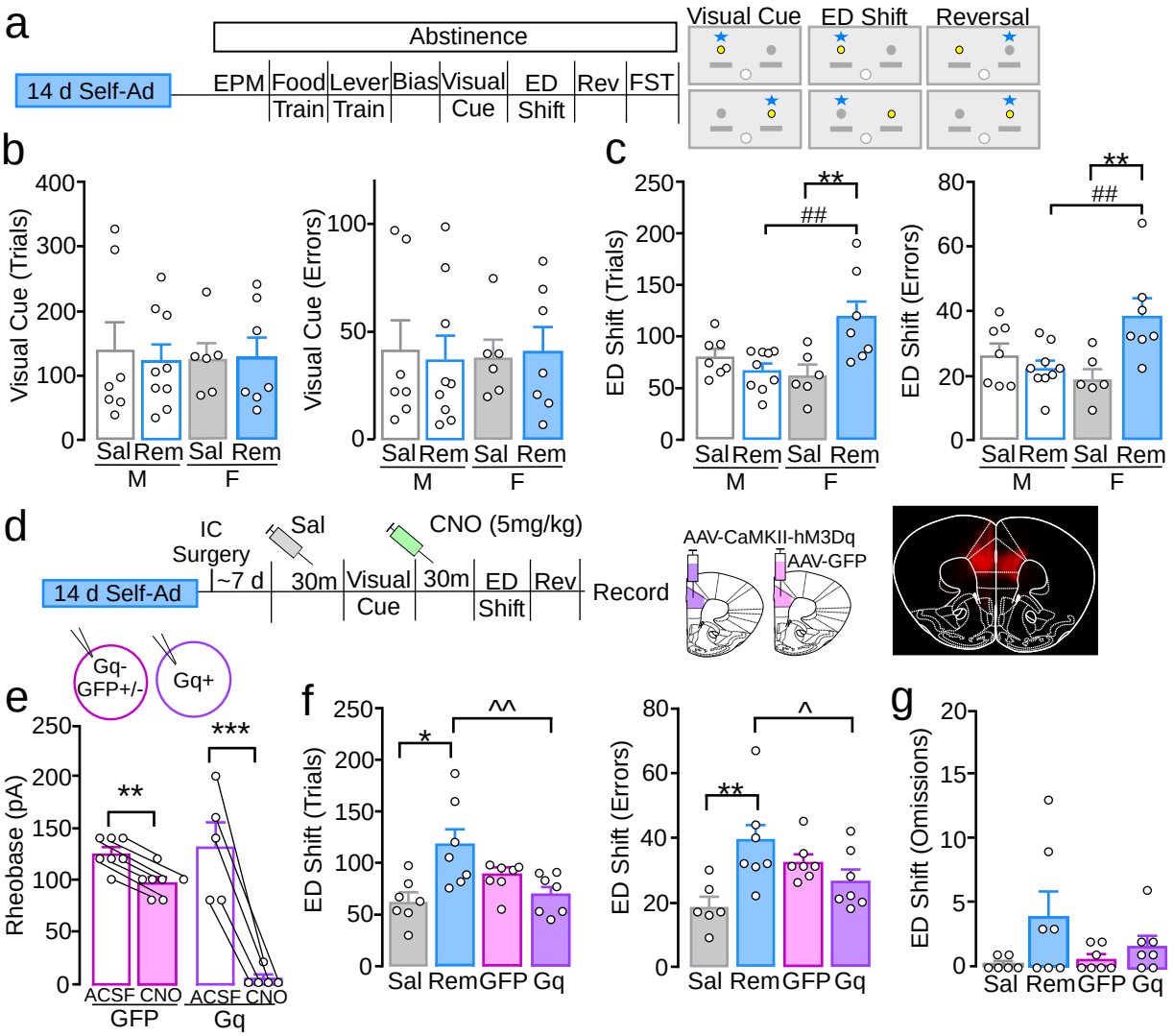
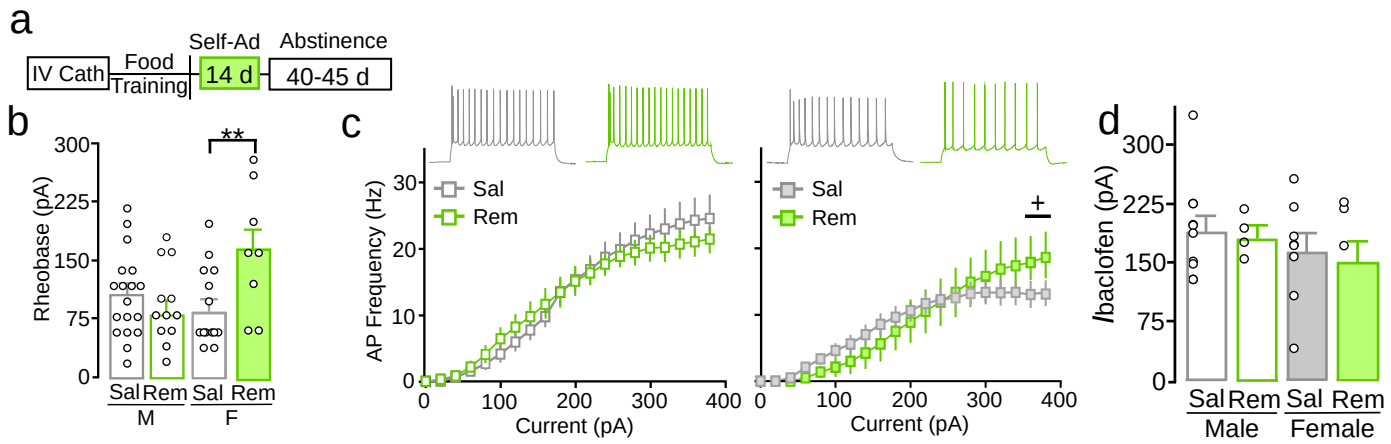
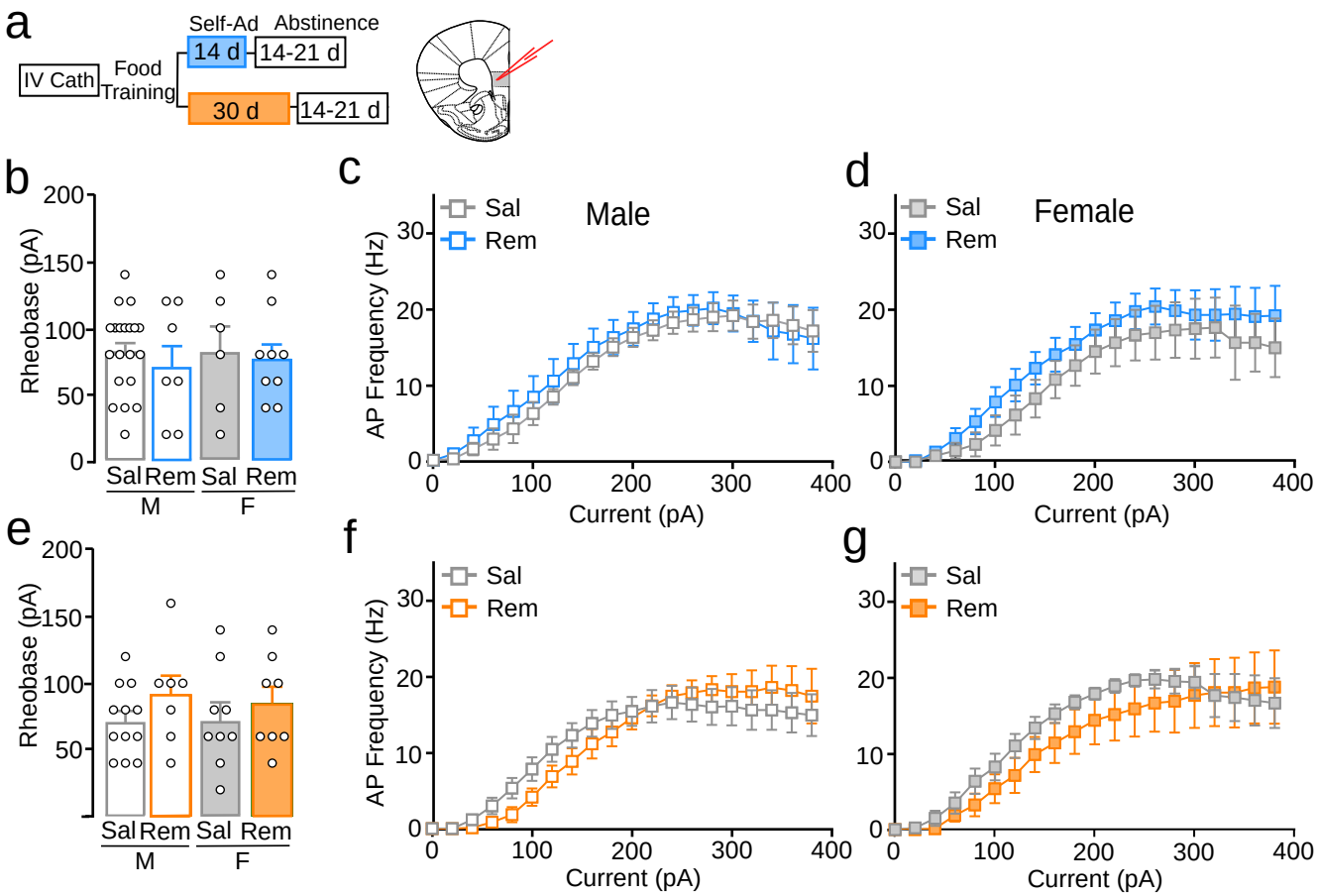


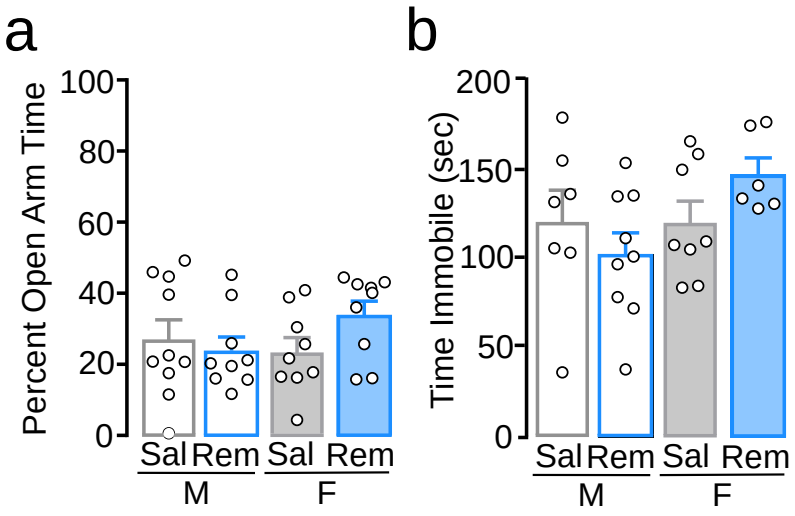
Figure 3. **Opioid-induced PrL L5/6 PN hypoactivity produces deficits in cognitive flexibility but not affect.** a, Timeline of behavioral assessments following self-administration and schematic of operant set-shifting paradigm depicting levers (gray), dipper (white), cue location (yellow) and correct response (star). b, Mean trials (left) and errors (right) to criteria for visual cue test. No significant main effect of sex or treatment, or interaction was observed for trials or errors to criteria (sex: trials ($F_{(1,25)}$=0.02, p=0.89), errors ($F_{(1,25)}$=0.00, p=0.99); treatment: trials ($F_{(1,25)}$=0.06, p=0.81), errors ($F_{(1,25)}$=0.01, p=0.94); interaction: trials ($F_{(1,25)}$=0.09, p=0.76), errors ($F_{(1,25)}$=0.10, p=0.76). c, Mean trials and errors to criteria for extradimensional (ED) cue-to-response shift. A significant interaction was observed for trials ($F_{(1,28)}$=10.19, p=0.004) and errors ($F_{(1,28)}$=10.48, p=0.003). Remifentanil females (N=7) required significantly more trials and exhibited more errors compared to saline females (N=6, trials: **p=0.003, errors: **p=0.002) and remifentanil males (N=9; trials: ##p=0.002, errors: ##p=0.004), whereas performance in remifentanil males did not differ compared to saline males (N=7). d, Timeline of DREADD experiments involved remifentanil female mice expressing a CaMKII-hM3Dq(Gq)-DREADD or control GFP AAV in the PrL that received a saline and Clozapine-N-oxide (CNO; 5 mg/kg, i.p.) injection 30 minutes prior to the visual cue (Supplemental 6) and ED shift test, respectively. e, Ex vivo bath application of CNO (5 µM) reduced rheobase in PrL L5/6 PN ($F_{(1,10)}$=78.01, p<0.001), producing spontaneous firing (Rheobase = 0) in 4 out of 5 PN positive for Gq-DREADD (N/n= 3/5, ***p<0.001) and reducing rheobase in PN negative for Gq-DREADD or positive for GFP-control compared to baseline (N/n=5/7, *p=0.032), albeit to a lesser extent (interaction: $F_{(1,10)}$= 31.46, ***p<0.001). f, During the ED Shift, Gq- DREADD remifentanil females (purple, N= 7) required fewer trials (left) and a trend towards fewer errors (right) to criteria versus remifentanil females with no virus (REM, blue; trials: ^^p=0.017; errors: ^p<0.095), with no difference versus GFP controls (pink, N=7, trials: p=0.32, errors: p=0.29) or saline (gray, trials: p=0.66, errors: p=0.17). g, No difference in omissions were observed (p=0.26).



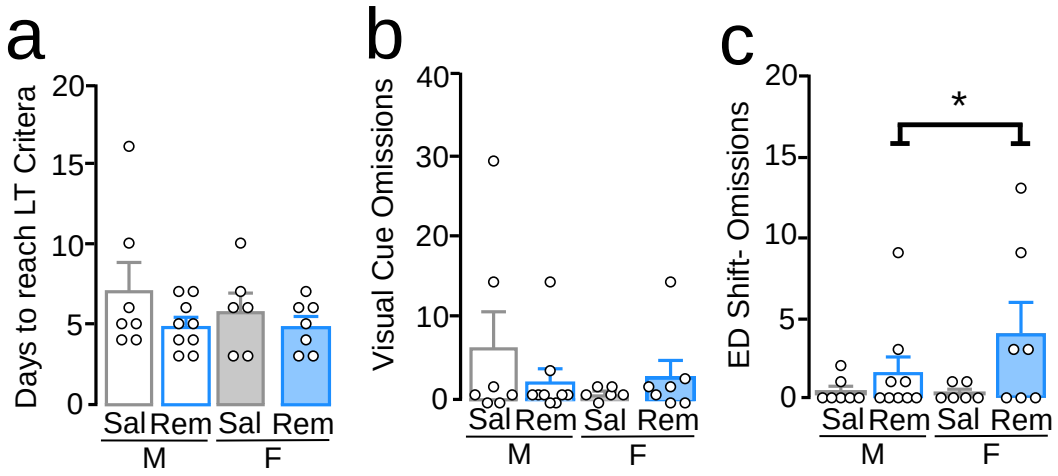
Supplementary Figure 1. **Increased rheobase in females following remifentanil self-administration is enduring.** a, Self-administration and abstinence timeline. b, Following 45 day abstinence, no significant difference in rheobase was observed in remifentanil males (Sal: N/n=7/12, Rem: N/n=10/18, p=0.50) while rheobase remained increased in remifentanil females (N/n=5/9) versus saline (N/n=7/14, **p=0.009; interaction: $F_{(1,48)}$=6.37, p=0.02). c, Current-spike analysis showed no difference in firing frequency across current in remifentanil males (N/n=7/12) compared to saline (left; N/n=9/16; treatment: $F_{(19,494)}$= 0.69, p=0.83), and a significant treatment by current interaction in females ($F_{(19,361)}$= 2.46, p<0.001), with a trend towards increased firing at higher currents in remifentanil mice (N/n=4/8) compared to saline (N/n=6/13; +p<0.10). d, No significant effect of sex, treatment, or interaction on IBaclofen was observed following 40-45 days of abstinence (right; sex: $F_{(1,24)}$=1.88, p=0.18; treatment: F=0.24, p=0.63; interaction: F=0.04, p=0.85).



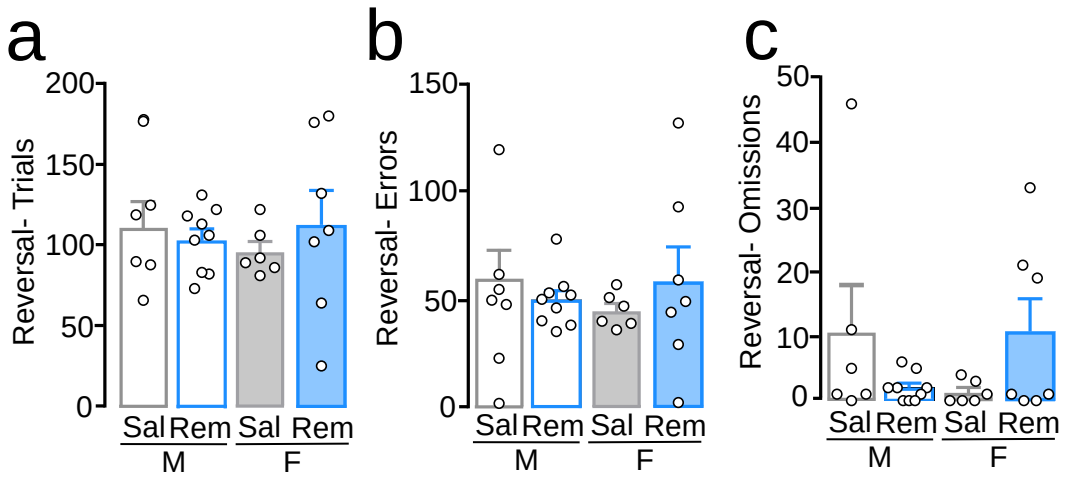
Supplementary Figure 2. **Remifentanil self-administration does not alter L5/6 PN membrane excitability in the ILC.** a, Self-administration and abstinence timeline and location of current-injection whole-cell recordings, in the infralimbic cortex (ILC). b, Mean action potential (AP) threshold (Rheobase) in ILC L5/6 PN following 14-21 d abstinence from 10-16 d self-administration showed no interaction or main effects of sex or treatment (interaction: $F_{(1, 38)}$= 0.06, p=0.81; sex: F=0.07, p=0.79; treatment: F=0.48, p=0.49; Sal-M: N/n= 9/20, Rem-M: N/n= 5/7, Sal-F: N/n= 5/6, Rem-F: N/n=5/9). c, Current-spike analysis showed no differences in mean firing frequency in remifentanil (N/n=5/7) versus saline (N/n=7/15) males (left, treatment x current step: $F_{(19, 380)}$= 0.20, p=1.00; treatment: $F_{(1, 20)}$= 0.31, p=0.59). d, Current-spike analysis showed no differences in mean firing frequency in remifentanil (N/n=5/9) versus saline (N/n=5/6) females (right, treatment x current step: $F_{(19, 247)}$= 0.21, p=1.00; treatment: $F_{(1, 13)}$= 0.97, p=0.34). e, Following ~30 days of remifentanil, ILC L5/6 PYR exhibited similar firing thresholds in males (N/n=3/7) and females (N/n=3/8) compared to respective saline controls (Male: N/n=6/12, Female: N/n=4/9; Interaction: $F_{(1,32)}$= 0.13, p=0.73, sex: F=0.04, p=0.84; treatment: F=1.90, p=0.18). f, Current-spike analysis showed no differences in firing frequency across current in remifentanil males (N/n=3/7) compared to saline (left, N/n=6/12; treatment: F(1,19)= 0.02, p=0.90, interaction: ($F_{(19,323)}$= 1.25, p=0.22). g, No differences in remifentanil females (N/n=3/8) compared to saline were obseved (right, N/n=4/8; treatment: $F_{(1,14)}$= 0.50, p=0.49, interaction: ($F_{(19,266)}$= 0.61, p=0.90).



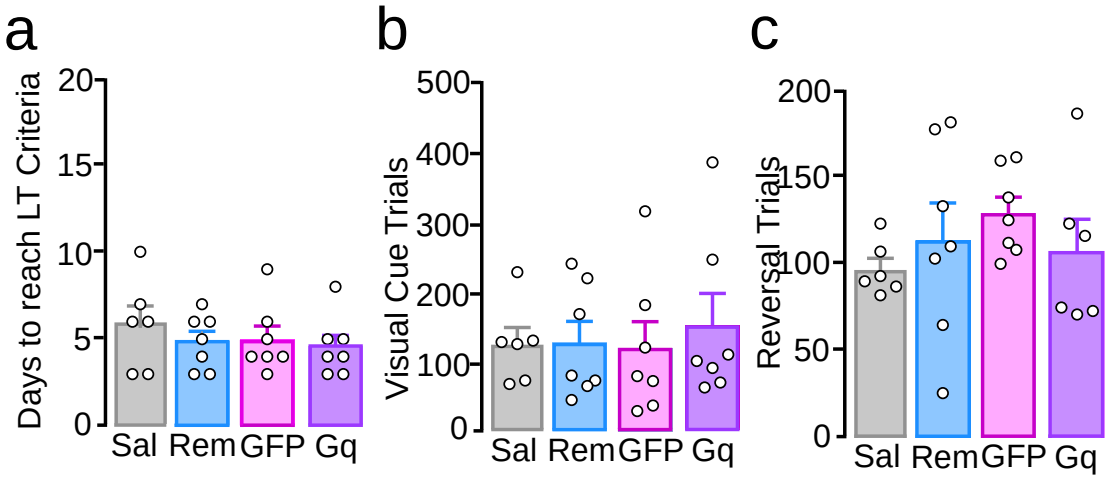
Supplementary Figure 3. **Remifentanil self-administration does not alter elevated plus maze or forced swim test behaviors.** Assessment of anxiety-like behavior following 10-14 d self-administration. There was no significant effect of sex, treatment, or interaction of sex by treatment on percent open arm time in the elevated plus maze (sex: $F_{(1,33)}$=0.54, p=0.47; treatment: F=0.64, p=0.43; interaction: F= 2.54, p=0.12; N= Sal-M (10), Rem-M (9), Sal-F (9), Rem-F (9). b, There was no significant effect of sex, treatment, or interaction on time immobile during a forced swim test (sex: $F_{(1,26)}$=2.86, p=0.10; treatment: F=0.10, p=0.76; interaction: F=2.96, p=0.10; N= Sal-M (7), Rem-M (9), Sal-F (8), Rem-F (6)).



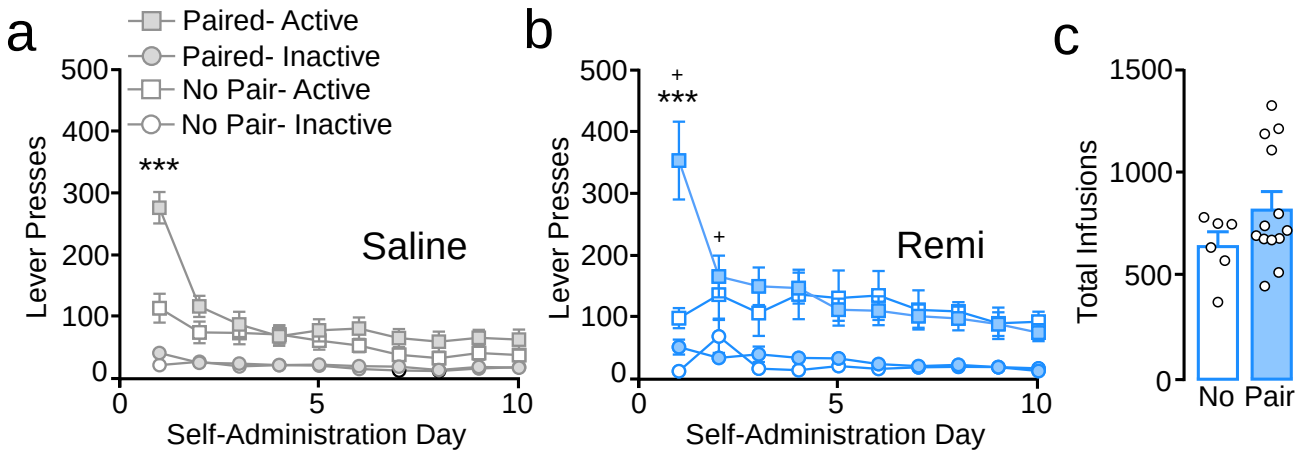
Supplementary Figure 4. **Differences in ASST performance were not influenced by differences in acquisition of lever training or omissions.** a, All groups took similar amount of time to acquire lever training (sex: $F_{(1,25)}$=0.43, p=0.52; treatment: $F_{(1,25)}$=2.50, p=0.13; sex by treatment: $F_{(1,25)}$=0.39, p=0.44). b, There were no differences in omissions during the visual cue test (sex: $F_{(1,25)}$=1.06, p=0.31; treatment: $F_{(1,25)}$=0.19, p=0.67; sex by treatment: $F_{(1,25)}$=1.62, p=0.22). c, There was not a significant difference in omissions during extradimensional set shift testing between sex ($F_{(1,25)}$=1.06, p=0.31) or a sex by treatment interaction ($F_{(1,25)}$=1.24, p=0.28) however there was a main effect of treatment ($F_{(1,25)}$=4.41, p=0.05) but both saline and remi mice had overall low omissions (saline: 0.38 ± 0.18 omissions; remi: 4.08 ± 2.63 omissions).



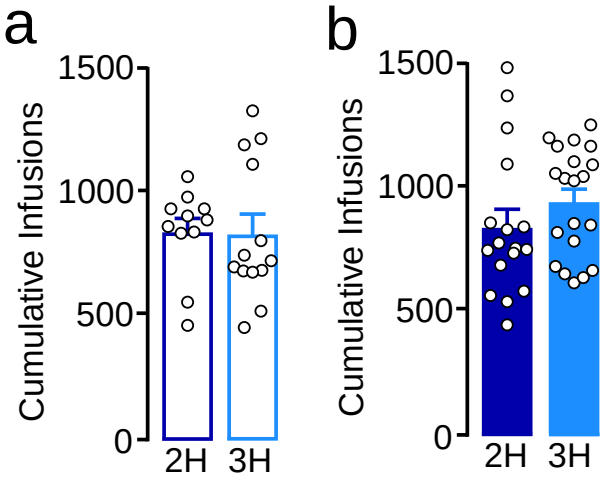
Supplementary Figure 5. **Remifentanil self-administration does not alter performance in the reversal test.** a, No significant effects of sex, treatment, or interaction of sex by treatment were observed for trials to criteria during the reversal test (sex: $F_{(1,24)}$=0.06, p=0.81; treatment: F=0.09, p=0.77; interaction: F=0.82, p=0.38. b, No significant effects of sex, treatment, or interaction of sex by treatment were observed for errors to criteria during the reversal test (sex: $F_{(1,24)}$=0.09, p=0.77; treatment: F=0.03, p=0.87; interaction: F=1.23, p=0.28). c, There was a significant sex by treatment interaction in omissions during the reversal test ($F_{(1, 24)}$=4.89, p=0.04, however no post-hoc analyses were significant.



Supplementary Figure 6. **No differences in lever training, visual cue test performance, or reversal test performance in Gq-DREADD attention set-shifting.** a, There were no differences in days to reach lever training criterion between saline-exposed, remi-exposed, remi-exposed with Gq-DREADD, and remi-exposed with GFP-control virus ($F_{(3,23)}$= 0.47, p=0.71). b, An injection of saline prior to the visual cue test did not influence trials to reach criteria ($F_{(3,23)}$=0.17, p=0.92), errors to criteria ($F_{(3,23)}$=0.23, p=0.87; not shown), or the number of omissions during testing ($F_{(3,23)}$= 1.50, p=0.24; not shown). c, Exposure to CNO the day prior did not significantly influence trials to criteria during the reversal test ($F_{(3,22)}$=0.76, p=0.53), errors ($F_{(3,22)}$=0.49, p=0.69; not shown), or omissions ($F_{(3,22)}$=1.71, p=0.20).



Supplementary Figure 7. **Paired Ensure-Infusion training increases active lever pressing in saline, but not remifentanil, male mice.** A portion of mice were trained using Ensure paired with remifentanil or saline for three days on an increasing fixed ratio schedule (Paired) whereas others received a single day of Ensure that was not paired with an infusion (Unpaired). a, Paired male mice receiving saline infusions prior to saline self-administration had significantly more active lever presses (gray filled, square) than saline Unpaired males (interaction: $F_{(9, 261)}= 7.44$, $p<0.001$, training type: $F_{(1, 29)}= 4.83$, $p=0.04$; ***$p<0.001$) but no differences were observed with inactive lever pressing (circles) (interaction: $F_{(9,261)}= 0.95$, $p=0.48$, training type: $F_{(1, 29)}= 0.52$, $p=0.48$). b, There was no main effect of training type on active lever pressing in males self-administering remifentanil ($F_{(1, 17)}= 0.58$, $p=0.46$), however there was a day by training interaction ($F_{(9, 153)}=5.41$, $p<0.001$). Higher active lever responding was observed in Paired versus Unpaired remifentanil mice only on the first day following ensure training (***$p<0.001$). Paired mice also had greater inactive lever pressing on the two days following ensure training (interaction: $F_{(9, 153)}= 2.95$, $p<0.01$, training type: $F_{(1, 17)}= 1.42$, $p=0.25$; +$p<0.05$). c, Total remifentanil intake (infusions) did not differ in Paired versus Unpaired mice ($t_{(17)}=-1.58$, $p=0.13$), and did not correlate to average PrL rheobase for Unpaired ($p=0.88$) or Paired ($p=0.73$) mice.



Supplementary Figure 8. **Duration of self-administration session does not contribute to overall remifentanil infusions earned.** In order to determine if length of self-administration contributed to overall responding and intake (infusions), male and female mice were ran for 2- or 3-hour daily self-administration sessions. a, Mean total infusions across 10 days in 2 hour and 3 hour self-administering males show no significant difference ($t_{(22)}$=0.08, p=0.94). b, Similar to males, no differences in total remifentanil intake was observed between 2 and 3 hour females ($t_{(35)}$=-1.24, p=0.22).