Sex-specific behavioral and thalamo-accumbal circuit adaptations after oxycodone abstinence.

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Abstract

Opioid use disorder is marked by a progressive change in the motivation to administer the drug even in the presence of negative consequences. After long periods of abstinence, the urge to return to taking the drug intensifies over time, known as incubation of craving. Conditioned responses to drug-related stimuli, can acquire motivational properties and exert control over motivated behaviors leading to relapse. Although, preclinical data suggest that the behavioral expression of opioid use is similar between male and female rodents, we do not have conclusive results on sex differences on craving and relapse across abstinence periods. Here, we investigated the effects of abstinence from oxycodone self-administration on neurotransmission in the paraventricular thalamus (PVT) to nucleus accumbens shell (NAcSh) pathway in male and female rats. Using optogenetics and *ex vivo* electrophysiology, we assessed synaptic strength and glutamate release probability in this pathway, as well as NAcSh medium spiny neurons (MSN) intrinsic excitability, in slices from rats which were subjected to either 1 (acute) or 14 (prolonged) days of forced abstinence after self-administration. Our results revealed no sex differences in oxycodone self-administration or somatic withdrawal symptoms following acute abstinence. However, we found a sex-specific enhancement in cue-induced relapse after prolonged, but not acute, abstinence from oxycodone self-administration, with females exhibiting higher relapse rates. Notably, prolonged abstinence led to similar increases in synaptic strength at PVT-NAcSh inputs compared to saline controls in both sexes, which was not observed after acute abstinence. Thus, prolonged abstinence results in a time-dependent increase in PVT-NAcSh synaptic strength and sex-specific effects on cue-induced relapse rates. These findings suggest that prolonged abstinence leads to significant synaptic changes, contributing to heightened relapse vulnerability, highlighting the need for targeted therapeutic strategies in opioid use disorder.

Introduction

Relapse is a hallmark of substance use disorder[1,2]. Relapse-inducing triggers include cues previously associated with the drug, stressful events and/or environments, and taking the drug itself (i.e., drug priming). These triggers can result in resumption of drug-taking (relapse), despite negative consequences[3–6]. Several preclinical models of relapse have been developed, and the model most closely mimicking the human conditions involves drug self-administration, an operant behavior in which the animal is required to press a lever to obtain drug and can therefore regulate its drug intake. To specifically assess relapse-like behavior, animals undergo a period of "forced abstinence" after several weeks of drug self-administration.

During forced abstinence, the animals are returned to their home cage without access to the drug[7]. When animals are re-introduced to the self-administration behavioral chambers after forced abstinence, they engage in rapid lever pressing - even though no drug is delivered in response. This time-dependent increase in motivation to seek out drug is referred to as 'incubation of craving' that leads to relapse[8–12]. Following extended periods of abstinence, incubated craving generally remains elevated before gradually stabilizing and declining[10]. The use of extinction training is also a common approach to study relapse after self-administration. In contrast to forced abstinence, extinction training prompts animals to learn that the drug is no longer available based on their actions (instrumental responding, e.g. lever presses or nose pokes), leading to a gradual reduction in drug-seeking behavior. Both models can trigger relapse when animals are reintroduced to drug-associated cues, but the neural pathways involved and the behavioral outcomes differ[7,13,14]. In these models, extinction training teaches animals that lever pressing no longer results in drug delivery. However, the drug-paired cues (contextual or discrete) are not extinguished. When presented during relapse, the cues elicit increased lever pressing based on the prior drug association formed during self-administration.

Recent studies have suggested that glutamatergic projections from the paraventricular nucleus of the thalamus (PVT) to the nucleus accumbens shell (NAcSh) are necessary for the expression of opioid withdrawal signs[15,16] and cue-induced relapse after abstinence but not extinction[13]. The nucleus accumbens (NAc) is an important node involved in cue-driven reward-seeking behaviors[17–20]. Multiple factors including synaptic plasticity contribute to changes in behavior over time. For example, increased synaptic strength in projections from the PVT to D2R-expressing NAc medium spiny neurons (MSNs) contributes to naloxone-induced withdrawal behaviors following morphine administration[15], which are evident in the first few days of abstinence and then dissipate. In addition, increased synaptic strength in projections from the PVT to D1R-expressing NAc MSNs is associated with relapse to heroin-seeking after 14 days of abstinence[13]. Although it is clear that changes in glutamatergic inputs onto MSNs may contribute to opioid withdrawal and reinstatement of drug-seeking[13,15,21,22], the relationships between NAcSh MSN circuitry functions, length of abstinence from oxycodone self-administration, level of drug-seeking, and sex specificity remain unclear.

Our goal here was to test the hypothesis that increased synaptic strength in PVT-NAcSh projections may be necessary for cue-induced relapse and drug-seeking. Additionally, we aimed to determine if there are sex-specific differences in either cue-induced relapse or PVT-NAcSh synaptic transmission after either 1 (acute) or 14 (prolonged) days of forced abstinence. Finally, in this study we also tested the hypothesis that increased synaptic strength in PVT-NAcSh projections may be necessary for cue-induced relapse and drug-seeking. Our results

demonstrate that sex-specific enhancement in cue-induced relapse emerges after prolonged abstinence and not during acute abstinence from oxycodone self-administration. Although, males and females have an increase in cue-induced relapse after prolonged abstinence, females exhibited a greater relapse rate compared to males. Additionally, both males and females had similar increases in PVT-NAcSh synaptic strength after prolonged abstinence. However, PVT-NAcSh synaptic strength was not altered after acute abstinence compared to saline controls. Thus, we found a time-dependent increase in synaptic strength in PVT-NAcSh projections and a sex-specific effect of prolonged abstinence on cue-induced relapse rates after oxycodone self-administration in rats, whereas synaptic enhancements in PVT-NAcSh projections after prolonged abstinence were not sex-specific.

Methods

Subjects

Adult male (250-275g; Total N = 42) and female (200-225g; Total N = 45) Sprague Dawley rats (Charles River Laboratory, Wilmington, MA) were used in this study. Upon arrival, rats were group housed (4 rats per cage) and were habituated for 1 week to the animal colony kept on a 12-h light/dark cycle (lights on 7:00AM) with food and water *ad libitum*. Following surgeries, rats were singly-housed for the rest of the experiment. The Institutional Animal Care and Use Committee at Mclean Hospital and the National Institutes of Health for the care and use of laboratory animals' guidelines were followed.

Surgeries

Stereotaxic surgery and viral injections

All surgeries were performed according to AAALAC guidelines. Rats were first anesthetized with ketamine and xylazine (80 mg/kg and 8 mg/kg, respectively,I.P.). A craniotomy was made to target the PVT using the following stereotaxic coordinates, based on Paxinos and Watson 6th edition rat brain atlas [23]: AP: -2.6mm from Bregma, ML: +2.0mm from Bregma (20° angle ML), and DV: -6.3mm from skull surface at injection site. A total of 1µL of the AAV5 vector carrying CaMKIIα-ChR2(H143R)-eYFP was injected unilaterally into PVT at a rate of 125 nl/min using a 10µl Hamilton syringe with a 29-gauge needle under the control of a micro-syringe pump (Harvard Instruments). Viral vectors (titers, ~10.0 x 10^{12} particles/ml) were purchased from the University of North Carolina viral vector facility.

Intravenous catheter implantation surgery

After recovery from stereotaxic surgery (~7 days), rats were implanted with indwelling silastic intravenous jugular catheters (SAI infusions; RSB-SA-7.5CF and RSB-SA-7.5CM), as described in [24–26]. Rats were anesthetized with ketamine and xylazine (80 mg/kg and 8 mg/kg, respectively, I.P.), and catheters were implanted into the right jugular vein, secured to the vein with non-absorbable suture thread and passed subcutaneously through the rat's back. All rats

received an injection of ketofen (5mg/kg; S.C.) and gentamicin (0.1ml; 10mg/ml, I.V.) during catheter implantation. Catheters were flushed daily with 0.2ml of heparinized saline (30 units/ml; I.V.) and once a week with 0.2ml of gentamicin (10 mg/ml I.V.). Catheter patency was checked once per week using methoexital (Brevital; 0.1 ml females; 0.2 males of 10 mg/ml I.V.).

Behavioral methods

Oxycodone self-administration

Med Associates operant conditioning chambers (30.5 (*l*) × 24.1 (*w*) × 29.2 (*h*) cm), kept within soundproofed outer chambers with ventilation fans, were equipped with two retractable levers, each with a cue light above them, a house light, a counterbalance swivel and tether, and an infusion pump. Rats (males: n = 15 saline, 27 oxycodone; females: n= 22, saline, 23 oxycodone) underwent 8 days of short access (ShA) oxycodone self-administration training (0.06mg/kg/infusion; 1h/day) followed by 14 days of long access (LgA) regimen (0.06 mg/kg/infusion; 6h/day), similar to Mavrikaki et al 2019). Self-administration sessions were run 7 days/week, at approximately 9:00 am each day. All self-administration was conducted during the light phase of a 12:12 light/dark cycle (lights on at 7:00am; lights off at 7:00pm). A fixed-ratio 1 (FR1) schedule of reinforcement was used such that a press on the active lever resulted in a 4-s oxycodone infusion (100 µl) followed by a 6-s time out period where a press on the active lever produced no consequences.

Assessing oxycodone dependence

To demonstrate that our oxycodone self-administration protocol induced dependence in both male and female rats, we measured spontaneous somatic withdrawal signs 24-h after the last oxycodone self-administration session. After removal from self-administration chambers and catheter flushing, rats were placed back in their home cages and brought to a quiet, temperature-maintained (20°C) room and allowed to habituate for ~15-min. Rats were then individually placed into clear, 65-cm-high by 25-cm-diameter Plexiglas cylinders that contained a small amount of bedding. Rats were allowed to habituate to the cylinders for ~15 min. At this point, a digital video system (Swann Communications, Sante Fe, CA) was used to record the rats in the cylinders for 20 minutes. Upon completion of recording, somatic withdrawal behaviors were scored for the first 15 min of the recording by a researcher who was unaware of the treatments. Every 15 seconds, the following behaviors were marked as either present or absent: diarrhea, ptosis, jumping, walking, rearing, digging, flat posture, “wet dog shakes,” grooming and teeth chattering [25,27]. The number of occurrences of each behavior was summed. In addition, a Total Withdrawal Score was calculated by summing weighted frequencies of those behaviors most commonly and specifically observed in opioid withdrawal: Total Withdrawal = Grooming (x1.0) + Wet Dog Shakes (x1.5) + Ptosis (x1.2)[27,28]. Wet Dog Shakes and Ptosis were multiplied by previously determined weighting factors to account for their high importance, but low prevalence, to withdrawal signs.

Forced abstinence and cue-induced oxycodone-seeking

a) *1-day abstinence* (acute abstinence): From the total rats above, male (Saline, N = 9; Oxycodone, N = 18) and female (Saline, N = 13; Oxycodone, N = 15) rats underwent 1d of forced abstinence (rats returned to the vivarium in their home cages for 24-h) from oxycodone self-administration. A subset of 1d abstinence male (Saline, N=5; Oxycodone N=10) and female (Saline, N=8; Oxycodone N=7) rats were used to measure somatic withdrawal signs, and another subset of males (Saline, N=4; Oxycodone, N=8) and females (Saline, N=6; Oxycodone, N=8) was used to measure cue-induced oxycodone-seeking after the 1d abstinence period. After 1d of abstinence, rats were reintroduced to the operant chamber for a 2-h relapse test. The cues associated with oxycodone were presented, but no drug was delivered.

b) *14-day abstinence* (prolonged abstinence): From the total rats above, male (Saline, N=7; Oxycodone N=8) and female (Saline, N=8; Oxycodone, N=8) rats underwent 14d of abstinence from oxycodone self-administration. After 14d of abstinence, rats were reintroduced to the operant chambers for a 2-h relapse test as described above.

The number of active and inactive lever presses were recorded during incubation/ cue-induced oxycodone-seeking testing following acute and prolonged abstinence periods. Active lever presses were compared between saline and oxycodone groups for cue-induced oxycodone-seeking. Rat brains were extracted thirty minutes to one hour after cue-induced oxycodone-seeking, and electrophysiological recordings of MSNs of the NAcSh were conducted from brain slices containing the PVT and NAcSh.

Ex-vivo electrophysiology and optogenetic stimulation

Coronal slices (300 μm in thickness) containing the NAc were obtained using a vibratome in cold cutting solution containing the following in mM: 252.0 sucrose, 1.0 $CaCl_2$, 5.0 $MgCl_2$, 2.5 KCl, 1.25 NaH_2PO_4, 26.0 $NaHCO_3$ and 10.0 glucose and equilibrated with 95% O_2 and 5% CO_2. Slices were then incubated in artificial cerebrospinal fluid (ACSF) containing the following in mM: 125 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.0 $MgSO_4$, 1.25 NaH_2PO_4, 26.0 $NaHCO_3$, and 10.0 glucose at room temperature for at least 1 hr before recordings started. Whole-cell recordings were obtained from the NAcSh neurons with patch electrodes (3-5 MΩ resistance) containing the following in mM: 135.0 Cs-methane-sulfonate, 5.0 NaCl, 1.0 $MgCl_2$, 10.0 BAPTA, 10.0 HEPES, 2.0 ATP and 0.20 GTP adjusted to pH 7.2 with CsOH. Neurobiotin (0.2%; Vector Laboratories) was also added to the internal solution before the recordings to allow subsequent histochemical localization of the recorded neurons in the NAcSh.

Synaptic responses were induced by photostimulation of ChR2-expressing PVT projecting terminals in the NAcSh with a LED light source (excitation wavelength: 470 nm, 5 ms in duration, Thorlabs). All whole-cell recordings were performed at 30-32°C. After recordings, slices were placed in PBS containing 4% paraformaldehyde and kept in the refrigerator until histological processing.

The pharmacological reagents used in electrophysiological experiments included NBQX disodium salt, D-AP5, NBQX and (-)-bicuculline methobromide were prepared as stock solution in water at 1000- to 5000-fold concentrations and stored at -20°C.

Statistical Analysis

Male and female rats were randomly assigned to either saline or oxycodone groups. In electrophysiological experiments, ~2-3 neurons were recorded per animal. The numbers of rats and recorded neurons for the analysis of the different experiments are indicated in the results section. Data are reported as mean +/- SEM. All electrophysiology data were collected using Patch Master (Heka systems). sEPSCs were analyzed using MiniAnalysis (Synaptosoft version 6.0.7) as previously described[29,30]. We used Prism 9 (GraphPad) for statistical analysis using Two-tailed t-tests, Mixed effects model (Restricted Maximum Likelihood/REML), and Two- or Three-way ANOVAs with Sidak's multiple comparisons, as appropiate. The number of animals and cells used per experiment, as well as the results of the statistical analysis are reported in the text or in the figure legends.

Results

Both male and female rats escalate oxycodone intake under long access self-administration conditions

Female (Saline, N=22; Oxycodone N=23) and male (Saline, N=15; Oxycodone, N=27) rats were trained to self-administer oxycodone (or saline) under short access (ShA) conditions (1h/day for 8 days), followed by 14 days of long access (LgA) conditions (6 h/day) (**Fig. 1A Experimental Design**). Males and females escalated their oxycodone intake under LgA conditions: there was an increase in the number of infusions over time (**Fig. 1B**: Mixed-effects model (REML): days x treatment interaction: $F_{(63,1463)} = 13.14$, $p < 0.0001$; main effect of days: $F_{(21,1463)} = 38.91$, $p < 0.0001$; main effect of treatment: $F_{(3,71)} = 31.29$, $p < 0.0001$; no sex difference: $F_{(1,71)} = 0.04$, $p = 0.84$), and there was an increase in active lever presses (**Fig. 1C**: Mixed effects model (REML): days x treatment interaction: $F_{(63,1149)} = 5.210$, $p < 0.0001$; main effect of days: $F_{(21,1149)} = 14.59$, $p < 0.0001$; main effect of treatment: $F_{(3,56)} = 12.48$, $p < 0.0001$; no sex differences: $F_{(1,56)} = 0.40$, $p = 0.53$). There was no change in inactive lever presses in either sex and lever pressing did not increase across days (**Fig. 1D**: Mixed effects model; main effect of treatment: $F_{(3,56)} = 3.909$, $p = 0.01$; no change across days: $F_{(21,1148)} = 1.47$, $p = 0.08$; no sex difference: $F_{(1,56)} = 0.41$, $p = 0.52$). These findings are consistent with the earlier published results [31], showing that escalation of oxycodone self-administration behavior did not differ between male and female rats under LgA conditions.

To assess whether our LgA oxycodone self-administration protocol produced similar levels of dependence in male and female rats, we measured spontaneous somatic withdrawal 1 day after the last LgA oxycodone SA session in female (n = 7 saline, 7 oxycodone) and male (n = 5 saline, 10 oxycodone) rats. Since opioid dependence is characterized by somatic withdrawal signs upon withdrawal, we predicted an increase in somatic withdrawal signs in both sexes. Indeed, we found a significant increase in expression of somatic withdrawal behaviors

(combined into a Total Withdrawal Score) in female and male rats that had self-administered oxycodone compared to saline **(Fig. 1E**: Two-way ANOVA; main effect of treatment: $F_{(1, 25)}$ = 12.68, p = 0.002). There were no sex differences in the somatic withdrawal score (p = 0.67), consistent with the similar level of escalation of oxycodone self-administration. This is consistent with previously published studies that have shown similar increases in somatic withdrawal symptoms after 24-hours between male and female rats [32].

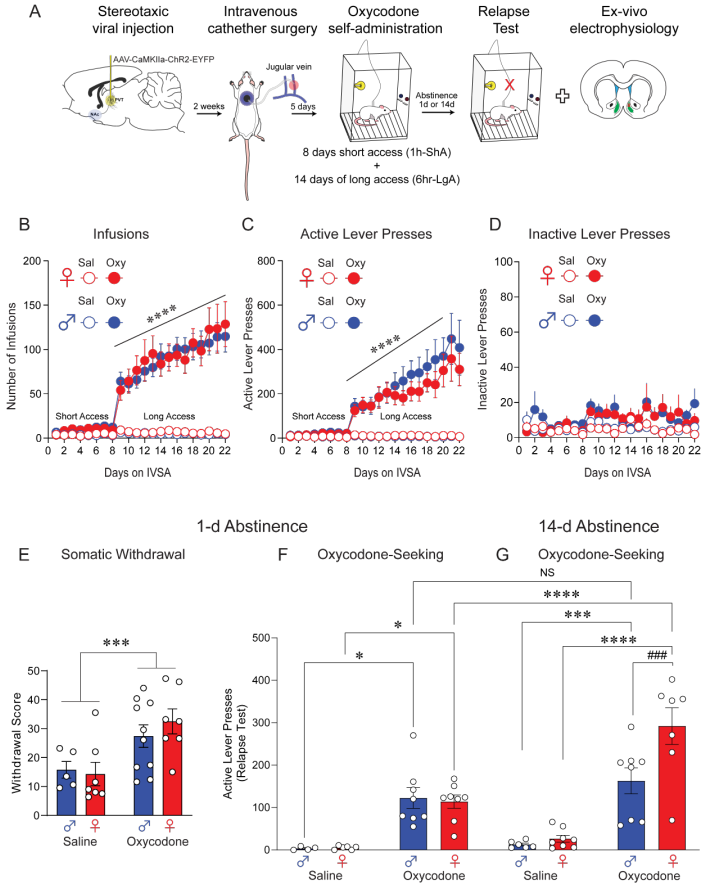


Figure 1. Oxycodone self-administration and oxycodone-seeking after short and prolonged abstinence. A: Experimental Timeline. PVT intracranial injections of AAV5-CAMKIIα-ChR2-EYFP, followed by intravenous catheter surgery and 22 days of oxycodone self-administration. Rats then received short (24 hours) or long (14 days) abstinence from oxycodone self-administration and tested for cue-induced oxycodone relapse test. Rats were euthanized, and brain slices were prepared for ex-vivo electrophysiological recordings. **B**: Number of infusions across short and long-access oxycodone self-administration in male and female rats. Mixed-effects model: main effect of days (increased infusions over time): p> 0.0001, main of treatment: p < 0.0001, no effect of sex: p = 0.84. **C**: Active lever presses across short- and long-access oxycodone self-administration. Mixed-effects model: main effect of days (increased infusions over time): p> 0.0001, main of treatment: p < 0.0001, no effect of sex: p = 0.53. **D**: Inactive lever presses across short- and long-access oxycodone self-administration. No significant differences between males and females or treatments were observed. **E**: Somatic withdrawal signs increased in both males and females. Two-way ANOVA; main effect of treatment: ***p = 0.002. **F**: Oxycodone-seeking (relapse test) after acute abstinence. Similar oxycodone-seeking behaviors between males and females but increased oxycodone-seeking compared to saline after acute abstinence in males and females. Two-way ANOVA; Sidak's multiple comparisons females saline vs. oxycodone *p = 0.01; males saline vs. oxycodone *p = 0.02. **G**: Oxycodone-seeking after prolonged abstinence. Both males and females exhibited increased drug-seeking compared to saline. Two-way ANOVA: main effect of drug: F(3,47) = 36.3 *** = p < 0.0001. Females exhibited significantly higher oxycodone-seeking behaviors compared to males: Sidak's multiple comparisons ### p = 0.006. Data is shown as mean ± SEM.

Acute abstinence (1-day) from oxycodone self-administration increased cue-induced oxycodone-seeking in female and male rats

We also assessed cue-induced drug-seeking after acute abstinence from LgA oxycodone SA. Female (n = 7 saline, 8 oxy) and male rats (n = 4 saline, 9 oxy) remained in their home cages without any access to oxycodone (forced abstinence) for 1-day. After 1-day of abstinence, rats returned to the operant box where only the house-cue, lever-cue and lever were available for 2 hrs. No infusions were given. We measured the number of active lever presses during cue presentation without drug delivery. We found that 1-day of abstinence increased cue-induced oxycodone seeking in males and females compared to the saline counterpart (**Fig. 1F**: Two-way ANOVA; significant sex x treatment interaction: $F_{(3,47)} = 3.9$, $p = 0.02$; no effect of sex: $F_{(1,47)} = 3.9$, $p = 0.06$; main effect of drug: $F_{(3,47)} = 36.3$, $p < 0.0001$). Sidak's multiple comparison: female saline group vs. oxycodone group, $p = 0.01$; male saline group vs. oxycodone group, $p = 0.02$.

Cue-induced drug-seeking after prolonged abstinence (14-days) exhibited sex-specific differences

Cue-induced drug-seeking was assessed after 14-days of abstinence from LgA oxycodone SA. Female (n = 8 saline, 8 oxycodone) and male (n = 6 saline, 8 oxycodone) rats underwent 14-days of forced abstinence in their home cages. On the 14th day of abstinence, rats were returned to the operant box where only the house-cue, lever-cue and lever were available for 2 hrs. No infusions were given. We found that both male and female rats on oxycodone pressed the active lever more frequently than rats in the saline control group (**Fig. 1G**: Two-way ANOVA: main effect of drug: $F_{(3,47)} = 36.3$, $p < 0.0001$. Additionally, females made significantly more active lever presses than males (**Fig. 1G**: Two-way ANOVA: significant drug x sex interaction: $F_{(3,47)} = 3.9$, $p = 0.02$; Sidak's multiple comparisons test female oxycodone greater than male oxycodone, $p = 0.006$). In addition, when comparing oxycodone-seeking in males and females during acute or prolonged abstinence we found that females but not males show an increase in seeking over time (**Fig. 1F-G**: Two-way ANOVA: significant day x sex interaction: $F_{(3, 47)} = 3.9$, $p = 0.02$. Sidak's multiple comparisons: oxycodone male acute vs. prolonged: $p = 0.9$; oxycodone female acute vs. prolonged: $p < 0.0001$.

These findings suggest that prolonged forced abstinence increased oxycodone-seeking, in both males and females, with a stronger effect in females than males. Moreover, females exhibit greater craving over time compared to males.

Electrophysiological characterization of PVT-NAcSh projections

To test whether PVT-arising inputs to NAcSh contribute to cue-induced relapse and drug-seeking, we targeted PVT-NAcSh projections optogenetically by expressing ChR2 in the PVT via stereotaxic injections of AAV5-CaMKIIα-ChR2-eYFP viral construct in rats from all experimental groups. 7-8 weeks after viral transfection, eYFP-tagged ChR2 was expressed at the injection site (PVT) and ChR2-eYFP-expressing PVT-arising projections were found in the NAcSh (**Fig. 2A**). Using whole-cell patch clamp recordings (**Fig. 2B**), we confirmed that PVT

projections form functional synapses on the NAcSh neurons (**Fig. 2C, D**). Photostimulation-induced (470 nm. 5 ms-long pulses of blue light) excitatory postsynaptic currents (EPSCs) were recorded under voltage clamp recording conditions at a holding potential of –70 mV. EPSCs at the PVT-NAcSh synapses are glutamatergic, as demonstrated by their sensitivity to NBQX (10μM), an AMPA/Kainate receptor antagonist, and D-AP5, an NMDA receptor antagonist (**Fig. 2D, E**). Glutamatergic EPSCs in PVT-NAcSh projections were monosynaptic in nature as they were rescued by the potassium channel blocker, 4-AP (1 mM) after they were blocked by tetrodotoxin (TTX, 1 μM), a sodium channel blocker (**Fig. 2G, H**) [33].

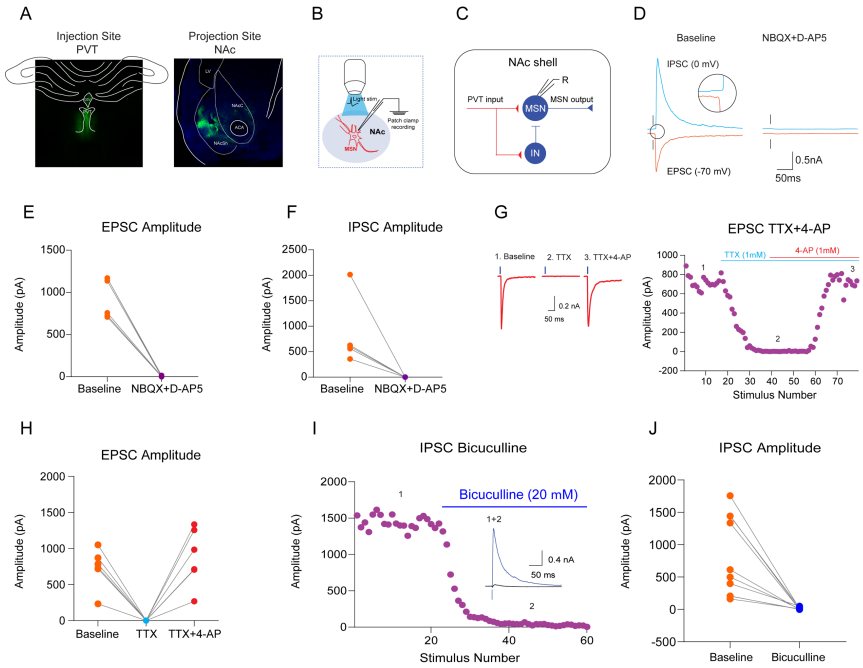


Figure 2. **Synaptic Properties in PVT-to-NAcSh Projections. A**: Representative images showing expression of ChR2-EYFP at the injection site in PVT and ChR2-EYFP-expressing projecting fibers in NAcSh. **B**: Schematic of optical stimulation of PVT terminals in NAcSh and recording of optically-evoked EPSCs in MSNs. **C**: Schematic representation of the local circuit within NAcSh with PVT afferents forming monosynaptic glutamatergic contacts on both MSNs and GABAergic interneurons, resulting in of feedforward inhibitory responses in MSNs. **D:** Photostimulation-induced (10.5 mW/mm^2; 5 ms) EPSC (orange) and IPSC (cyan) recorded from NAcSh MSN at holding potentials of -70 mV or 0 mV, respectively. Recordings were performed under control conditions first (left; baseline) and 10 min after NBQX (10 μM) and D-APV (50 μM; right) were added to the bath solution. The inset shows a delayed onset (synaptic latency) of the IPSC recorded at 0 mV. **E-F:** Summary plot of the amplitude of EPSC (panel E) and IPSC (panel F) recorded in MSNs under control conditions (baseline) and after bath application of NBQX and D-APV. The symbols represent individual experiments. **G:** Rescue of light-induced and TTX-blocked EPSCs at PVT-NAcSh projections by 4-AP. Left, an example of recordings shows EPSC (average of 10 traces) recorded at -70 mV under control conditions (baseline; 1), the EPSC was blocked by TTX (1 μM, 2), and application of 4-AP (1 mM) in the continuing presence of TTX restored the EPSC (3), thus confirming the monosynaptic nature of the PVT-NAcSh projections. Right, the time course of the EPSC amplitude changes. **H:** Summary plot of the experiments showing the EPSC amplitudes in NAcSh MSNs under three conditions (baseline, TTX, and TTX + 4-AP. **I:** Feed-forward IPSCs in NAcSh MSNs, recorded at 0 mV, were blocked by the $GABA_A$ receptor antagonist bicuculline (20 μM). **J:** IPSC amplitudes recorded from NAcSh under control conditions (baseline) and after application of bicuculline.

Stimulation of glutamatergic PVT projections resulted in activation of GABAergic interneurons in NAcSh **(Fig. 2C)**, triggering feed-forward inhibitory postsynaptic currents (IPSCs) in recorded MSNs **(Fig. 2D).** The IPSCs, recorded at a holding potential of 0 mV, were GABAergic as they were blocked by the $GABA_A$ receptor antagonist, bicuculline (20 μM) (**Fig. 2I, J**), and they were disynaptic in nature (**Fig. 2C**) as they were sensitive to glutamate receptors antagonists NBQX and D-AP5 (**Fig. 2F**). Based on these findings, we conclude that PVT sends monosynaptic projections to NAcSh, forming glutamatergic synapses on MSNs, as well as triggering feedforward inhibition in the PVT-NAcSh circuits.

Synaptic strength in PVT-NAcSh projection is increased after prolonged but not acute abstinence in both male and female rats

To examine the effect of acute abstinence from oxycodone self-administration on synaptic transmission in the PVT-NAcSh projections, we performed whole-cell patch-clamp recordings of light-induced EPSCs in medium spiny neurons in NAcSh in slices from rats which self-administered oxycodone at both abstinence time points. To assay the effects of acute abstinence on the efficacy of excitatory synaptic transmission, we obtained input-output curves for the light-induced EPSCs in all experimental groups (male saline group: 5 rats, 18 cells; male oxycodone group: 8 rats, 36 cells; female saline group: 7 rats; 26 cells; female oxycodone group: 6 rats, 20 cells) by recoding the EPSCs at a holding potential of –80 mV evoked by photostimuli of increasing intensity (**Fig. 3A**). Although the EPSC amplitude increased with light density (**Fig. 3A**: Three-way ANOVA: main effect of light intensity $F_{(5,582)} = 52.69$, $p < 0.0001$), we did not find any treatment effect on synaptic efficacy in the PVT-NAcSh pathway (**Fig. 3A**: Three-way ANOVA: no effect of treatment: $F_{(1,582)} = 1.5$, $p = 0.2$) or sex specificity (**Fig. 3A**: Three-way ANOVA: no effect of sex: $F_{(1,582)} = 0.5$, $p = 0.5$). This finding indicates that 1-day abstinence from oxycodone self-administration did not affect the efficacy of glutamatergic synaptic transmission in PVT-NAcSh projections in both male and female rats.

Consistent with the lack of changes in synaptic strength, the magnitude of paired-pulse ratio (PPR), and index of presynaptic function[33] of EPSC amplitude at the PVT-NAcSh synapses did not differ between the treatment groups (male saline: 5 rats, 28 cells; male oxycodone: 10 rats, 25 cells; female saline: 7 rats, 30 cells; female oxycodone: 6 rats, 22 cells) (**Fig. 3B**: Three-way ANOVA: $F_{(1,443)} = 1.19$, $p = 0.3$) or sex specificity (**Fig. 3B**: Three-way ANOVA: $F_{(1,443)} = 2.20$, $p = 0.1$), indicating that the probability of neurotransmitter release at the PVT-NAcSh synapses was unaffected by acute abstinence.

In analogously designed experiments, we assayed the effects of prolonged abstinence on synaptic transmission in PVT-NAcSh projections. As above, input-output curves for PVT-NAcSh EPSCs were obtained by recording photostimulation-induced EPSCs in MSNs at a holding potential of –80 mV **(Fig. 3C)** (male saline group: 5 rats, 16 cells; male oxycodone group: 7 rats, 20 cells; female saline group: 7 rats; 21 cells; female oxycodone group: 5 rats, 21 cells). We found that the EPSC amplitude increased with light intensity (**Fig. 3C**: Three-way ANOVA: main effect of light intensity; $F_{(5,442)} = 40.5$, $p < 0.0001$), and, unlike 1-day abstinence, long abstinence from oxycodone self-administration was associated with increases in the EPSC amplitude in both male and female rats compared to control group (**Fig. 3C**: Three-Way

ANOVA: main effect of treatment; $F_{(1,442)} = 18.3$, $p < 0.0001$). The effects of prolonged abstinence on synaptic strength in the PVT-NAcSh pathway was similar between males and females.

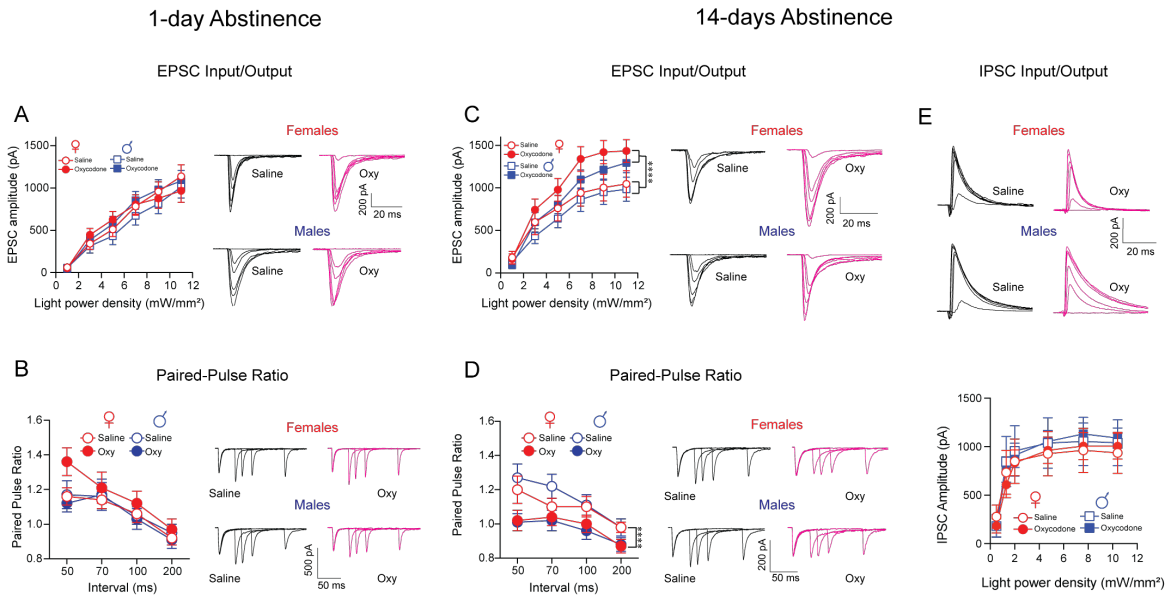


Figure 3. Prolonged abstinence from oxycodone self-administration is associated with increased synaptic strength in glutamatergic PVT projections to MSNs in NAcSh in male and female rats. A: Right, 1-day of abstinence from oxycodone self-administration (acute abstinence) had no effect on the efficacy of glutamatergic synaptic transmission in PVT projections to MSNs in NAcSh, as assessed with synaptic input-output curves for light-induced EPSCs which were triggered by the pulses of blue light of increasing intensity. There was no significant difference between saline (n = male: 5 rats, 18 cells; females: 7 rats, 26 cells) and oxycodone (n = male: 8 rats, 36 cells; females: 6 rats, 20 cells) groups. Three-way ANOVA no effect of treatment: p = 0.2, no effect of sex: p = 0.5. **Right**, example traces of EPSCs triggered by light pulses of increasing intensity from different experimental groups. **B: Left**, the magnitude of the paired-pulse ratio remained unchanged in the oxycodone (n = males: 10 rats, 25 cells; females: 6 rats, 22 cells) compared to saline control (n = males: 5 rats, 28 cells; females: 7 rats, 30 cells) groups, indicating that the probability of glutamate release was not affected after acute abstinence from oxycodone self-administration. Three-way ANOVA no effect of drug, p = 0.3, no effect of sex, p = 0.1. **Right**, representative traces of EPSCs evoked by paired-pulses of blue light (10.5 mW/mm²) at different interpulse intervals (50, 70, 100 and 200 ms). **C: Left**, the efficacy of glutamatergic synaptic transmission in PVT projections to MSNs in NAcSh, assessed as in **A** with synaptic input-output curves, was enhanced in both male and female rats in the oxycodone group (n = males: 7 rats, 20 cells; females: 5 rats, 21 cells) compared to saline control group (n = males: 5 rats, 16 cells; females: 7 rats, 21 cells) after 14-days of abstinence from oxycodone self-administration (prolonged abstinence). Three-way ANOVA main effect of drug ****p < 0.0001. **Right**, example traces of EPSCs from different experimental groups. **D: Left**, the magnitude of the paired-pulse ratio decreases in the oxycodone groups (n = males: 7 rats, 33 cells; females: 6 rats, 22 cells) compared to saline control groups (n = males: 5 rats, 23 cells; females: 7 rats, 33 cells). Three-way ANOVA, main effect of drug **** p < 0.0001. **E: Top**, example traces of IPSCs from different experimental groups triggered by photostimuli of increasing intensity. The IPSCs were recorded at a holding potential of 0 mV. **Bottom**, the efficacy of feed-forward inhibition in the PVT-NAcSh pathway, as assessed with the input-output curves for light-induced IPSCs, was unaffected by prolonged oxycodone abstinence. There was no significant difference between saline (n = male: 5 rats, 11 cells; females: 7 rats, 14 cells) and oxycodone (n = male: 7 rats, 16 cells; females: 7 rats, 14 cells) groups. Mixed effects model: no effect of treatment: p = 0.9, no effect of sex: p = 0.3. Data is shown as mean ± SEM.

Notably, the magnitude of PPR at the studied synapses was found to be decreased after prolonged abstinence from oxycodone self-administration in both male and female rats (**Fig. 3D**: Three-Way ANOVA: main effect of treatment: $F_{(1,388)} = 25.8$, $p < 0.001$; male saline: 5 rats, 23 cells; male oxycodone: 7 rats, 33 cells; female saline: 7 rats, 23 cells; female oxycodone: 6 rats, 22 cells). Thus, the observed synaptic potentiation in PVT-NAcSh projections after longer abstinence periods was at least in part due to increased glutamate release probability [34].

Remarkably, the efficiency of feed-forward inhibition in the studied pathway (Fig. 2) was unaffected in both male and female rats following prolonged abstinence from oxycodone self-administration as we did not observe differences in synaptic input-output curves for light-induced IPSCs between the experimental groups (**Fig. 3E**). As excitatory synaptic transmission was strengthened in PVT-NAcSh projections (**Fig. 3C**), the lack of changes in PVT-driven GABAergic inputs to MSNs indicates that the balance between excitation and inhibition in this pathway was shifted towards a greater functional efficiency of excitation, thus possibly facilitating the synaptically-triggered output of MSNs to other components of the relapse circuitry and contributing to the observed behavioral effects in our study.

Abstinence from oxycodone self-administration does not change AMPAR subunit composition or AMPAR/NMDAR EPSC amplitude ratio in glutamatergic PVT projections to MSNs in NAcSh

To explore whether changes in glutamatergic PVT projections to MSNs in NAcSh correlated with abstinence from oxycodone self-administration, we recorded light-induced AMPA receptor-mediated (AMPAR) EPSCs at the PVT-NAcSh synapses in slices from all experimental groups at holding potentials of −70 mV, 0 mV or +40 mV. In these experiments, we included endogenous polyamine spermine (200 μM), in the internal recording solution. We then calculated the rectification index for AMPAR EPSCs at the studied PVT-NAcSh projections[35] by dividing the peak amplitude of AMPAR EPSC at +40 mV by the EPSC amplitude at -70 mV. The changes in rectification index associated with experimental interventions would indicate that the AMPAR subunit composition was modified, as the GluR1 subunit trafficking to synapses was shown to affect the rectification index[35]. However, after acute abstinence, we did not observe significant changes in the rectification index (male saline: 5 rats, 23 cells; male oxycodone: 8 rats, 28 cells; female saline: 7 rats, 14 cells; female oxycodone: 6 rats, 18 cells) by sex (**Fig. 4A**, inset bar graph: Two-way ANOVA: $F_{(1,68)} = 0.83$, $p = 0.4$) or treatment (**Fig.4A**, inset bar graphs: Two-way ANOVA: $F_{(1,68)} = 0.35$, $p = 0.6$).

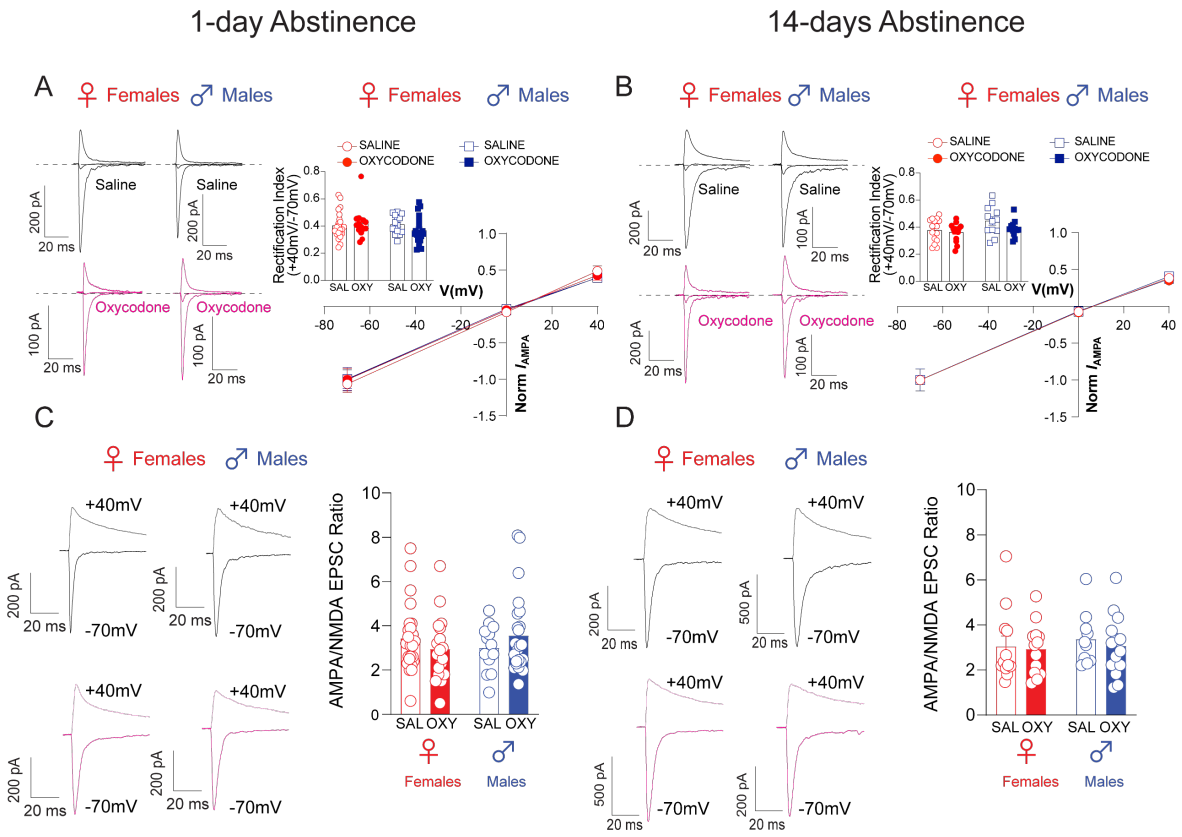


Figure 4. Abstinence from oxycodone self-administration does not change AMPAR subunit composition or AMPAR/NMDAR EPSC amplitude ratio in glutamatergic PVT projections to MSNs in NAcSh. A-B: Rectification index and current-voltage relationship for AMPAR EPSCs for short (A; 1-day) and long (B; 14-days) abstinence periods. **A: Left**, representative traces of AMPAR EPSCs recorded at holding potentials of -70, 0 and +40 mV during acute abstinence. **Right**, current/voltage relationship of AMPAR EPSCs recorded at holding potentials of -70, 0 and +40 mV of saline (n = males: 9 rats, 23 cells; females: 7 rats, 14 cells) vs. oxycodone (n = males: 13 rats, 28 cells; females: 4 rats, 10 cells) rats. **Inset:** Rectification index for EPSCs (calculated as the ratio of peak EPSC amplitudes at +40/-70 mV [$EPSC_{+40}/EPSC_{-70}$]). **B: Left**, representative traces of AMPAR EPSCs recorded at holding potentials of -70, 0 and +40 mV during prolonged abstinence. **Right**, current/voltage relationship of AMPAR EPSCs recorded at holding potentials of -70, 0 and +40mV of saline (n = males: 4 rats, 14 cells; females: 5 rats, 15 cells) vs. oxycodone (n = males: 5 rats, 13 cells; females: 4 rats, 14 cells) rats. The recordings were performed in the presence of the NMDA receptor antagonist D-APV (50 μM) in the external medium and spermine (200 μM) in the pipette solution. There were no significant differences between groups. Data is shown as mean ± SEM. Comparisons were made using two-way ANOVAs $p > 0.05$. **C-D:** AMPA/NMDA ratios of AMPAR EPSCs for short (C) and long (D) abstinence periods. **C: Left**, representative traces of light-induced EPSCs in projections from PVT to MSNs in NAcSh at +40mV (light-colored traces) and -70mV (dark-colored traces) in slices from male and female rats during acute abstinence. **Right**, AMPAR/NMDAR EPSC amplitude ratios for saline (n = males: 5 rats, 14 cells; females: 7 rats, 24 cells) and oxycodone (males: 8 rats, 24 cells; females: 6 rats, 18 cells) groups during acute abstinence. **D: Left**, representative traces of light-induced EPSCs in projections from PVT to MSNs in NAcSh at +40mV (light-colored traces) and -70mV (dark-colored traces) in slices from male and female rats during prolonged abstinence. **Right**, AMPAR/NMDAR EPSC amplitude ratios for saline (n = males: 5 rats, 12 cells; females: 5 rats, 15 cells) and oxycodone (males: 5 rats, 12 cells; females: 5 rats, 15 cells) groups. Data is shown as mean ± SEM. Comparisons were made using two-way ANOVA.

Similarly, after prolonged abstinence we did not observe significant changes in the rectification index (male saline: 4 rats, 15 cells; male oxycodone: 5 rats, 23 cells; female saline: 5 rats, 22 cells; female oxycodone: 5 rats, 15 cells) by treatment (**Fig. 4B** (inset bar graph): Two-way ANOVA: $F_{(1,50)} = 2.7$, $p = 0.1$). These results suggest that neither acute nor prolonged abstinence from oxycodone self-administration was associated with changes in the AMPAR subunit composition at synapses formed by PVT projecting fibers on MSNs in NAcSh**.** Thus, synaptic potentiation in PVT-NAcSh projections, observed after 14-days abstinence (see **Fig. 3C**), was unlikely due to increased GluR1 trafficking at studied synapses.

Consistent with the latter notion, the AMPAR/NMDAR EPSC amplitude ratio was not affected by acute abstinence (**Fig. 4C**: Two-way ANOVA: no effect of sex: $F_{(1,76)} = 0.06$, $p = 0.8$; no effect of treatment: $F_{(1,76)} = 0.008$, $p = 0.9$; n = male saline: 5 rats, 14 cells; male oxycodone: 8 rats, 24 cells; female saline: 7 rats, 24 cells; female oxycodone: 6 rats, 18 cells). The AMPAR/NMDAR EPSC ratio was assessed by measuring the amplitude of the NMDAR-mediated EPSC at a holding potential of +40mV, 40ms after the peak of AMPAR-mediated EPSC at -70mV. Similarly, the AMPAR/NMDAR EPSC amplitude ratio was also unaffected after prolonged (14-days) abstinence (**Fig. 4D**: Two-way ANOVA: no effect of sex: $F_{(1,45)} = 0.4$, $p = 0.5$; no effect of treatment: $F_{(1,45)} = 0.3$, $p = 0.6$; (n = male saline: 5 rats, 12 cells; male oxycodone: 5 rats, 12 cells; female saline: 5 rats, 15 cells; female oxycodone: 5 rats, 15 cells), suggesting that acute or prolonged abstinence from oxycodone self-administration might have no detectable postsynaptic effects in PVT-NAcSh projections.

Prolonged but not acute abstinence from oxycodone self-administration increased NAcSh MSN intrinsic excitability in both male and female rats

To determine whether abstinence from oxycodone self-administration affects MSN intrinsic excitability in the NAcSh independently of the PVT to NAcSh circuit effects, we performed current-clamp recordings from NAcSh MSNs in slices from saline- (males: 7 rats, 16 cells; females: 4 rats, 7 cells) and oxycodone-treated (males: 6 rats, 18 cells; females: 8 rats, 15 cells) rats after acute abstinence (**Fig. 5A; C-E**) or saline- (males: 3 rats, 8 cells; females: 6 rats, 14 cells) and oxycodone-treated (males: 4 rats, 14 cells; females: 6 rats, 15 cells) rats after prolonged abstinence (**Fig. 5B; F-H**). We did not observe any sex differences in the oxycodone-treated rats after acute (**Fig. 5C**: Two-way ANOVA: $F_{(1,302)} = 0.74$, $p = 0.4$) or prolonged abstinence (**Fig. 5F**: Two-way ANOVA: $F_{(1,261)} = 2.2$, $p = 0.14$).

Notably, acute abstinence from oxycodone self-administration does not affect intrinsic excitability in males (**Fig. 5D**: Two-way ANOVA: $F_{(1,320)} = 0.2$, $p = 0.7$) or females (**Fig. 5E**: Two-way ANOVA: $F_{(1,192)} = 2.8$, $p = 0.1$). However, prolonged abstinence increases excitability in both male (**Fig. 5G**: Two-way ANOVA: $F_{(1,191)} = 55.1$, $p < 0.0001$) and female (**Fig. 5H**: Two-way ANOVA: $F_{(1,265)} = 4.8$, $p = 0.03$) rats. Thus, acting in concert with synaptic enhancements in the PVT to NAcSh projections (shown in **Fig. 3**), the observed increases in MSN's excitability could facilitate the signal flow in PVT-NAcSh circuits after prolonged abstinence, possibly contributing to the enhanced drug-seeking.

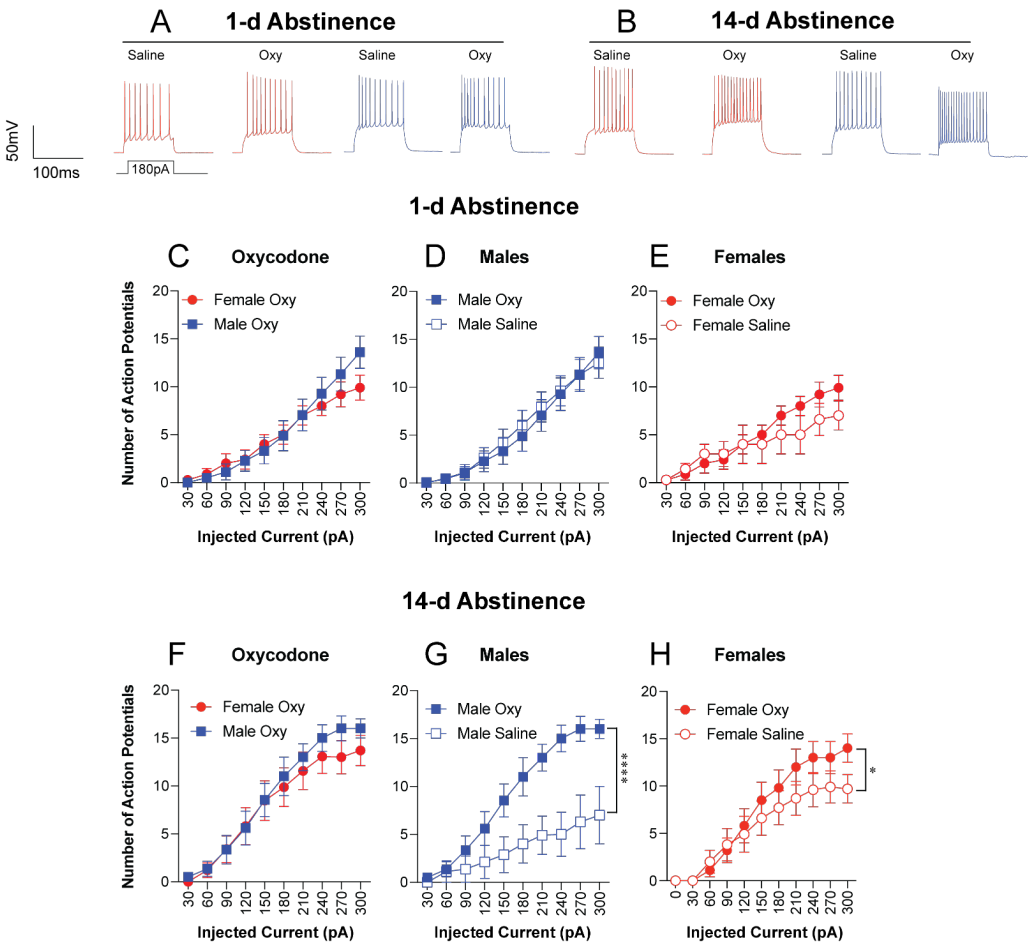


Figure 5. Prolonged abstinence from oxycodone self-administration increases NAcSh MSN intrinsic excitability in both male and female rats. A: Example traces of current-clamp recordings from NAcSh MSNs from saline- (n = males: 7 rats, 16 cells; females: 4 rats, 7 cells) and oxycodone-treated (n = males: 6 rats, 18 cells; females: 8 rats, 15 cells) rats after 1 day of abstinence. **B:** Example traces of current-clamp recordings from NAcSh MSNs from saline- (n = males: 3 rats, 8 cells; females: 6 rats, 14 cells) and oxycodone-treated (n = males: 4 rats, 14 cells; females: 6 rats, 15 cells) rats after 14 days of abstinence. **C:** Number of action potentials fired at increasing current injections (increments of 30pA) in male and female rats after 1 day of abstinence from oxycodone self-administration. No significant difference in excitability between males and females after 1 day of abstinence from oxycodone self-administration. Two-way ANOVA p = 0.39. **D:** Comparison of number of action potentials fired at increasing current injections between saline-treated vs. oxycodone-treated males after 1 day of abstinence from self-administration. No significant difference in excitability between males that self-administered saline vs. oxycodone after 1 day of abstinence from self-administration. Two-way ANOVA p = 0.67 **E:** Comparison of number of action potentials fired at increasing current injections between saline-treated vs. oxycodone-treated females after 1 day of abstinence from self-administration. No significant difference in excitability between females that self-administered saline vs. oxycodone after 1 day of abstinence from self-administration. Data shown as mean ± SEM. Two-way ANOVA p = 0.09. **F:** Number of action potentials fired at increasing current injections in male and female rats after 14 days of abstinence from oxycodone self-administration. No significant difference in excitability between males and females after 14 days of abstinence from oxycodone self-administration. Two-way ANOVA p = 0.14. **G:** Comparison of number of action potentials fired at increasing current injections between saline-treated vs. oxycodone-treated males after 14 days of abstinence from self-administration. Oxycodone-treated males have greater MSN excitability after 14 days of abstinence from oxycodone self-administration compared to saline males. Two-way ANOVA ****p < 0.0001. **H:** Comparison of number of action potentials fired at increasing current injections between saline-treated vs. oxycodone-treated females after 14 days of abstinence from self-administration. Oxycodone-treated females have greater MSN excitability after 14 days of abstinence from oxycodone self-administration compared to saline females. Two-way ANOVA *p < 0.05. Data shown as mean ± SEM.

Discussion

In this study, we investigated sex differences in withdrawal symptoms, cue-induced oxycodone-seeking (relapse), and changes in PVT-NAcSh synaptic transmission following short and long periods of abstinence from oxycodone self-administration. Our results indicate that acute abstinence increased withdrawal symptoms and cue-induced oxycodone-seeking without affecting PVT-NAcSh glutamatergic transmission, and no significant sex differences were observed. In contrast, prolonged abstinence increased cue-induced oxycodone-seeking and the efficacy of synaptic transmission in PVT-NAcSh projections in both male and female rats, with a stronger effect on cue-induced oxycodone-seeking in females.

Oxycodone self-administration is similar between male and female rats

Escalation of drug taking in rats is defined as an increase in the amount and frequency of drug intake over time during self-administration protocols. It is used as a model of addiction, specifically to study tolerance to drugs taken and dependence on them. Our findings revealed that escalation of intravenous oxycodone self-administration under long access conditions was similar between female and male rats, indicating that sex does not significantly affect the development of oxycodone addiction using this protocol of self-administration. This is consistent with our previous observations when long-access self-administration was used[31,36]. However, it contrasts with other studies that have found that female rats orally self-administered more oxycodone than males in short access (1-hr) FR-1 schedule self-administration paradigm[37], whereas other studies from our lab have shown that males intravenously self-administered more oxycodone compared to females in the first three trials of an FR-1 schedule of reinforcement[24]. These results suggest that specific behavioral measurements and routes of administration may reveal differences in the development and maintenance of oxycodone use in male and female subjects.

Increased spontaneous withdrawal symptoms after acute abstinence in males and females

Spontaneous withdrawal symptoms from opioids use refer to the symptoms that occur when an individual who has been taking opioids regularly suddenly stops using them or reduces their dose. These symptoms occur as a result of the body's physical dependence on the drug. Here we found that both male and female rats exhibited increased spontaneous withdrawal signs after acute abstinence from oxycodone self-administration. These findings are consistent with the results of previous experiments with non-contingent morphine administration, which showed a similar increase in withdrawal symptoms after 1-day of abstinence in both males and females, with males showing a stronger effect [25]. However, we did not find a significant difference in the magnitude of spontaneous withdrawal signs between sexes following oxycodone self-administration in rats, highlighting the importance of considering the drug's mechanism of action and administration routes when assessing its potential to affect males and females differently.

Sex differences in cue-induced oxycodone-seeking after prolonged abstinence

In rats, abstinence periods from drug use have been shown to lead to the development of drug-seeking behaviors, which can intensify over time and lead to relapse. It has been shown that the longer abstinence periods result in a greater intensity of drug-seeking[10,38,39]. During this period, there are changes in the brain that can lead to the development of drug cravings, including alterations in synaptic function and changes in the expression of neurotransmitters and their receptors[10,22,40–44].

In addition, a recent study[45] found that male rats that underwent a longer abstinence period (30 days) following oxycodone self-administration showed significantly higher levels of drug-seeking behavior compared to rats that underwent a shorter abstinence period (1-day). This suggests that the incubation of oxycodone craving occurs in a time-dependent manner, with longer abstinence periods leading to increased drug-seeking behavior. Here we found that females but not males have an increased in cue-induced oxycodone-seeking after prolonged abstinence (14-days) compared to acute (1-day) abstinence. Females also exhibited increased drug-seeking during the prolonged abstinence period compared to males. This suggests that cue-induced cravings for oxycodone may develop faster and they are stronger in females compared to males.

PVT-NAcSh synaptic strength increases after prolonged abstinence in both male and female rats

Furthermore, we investigated the effects of abstinence on glutamatergic synaptic transmission in PVT-NAcSh projections. Both the PVT and the NAc are highly heterogeneous nuclei with distinctive anatomical subregions (anterior/posterior PVT; core/shell NAc) that contain different types of cells (Type I and Type II in the PVT; dopamine receptor 1 (D1)- and dopamine receptor 2 (D2)-expressing medium spiny neurons (MSNs; D1-MSN or D2-MSN) in the NAc). Recent studies have highlighted the role of the PVT in modulating reward-seeking behaviors, particularly in response to drugs of abuse[46–50]. Several studies have shown that the PVT is involved in retrieving and consolidating drug-associated memories[51,52]. For example, one study found that optogenetic activation of PVT inputs to the NAc was sufficient to reinstate drug-seeking behavior in rats[15]. Another study showed that silencing PVT neurons during the retrieval of opiate-associated memories impaired the expression of drug-seeking behavior[51]. The PVT has also been implicated in the regulation of drug-seeking behavior more broadly. For example, transient inactivation of the posterior PVT can block cocaine-seeking behavior in rats[53], which seems to be dependent on the type of reinstatement model and individual differences[54]. Additionally, the PVT has been shown to play a key role in cue-induced drug-seeking after abstinence[13], suggesting that it serves an important function in the neural circuitry underlying relapse.

Here, we found that acute abstinence from oxycodone self-administration did not appear to affect neurotransmission in the PVT-NAcSh projections, as assessed by the EPSC amplitude input-output curves and paired-pulse ratio. Furthermore, the rectification index for the AMPAR-

mediated EPSCs and AMPAR/NMDAR EPSC amplitude ratio at the PVT-NAcSh synapses were not significantly different between treatment groups or sexes, indicating that subunit composition of postsynaptic AMPA receptors or their sensitivity to glutamate were not affected by acute abstinence from oxycodone self-administration. These results suggest that a brief period of abstinence may not be sufficient to induce significant changes in synaptic properties of the PVT-NAcSh circuit.

In contrast, prolonged abstinence from oxycodone self-administration resulted in significant changes in glutamatergic synaptic transmission in the PVT-NAcSh circuits, as measured by EPSC input-output curves and paired-pulse ratio. Specifically, prolonged abstinence resulted in an increase in EPSC amplitude and a decrease in the paired-pulse ratio, suggesting that there is an increase in the probability of neurotransmitter release and/or alterations in presynaptic release mechanisms in the studied pathway. However, prolonged abstinence did not change the rectification index of the EPSCs or the AMPAR/NMDAR EPSC amplitude ratio, indicating that there is no change in the subunit composition of AMPA receptors at the PVT-NAcSh glutamatergic synapses. These results suggest that long-term abstinence from oxycodone self-administration is associated with significant changes in synaptic properties of the PVT-NAcSh circuit—presynaptically-expressed synaptic potentiation, specifically—potentially contributing to the persistent changes in reward processing and relapse vulnerability that are often observed in individuals with opioid use disorder.

NAcSh MSN intrinsic excitability increases after prolonged abstinence in male and female rats

The NAc plays an important role in regulating motivated behaviors such as reward-seeking and cravings[55,56]. Within the NAc, MSN intrinsic excitability regulates NAc responses to afferent inputs and controls the NAc outputs to other components of reward system in the brain. In our study, we investigated changes in NAcSh MSN intrinsic excitability following acute or prolonged abstinence from oxycodone self-administration.

Our findings suggest that while acute abstinence does not significantly alter NAcSh MSN intrinsic excitability, prolonged abstinence heightened excitability in both males and females. This is consistent with our PVT-NAcSh increase in synaptic strength data. One goal of this study was to look at the interplay between PVT-NAcSh glutamatergic transmission and NAcSh MSN changes in intrinsic excitability after acute or prolonged abstinence. While the standing theory is that MSN excitability decreases to compensate for an increase in glutamatergic input (in males[22,41,57], our results suggest that prolonged abstinence may lead to compensatory neuroadaptations that enhance intrinsic excitability, potentially acting in concert with the increases in synaptic strength observed at inputs from the PVT, thus potentially facilitating the signal flow in the PVT-NAcSh circuits and modifying the output of MSNs to the downstream targets, thus contributing to relapse mechanisms.

Implications of anatomical and functional distinctions in PVT-NAcSh circuits for relapse.

Our study was designed to target posterior PVT-NAcSh projections, based on previous studies[13,16,58]. The anterior/posterior PVT have been shown to have different subcortical targets[59] and increasing evidence demonstrates substantial functional differences in anterior vs posterior PVT outputs[60]. Posterior projections to the NAc are selectively tuned to aversive stimuli, while the anterior PVT is more involved in reward-seeking behaviors[61]. Furthermore, our goal was to determine the overall effects of acute versus prolonged abstinence on synaptic and neuronal properties in PVT-NAcSh circuits in male and female rats, as opposed to differentiating between the contributions of D1 and D2 receptor-expressing MSNs. However, recent studies demonstrated differences in the roles of PVT-to-D1 and PVT-to-D2 MSN projections in mediating drug-seeking or withdrawal after either acute or prolonged abstinence from opioid self-administration [13,58]. Thus, it would it be interesting to explore the effects of our oxycodone administration regiments on neurotransmission in PVT projections to D1- or D2-MSNs selectively in future studies, performing *ex vivo* optogenetic studies analogous to those described in the present work.

In addition, stimulation of PVT terminals in the NAcSh can increase opioid-seeking before but not after extinction of drug availability[13]. Given the complex and heterogeneous functions of PVT-NAc projections and their targets, conclusions about the role of these circuits cannot be generalized across studies that differ in anatomical-, projection-, cell type-, or molecular-specific targeting of the PVT-NAc pathway. Together, these studies suggest that the anatomical distinction of the PVT and its inputs to the NAcSh D1 or D2 MSNs play a key role in mediating behaviors that lead to relapse during acute abstinence (primarily through negative affective states driven by the D2 pathway) or prolonged abstinence (through incubation of craving driven by the D1 pathway).

Overall, our findings provide insights into the neural mechanisms underlying oxycodone relapse and indicate potential sex-specific differences in the long-term effects of abstinence on cue-induced oxycodone-seeking behaviors. Future studies may focus on differentiating the roles of anterior and posterior PVT innervation to D1 and D2 MSNs in the NAcSh and how manipulating these specific projections influences relapse behaviors based on abstinence duration and type of relapse (drug-, cue-, or stress-induced). Moreover, the differential contributions and interactions between cortical and thalamic projections into ventral striatal regions in abstinence and relapse remain an open direction of investigation.

References

1. Feltenstein, M. W., See, R. E. & Fuchs, R. A. Neural Substrates and Circuits of Drug Addiction. *Cold Spring Harb. Perspect. Med.* **11**, a039628 (2021).
2. Strang, J. *et al.* Opioid use disorder. *Nat. Rev. Dis. Primer* **6**, 1–28 (2020).
3. Golden, S. A., Jin, M. & Shaham, Y. Animal Models of (or for) Aggression Reward, Addiction, and Relapse: Behavior and Circuits. *J. Neurosci. Off. J. Soc. Neurosci.* **39**, 3996–4008 (2019).
4. Koob, G. F. & Volkow, N. D. Neurocircuitry of addiction. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* **35**, 217–38 (2010).
5. Self, D. W. Neural substrates of drug craving and relapse in drug addiction. *Ann. Med.* **30**, 379–389 (1998).
6. Self, D. W. & Nestler, E. J. Relapse to drug-seeking: neural and molecular mechanisms. *Drug Alcohol Depend.* **51**, 49–60 (1998).
7. Venniro, M., Caprioli, D. & Shaham, Y. Animal models of drug relapse and craving: From drug priming-induced reinstatement to incubation of craving after voluntary abstinence. in *Progress in Brain Research* vol. 224 25–52 (2016).
8. Grimm, J. W., Hope, B. T., Wise, R. A. & Shaham, Y. Incubation of cocaine craving after withdrawal. *Nature* **412**, 141–142 (2001).
9. Lu, L., Grimm, J. W., Dempsey, J. & Shaham, Y. Cocaine seeking over extended withdrawal periods in rats: Different time courses of responding induced by cocaine cues versus cocaine priming over the first 6 months. *Psychopharmacology (Berl.)* **176**, 101–108 (2004).
10. Pickens, C. L. *et al.* Neurobiology of the incubation of drug craving. *Trends Neurosci.* **34**, 411–420 (2011).

11. Reiner, D. J., Fredriksson, I., Lofaro, O. M., Bossert, J. M. & Shaham, Y. Relapse to opioid seeking in rat models: behavior, pharmacology and circuits. *Neuropsychopharmacology* **44**, 465–477 (2019).
12. Venniro, M. *et al.* Factors modulating the incubation of drug and non-drug craving and their clinical implications. *Neurosci. Biobehav. Rev.* **131**, 847–864 (2021).
13. Giannotti, G. *et al.* Extinction blunts paraventricular thalamic contributions to heroin relapse. *Cell Rep.* **36**, 109605 (2021).
14. Peters, J., Kalivas, P. W. & Quirk, G. J. Extinction circuits for fear and addiction overlap in prefrontal cortex. *Learn. Mem.* **16**, 279–288 (2009).
15. Zhu, Y., Wienecke, C. F. R., Nachtrab, G. & Chen, X. A thalamic input to the nucleus accumbens mediates opiate dependence. *Nature* **530**, 219–222 (2016).
16. Zhu, Y. *et al.* Dynamic salience processing in paraventricular thalamus gates associative learning. *Science* **362**, 423–429 (2018).
17. Floresco, S. B. The nucleus accumbens: An interface between cognition, emotion, and action. *Annu. Rev. Psychol.* **66**, 25–32 (2015).
18. Floresco, S. B., McLaughlin, R. J. & Haluk, D. M. Opposing roles for the nucleus accumbens core and shell in cue-induced reinstatement of food-seeking behavior. *Neuroscience* **154**, 877–84 (2008).
19. Floresco, S. B., Montes, D. R., Tse, M. M. T. & Holstein, M. van. Differential Contributions of Nucleus Accumbens Subregions to Cue-Guided Risk/Reward Decision Making and Implementation of Conditional Rules. *J. Neurosci.* **38**, 1901–1914 (2018).
20. Klawonn, A. M. & Malenka, R. C. Nucleus Accumbens Modulation in Reward and Aversion. *Cold Spring Harb. Symp. Quant. Biol.* **83**, 119–129 (2018).
21. Carlezon, W. A. & Thomas, M. J. Biological substrates of reward and aversion: a nucleus accumbens activity hypothesis. *Neuropharmacology* **56**, 122–132 (2009).

22. Hearing, M., Graziane, N., Dong, Y. & Thomas, M. J. Opioid and psychostimulant plasticity: targeting overlap in nucleus accumbens glutamate signaling. *Trends Pharmacol. Sci.* **39**, 276–294 (2018).
23. Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates: Hard Cover Edition*. (Elsevier, 2006).
24. Mavrikaki, M., Pravetoni, M., Page, S., Potter, D. & Chartoff, E. Oxycodone self-administration in male and female rats. *Psychopharmacology (Berl.)* **234**, 977–987 (2017).
25. Mavrikaki, M., Lintz, T., Constantino, N., Page, S. & Chartoff, E. Chronic opioid exposure differentially modulates oxycodone self-administration in male and female rats. *Addict. Biol.* **26**, e12973 (2021).
26. Thomsen, M. & Caine, S. B. Chronic Intravenous Drug Self-Administration in Rats and Mice. *Curr. Protoc. Neurosci.* **32**, (2005).
27. Chartoff, E. H., Mague, S. D., Barhight, M. F., Smith, A. M. & Carlezon, W. A. Behavioral and Molecular Effects of Dopamine D1 Receptor Stimulation during Naloxone-Precipitated Morphine Withdrawal. *J. Neurosci.* **26**, 6450–6457 (2006).
28. Chartoff, E. H., Barhight, M. F., Mague, S. D., Sawyer, A. M. & Carlezon, W. A. Anatomically dissociable effects of dopamine D1 receptor agonists on reward and relief of withdrawal in morphine-dependent rats. *Psychopharmacology (Berl.)* **204**, 227–239 (2009).
29. Li, Y. *et al.* Maternal and early postnatal immune activation produce dissociable effects on neurotransmission in mPFC–amygdala circuits. *J. Neurosci.* **38**, 3358–3372 (2018).
30. Nieto, A. M., Catalfio, A. M., Papacostas Quintanilla, H., Alonso-Caraballo, Y. & Ferrario, C. R. Transient effects of junk food on NAc core MSN excitability and glutamatergic transmission in obesity-prone female rats. *Obesity* **31**, 434–445 (2023).
31. Guha, S. *et al.* Ranking the contribution of behavioral measures comprising oxycodone self-administration to reinstatement of drug-seeking in male and female rats. *Front. Behav. Neurosci.* 470 (2022).

32. Kalamarides, D. J., Singh, A., Wolfman, S. L. & Dani, J. A. Sex differences in VTA GABA transmission and plasticity during opioid withdrawal. *Sci. Rep.* **13**, 8460 (2023).
33. Cho, J. H., Deisseroth, K. & Bolshakov, V. Y. Synaptic encoding of fear extinction in mPFC-amygdala circuits. *Neuron* **80**, 1491–1507 (2013).
34. Tsvetkov, E., Carlezon, W. A., Benes, F. M., Kandel, E. R. & Bolshakov, V. Y. Fear Conditioning Occludes LTP-Induced Presynaptic Enhancement of Synaptic Transmission in the Cortical Pathway to the Lateral Amygdala. *Neuron* **34**, 289–300 (2002).
35. Clem, R. L. & Huganir, R. L. Calcium-Permeable AMPA Receptor Dynamics Mediate Fear Memory Erasure. *Science* **330**, 1108–1112 (2010).
36. Mavrikaki, M. *et al.* Overexpression of miR-9 in the Nucleus Accumbens Increases Oxycodone Self-Administration. *Int. J. Neuropsychopharmacol.* **22**, 383–393 (2019).
37. Fulenwider, H. D. *et al.* Sex differences in oral oxycodone self-administration and stress-primed reinstatement in rats. *Addict. Biol.* **25**, e12822 (2020).
38. Fuchs, R. A., Lasseter, H. C., Ramirez, D. R. & Xie, X. Relapse to drug seeking following prolonged abstinence: the role of environmental stimuli. *Drug Discov. Today Dis. Models* **5**, 251–258 (2008).
39. Zhou, W. *et al.* Effects of training and withdrawal periods on heroin seeking induced by conditioned cue in an animal of model of relapse. *Psychopharmacology (Berl.)* **203**, 677–684 (2009).
40. De Vries, T. J., Schoffelmeer, A. N. M., Binnekade, R., Raasø, H. & Vanderschuren, L. J. M. J. Relapse to cocaine- and heroin-seeking behavior mediated by dopamine D2 receptors is time-dependent and associated with behavioral sensitization. *Neuropsychopharmacology* **26**, 18–26 (2002).
41. Dong, Y., Taylor, J. R., Wolf, M. E. & Shaham, Y. Circuit and synaptic plasticity mechanisms of drug relapse. *J. Neurosci.* **37**, 10867–10876 (2017).

42. Loweth, J. A., Tseng, K. Y. & Wolf, M. E. Adaptations in AMPA receptor transmission in the nucleus accumbens contributing to incubation of cocaine craving. *Neuropharmacology* **76**, 287–300 (2014).
43. McCutcheon, J. E. *et al.* Group I mGlur activation reverses cocaine-induced accumulation of calcium-permeable AMPA receptors in nucleus accumbens synapses via a protein kinase C-dependent mechanism. *J. Neurosci.* **31**, 14536–14541 (2011).
44. Scheyer, A. F. *et al.* AMPA Receptor Plasticity in Accumbens Core Contributes to Incubation of Methamphetamine Craving. *Biol. Psychiatry* **80**, 661–670 (2016).
45. Wong, B., Zimbelman, A. R., Milovanovic, M., Wolf, M. E. & Stefanik, M. T. GluA2-lacking AMPA receptors in the nucleus accumbens core and shell contribute to the incubation of oxycodone craving in male rats. *Addict. Biol.* **27**, e13237 (2022).
46. Aurélie De Groote & Alban de Kerchove d'Exaerde. Thalamo-Nucleus Accumbens Projections in Motivated Behaviors and Addiction. *Front. Syst. Neurosci.* **15**, 711350 (2021).
47. Kirouac, G. J. Placing the paraventricular nucleus of the thalamus within the brain circuits that control behavior. *Neurosci. Biobehav. Rev.* **56**, 315–329 (2015).
48. Matzeu, A., Zamora-Martinez, E. R. & Martin-Fardon, R. The paraventricular nucleus of the thalamus is recruited by both natural rewards and drugs of abuse: recent evidence of a pivotal role for orexin/hypocretin signaling in this thalamic nucleus in drug-seeking behavior. *Front. Behav. Neurosci.* **8**, 117 (2014).
49. Millan, E. Z., Ong, Z. Y. & McNally, G. P. Paraventricular thalamus: Gateway to feeding, appetitive motivation, and drug addiction. in *Progress in Brain Research* vol. 235 113–137 (Elsevier B.V., 2017).
50. Zhou, K. & Zhu, Y. The paraventricular thalamic nucleus: A key hub of neural circuits underlying drug addiction. *Pharmacol. Res.* **142**, 70–76 (2019).
51. Keyes, P. C. *et al.* Orchestrating Opiate-Associated Memories in Thalamic Circuits. *Neuron* **107**, 1113-1123.e4 (2020).

52. Pribiag, H. & Lim, B. K. Thalamic Retrieval of Opioid Memories. *Neuron* **107**, 992–994 (2020).

53. Matzeu, A., Weiss, F. & Martin-Fardon, R. Transient inactivation of the posterior paraventricular nucleus of the thalamus blocks cocaine-seeking behavior. *Neurosci. Lett.* **608**, 34–39 (2015).

54. Kuhn, B. N., Klumpner, M. S., Covelo, I. R., Campus, P. & Flagel, S. B. Transient inactivation of the paraventricular nucleus of the thalamus enhances cue-induced reinstatement in goal-trackers, but not sign-trackers. *Psychopharmacology (Berl.)* **235**, 999–1014 (2018).

55. Robbins, T. W. & Everitt, B. J. Neurobehavioural mechanisms of reward and motivation. *Curr. Opin. Neurobiol.* **6**, 228–236 (1996).

56. White, F. J. & Kalivas, P. W. Neuroadaptations involved in amphetamine and cocaine addiction. *Drug Alcohol Depend.* **51**, 141–153 (1998).

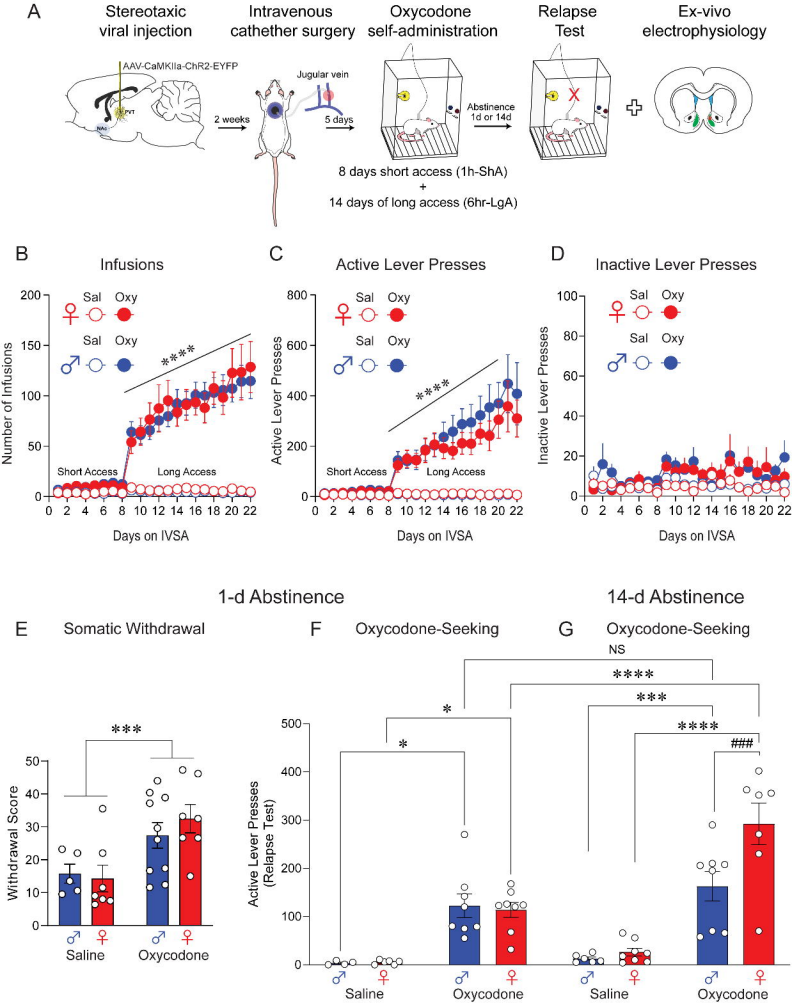
57. Hearing, M. Prefrontal-accumbens opioid plasticity: Implications for relapse and dependence. *Pharmacol. Res.* **139**, 158–165 (2019).

58. Paniccia, J. E. *et al.* Restoration of a paraventricular thalamo-accumbal behavioral suppression circuit prevents reinstatement of heroin seeking. *Neuron* **112**, 772-785.e9 (2024).

59. McGinty, J. F. & Otis, J. M. Heterogeneity in the Paraventricular Thalamus: The Traffic Light of Motivated Behaviors. *Front. Behav. Neurosci.* **14**, (2020).

60. Gao, C. *et al.* Two genetically, anatomically and functionally distinct cell types segregate across anteroposterior axis of paraventricular thalamus. *Nat. Neurosci.* **23**, 217–228 (2020).

61. Penzo, M. A. & Gao, C. The paraventricular nucleus of the thalamus: an integrative node underlying homeostatic behavior. *Trends Neurosci.* **44**, 538–549 (2021).


A
Stereotaxic viral injection
Intravenous cathether surgery
Oxycodone self-administration
Relapse Test
Ex-vivo electrophysiology
AAV-CaMKIIa-ChR2-EYFP
PVT
NAc
2 weeks
Jugular vein
5 days
Abstinence 1d or 14d
8 days short access (1h-ShA)
+
14 days of long access (6hr-LgA)
B
Infusions
Sal
Oxy
Sal
Oxy
****
Short Access
Long Access
Number of Infusions
Days on IVSA
C
Active Lever Presses
Sal
Oxy
Sal
Oxy
****
Short Access
Long Access
Active Lever Presses
Days on IVSA
D
Inactive Lever Presses
Sal
Oxy
Sal
Oxy
Inactive Lever Presses
Days on IVSA
1-d Abstinence
14-d Abstinence
E
Somatic Withdrawal
***
Withdrawal Score
Saline
Oxycodone
F
Oxycodone-Seeking
*
*
Active Lever Presses (Relapse Test)
Saline
Oxycodone
G
Oxycodone-Seeking
NS
****
***
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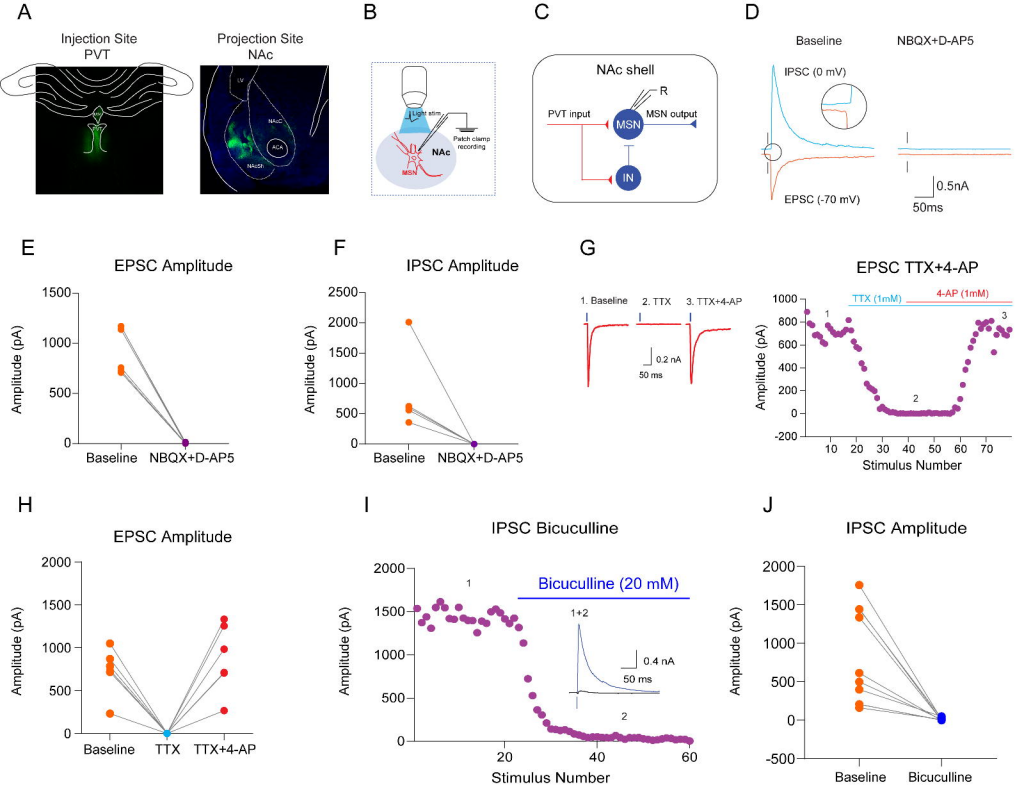
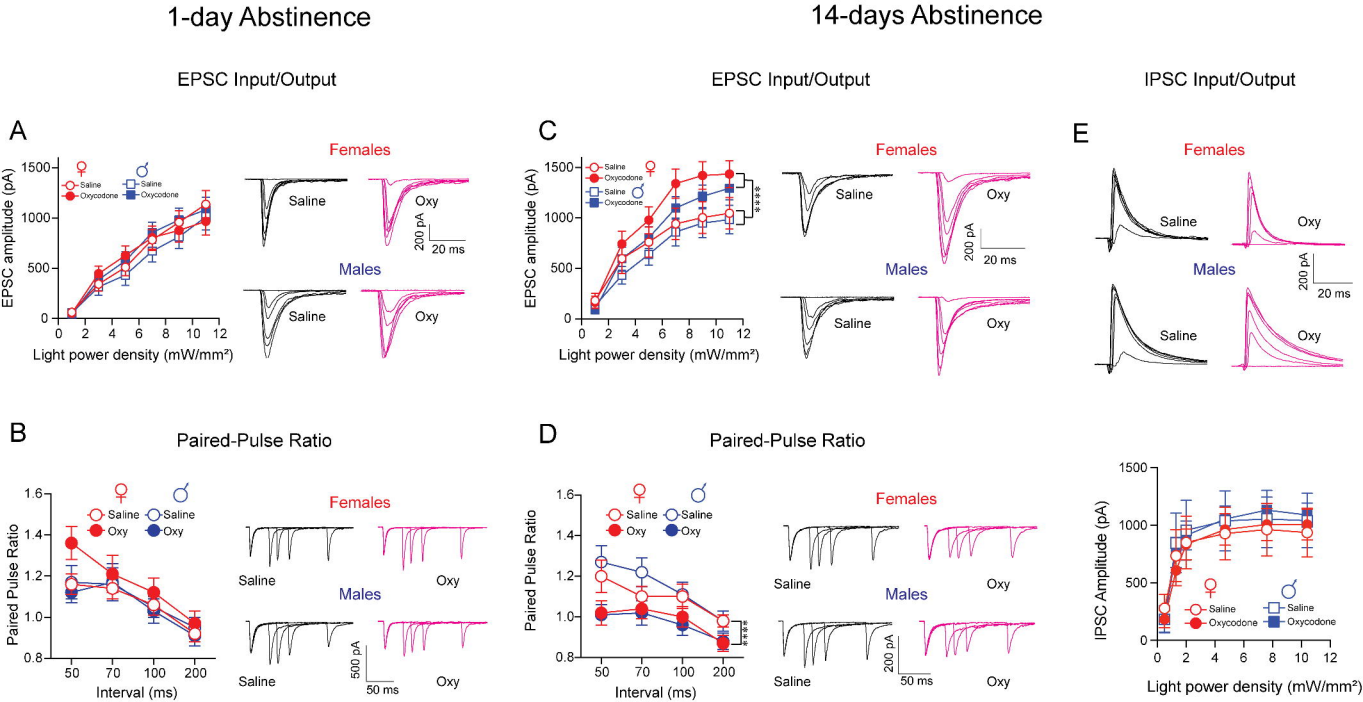
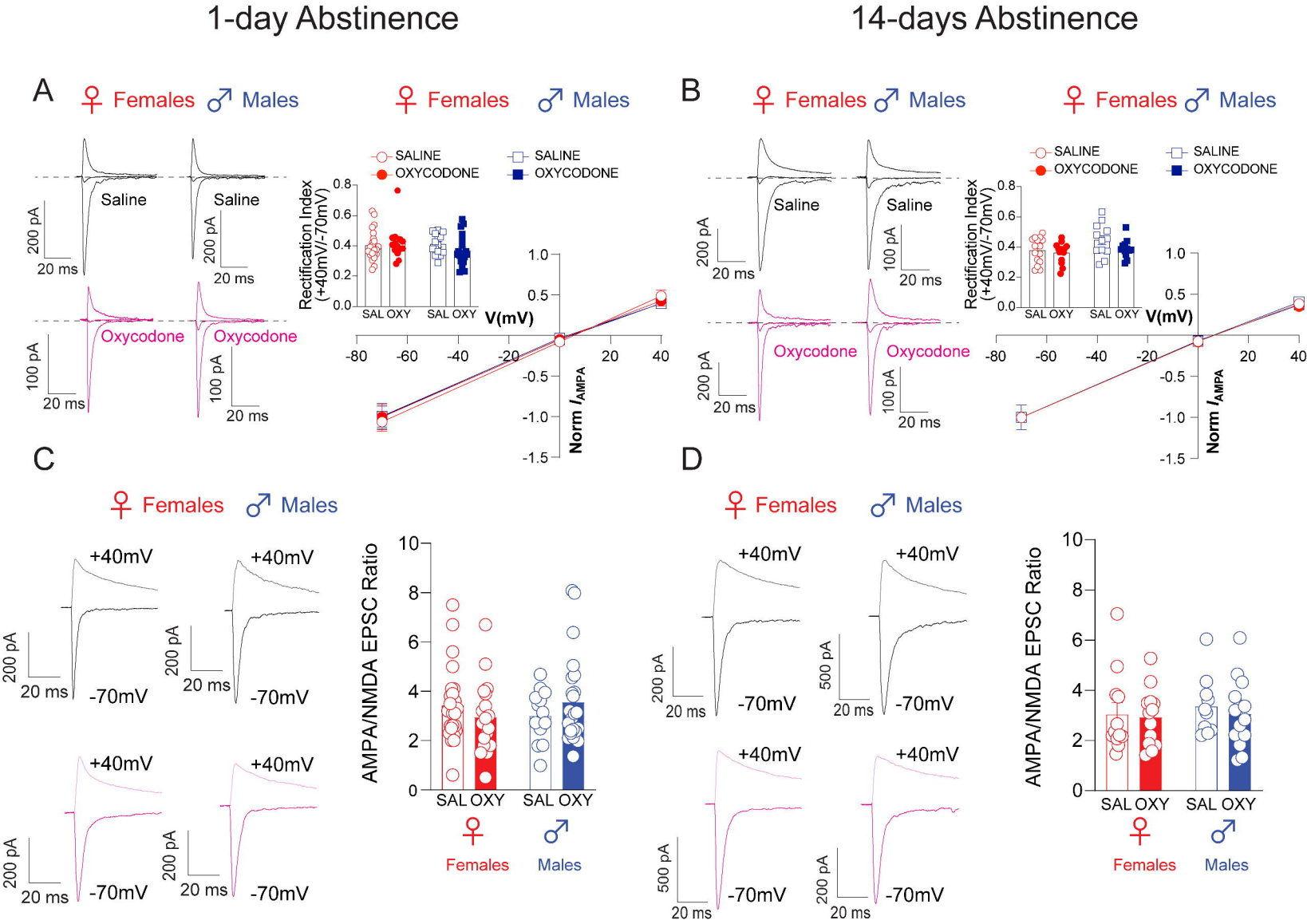
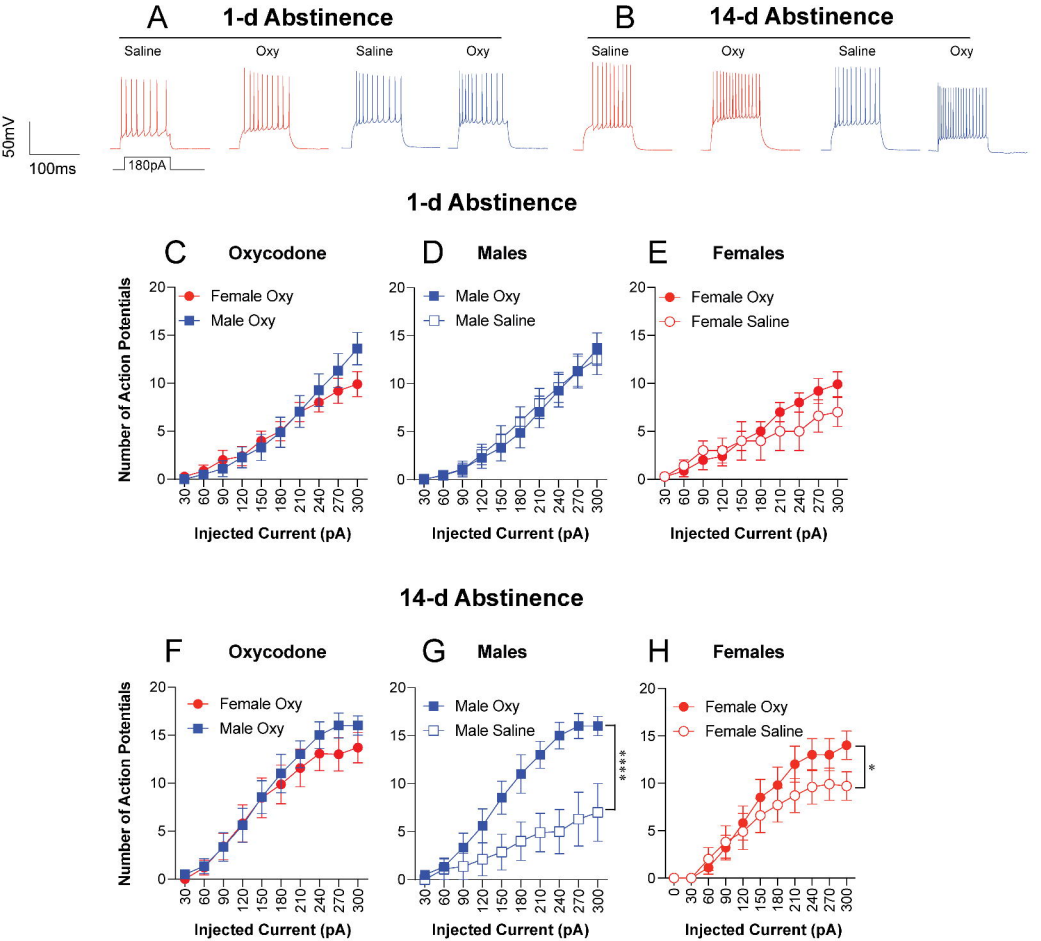
###
Saline
Oxycodone


A
Injection Site
PVT
Projection Site
NAc
LV
NAcC
ACA
NAcSh
B
Light stim.
Patch clamp recording
NAc
MSN
C
NAc shell
R
PVT input
MSN
MSN output
IN
D
Baseline
NBQX+D-AP5
IPSC (0 mV)
EPSC (-70 mV)
0.5nA
50ms
E
EPSC Amplitude
Amplitude (pA)
1500
1000
500
0
Baseline
NBQX+D-AP5
F
IPSC Amplitude
Amplitude (pA)
2500
2000
1500
1000
500
0
Baseline
NBQX+D-AP5
G
1. Baseline
2. TTX
3. TTX+4-AP
0.2 nA
50 ms
EPSC TTX+4-AP
TTX (1mM)
4-AP (1mM)
Amplitude (pA)
1000
800
600
400
200
0
-200
1
2
3
10
20
30
40
50
60
70
Stimulus Number
H
EPSC Amplitude
Amplitude (pA)
2000
1500
1000
500
0
Baseline
TTX
TTX+4-AP
I
IPSC Bicuculline
Amplitude (pA)
2000
1500
1000
500
0
1
Bicuculline (20 mM)
1+2
0.4 nA
50 ms
2
20
40
60
Stimulus Number
J
IPSC Amplitude
Amplitude (pA)
2000
1500
1000
500
0
-500
Baseline
Bicuculline


1-day Abstinence
14-days Abstinence
EPSC Input/Output
EPSC Input/Output
IPSC Input/Output
A
C
E
Females
Males
Saline
Oxycodone
EPSC amplitude (pA)
Light power density (mW/mm²)
Oxy
200 pA
20 ms
B
D
Paired-Pulse Ratio
Paired-Pulse Ratio
Interval (ms)
500 pA
50 ms
IPSC Amplitude (pA)


1-day Abstinence
14-days Abstinence
A
B
C
D
♀ Females ♂ Males
Saline
Oxycodone
200 pA
100 pA
20 ms
SALINE
OXYCODONE
Rectification Index (+40mV/-70mV)
SAL OXY
V(mV)
Norm I_{AMPA}
+40mV
-70mV
500 pA
AMPA/NMDA EPSC Ratio
Females
Males


A
1-d Abstinence
B
14-d Abstinence
Saline
Oxy
Saline
Oxy
Saline
Oxy
Saline
Oxy
50mV
100ms
180pA
1-d Abstinence
C
Oxycodone
D
Males
E
Females
Female Oxy
Male Oxy
Male Oxy
Male Saline
Female Oxy
Female Saline
Number of Action Potentials
Injected Current (pA)
14-d Abstinence
F
Oxycodone
G
Males
H
Females
****
*