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

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eLife Assessment

This study presents **valuable** evidence of sex differences in oxycodone relapse-related behavior alongside novel characterization of synaptic adaptations in the paraventricular thalamus - nucleus accumbens shell circuit. The authors show that females exhibit heightened cue-induced seeking after 14 days, but not 1 day, of abstinence, while both sexes display similar time-dependent strengthening of paraventricular thalamus - nucleus accumbens shell glutamatergic transmission. The revised manuscript strengthens the work through improved statistical analyses, clearer interpretation, and expanded integration with prior literature. The strength of evidence is **solid**. However, association among experiments is **incomplete**, as the sex-specific behavioral effect is not reflected in circuit-level plasticity, and no causal manipulations test pathway involvement in relapse. Future work could link these circuit adaptations to sex-specific relapse vulnerability.

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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 in 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 the intrinsic excitability of NAcSh medium spiny neurons (MSNs), in slices from rats subjected to either 1 (acute) or 14 (prolonged) days of forced abstinence following 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, with females exhibiting higher relapse rates. Prolonged but not acute abstinence led to comparable increases in PVT-NAcSh synaptic strength in both sexes, while inhibitory synaptic transmission in this pathway was not significantly altered at either abstinence time point. Intrinsic excitability of NAcSh MSNs was largely unaltered following oxycodone abstinence in both sexes; however, a

trend toward increased spike output was observed in males after prolonged abstinence. Together, these findings suggest that prolonged oxycodone abstinence produces time-dependent and pathway-selective increases in excitatory synaptic strength at PVT-NAcSh inputs, accompanied by sex-specific effects on 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 condition involves drug self-administration, an operant behavior in which the animal is required to press a lever to obtain the 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 suggest 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 for 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 behavioral changes over time. For example, increased synaptic strength in projections from the PVT to D2R-expressing NAc 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]. It has also been found that glutamatergic strength decreases from PVT to parvalbumin interneurons (PV-IN) after self-administration and during extinction and that rescuing this projection prevents heroin relapse [21]. Although it is clear that changes in glutamatergic inputs onto MSNs and other interneurons may contribute to opioid withdrawal and reinstatement of drug-seeking [13,15,22,23], 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 [24]: 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 [25–27]. 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 [28]). 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 [26,29]. 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) [29,30]. 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 following the cue-induced oxycodone-seeking test, such that electrophysiological recordings reflect synaptic properties associated with relapse after acute or prolonged abstinence.

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, which were prepared as stock solutions in water at 1000- to 5000-fold concentrations and stored at -20°C.

Morphological Analysis

Histology for Neurobiotin-filled cells

Brain slices containing Neurobiotin-filled neurons in NAcSh were washed in PBS for 20 min x 3 times and incubated with Streptavidin Alexa 568 conjugate (10-20 μg/ml, catalog number: S11226, Molecular Probes) in PBS containing 0.2% Triton X-100 at room temperature for 24 hours. The slices were then washed with PBS for 20 min x 3 times and mounted on gelatinized slides. The anti-fading mounting media with DAPI (Vectashield, Vector Laboratories) was applied to slices.

Analysis of dendritic morphology of NAcSh-MSNs

Acquisition of imaging data of Neurobiotin-stained neurons was performed using a Leica SP8 TCS confocal microscope under a 40X/1.30 NA oil-immersion objective lens. The image resolution (1024 X 1024 pixels) and z step (0.5 μm) of optical planes were kept constant throughout the study. To image entire dendritic trees of NAcSh neurons, the zoom was adjusted in a range of 0.75 – 1.0, which corresponds to the voxel size between 0.284 X 0.284 X 0.5 μm and 0.379 X 0.379 X 0.5 μm. Three-dimensional (3D) reconstruction of dendritic trees was conducted in stacked confocal images in the program NeuronStudio (version 0.9.92, Icahn School of Medicine at Mount Sinai, New York, NY; [31]. We used the manual tracing tool to reconstruct dendrites starting from the soma. Specifically, we started the manual tracing from the beginning of each primary dendrite and moved one node at a time to form an entire path along a branch through the views at XY, ZY and XZ orientations. The program provides numerical measurements of the reconstructed dendritic trees, including dendritic length, surface area, volume, number of branch points, and Sholl analysis. Soma size was estimated by measuring the maximal projection area of a neuron soma in a single optical coronal section (1.038 μm thick) using ImageJ. The spatial organization of the dendritic field was characterized by the ratio of the long axis to the short axis of the dendritic field at the coronal plane. The long axis of the dendritic field was defined as the distance from the soma to the most distal dendritic process, and the short axis as the distance from the soma to the most distal dendritic point located 90° from the long axis [32]. The measurements were performed on stacked images of MSNs using the Leica Application Suite X (LAS X, Leica Microsystems CMS GmbH, version 3.5.5). The Neurobiotin-stained neurons were primarily obtained from the medial division of NAcSh in two adjacent slices of caudal nucleus accumbens, corresponding to the bregma levels between 10.08 and 10.56 mm [24].

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 [33,34]. 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 appropriate.

For analyses of intrinsic excitability based on spike count–current relationships, spike output (number of action potentials per current step) was analyzed using nested hierarchical Poisson generalized linear mixed-effects models (GLMMs), fitted separately for each sex and abstinence duration. Each model included injected current (mean-centered), drug condition (Saline vs. Oxycodone), and their interaction as fixed effects. Random effects included random intercepts and slopes for injected current at the animal level, and random intercepts for cells nested within animals, to account for the non-independence of repeated current steps within cells and of multiple cells recorded from the same animal. Unique identifiers were assigned to each animal and cell by concatenating condition labels with animal and cell IDs, ensuring that animals across treatment conditions were treated as independent subjects. Models were fitted using maximum likelihood estimation with Laplace approximation. Overdispersion was assessed using the Pearson chi-squared statistic divided by the residual degrees of freedom; when this ratio exceeded 1.5, a dispersion correction was applied within the Poisson framework. Statistical significance of fixed effects was assessed using marginal F-tests with residual degrees of freedom. Sex comparisons among oxycodone-treated animals were conducted as separate analyses using identical model structures, with sex as the grouping condition. For visualization, marginal model-predicted values were overlaid on each plot as dashed lines, representing the population-level fixed-effects predictions from the fitted GLMM with random effects set to zero, back-transformed from the log scale to spike counts. To assess statistical power, a posthoc power analysis was conducted for each comparison using a two-sample t-test approximation at the rat level, with rat-level mean spike output as the unit of analysis and the pooled between-rat standard deviation as the denominator for effect size estimation (Cohen's d). This approach was used to estimate the number of animals per group required to achieve 80% and 90% statistical power, given the observed effect sizes. All analyses were performed in MATLAB (R2025b).

IPSC amplitudes were analyzed using linear mixed-effects models (LMEs) to account for the hierarchical structure of the data, with repeated measurements across light intensities nested within animals and multiple cells recorded per animal. Unique identifiers were assigned to each rat and cell by concatenating condition labels with animal and cell IDs, ensuring that animals across treatment conditions were treated as independent subjects. Models included fixed effects of light intensity (mean-centered across the six stimulation levels: 0.5, 1.3, 2.0, 4.7, 7.6, and 10.4 mW), treatment condition (Saline vs. Oxycodone), and their interaction. Random effects included random intercepts and light intensity slopes for individual animals, and random intercepts for cells nested within animals, capturing both between-animal variability in baseline responses and sensitivity to light, as well as within-animal variability across cells. Model parameters were estimated using restricted maximum likelihood (REML). Statistical significance of fixed effects was assessed using marginal F-tests with residual degrees of freedom, applied consistently across male and female datasets. Data are presented as mean ± SEM, computed from rat-level averages, in which responses from multiple cells within the same animal were first averaged to yield one value per animal per light intensity, consistent with the animal-level inference of the LME. Male and female datasets were analyzed separately using identical model structures to assess sex-specific effects. All analyses were performed in MATLAB (R2025b).

The number of animals and cells used per experiment, as well as the results of all statistical analyses, are reported in the text and figure legends.

Results

Male and female rats escalate oxycodone intake under long access self-administration

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 published results [35], 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 SA 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 females (n = 7 saline, 7 oxycodone) and males (n = 5 saline, 10 oxycodone). Since opioid dependence is characterized by somatic withdrawal signs upon withdrawal, we predicted an increase in somatic withdrawal signs in both sexes. We found a significant increase in expression of somatic withdrawal behaviors (combined into a Total Withdrawal Score) in females and males that received 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 SA. This is consistent with previously published studies that have shown increases in somatic withdrawal symptoms after 24-hours in male and female rats [36]

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 saline (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, $p = 0.02$.

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

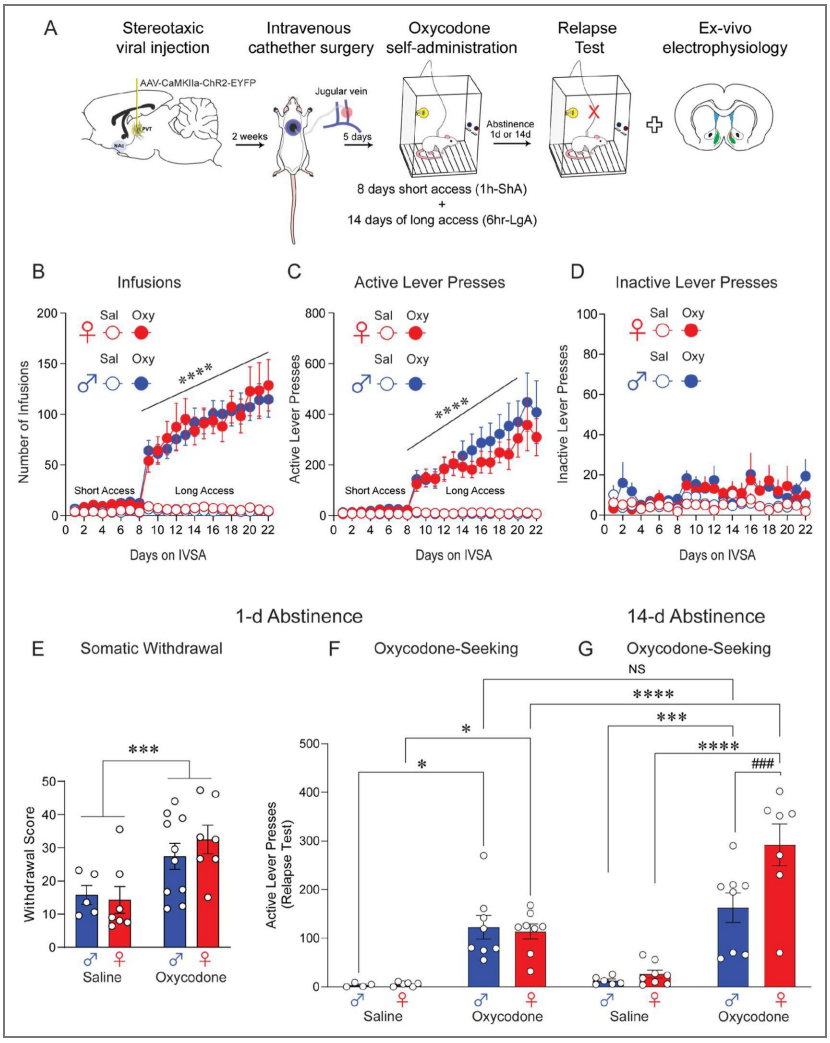


Figure 1. Oxycodone self-administration and oxycodone-seeking after short and prolonged abstinence.

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.

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) [37].

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 [37] 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.

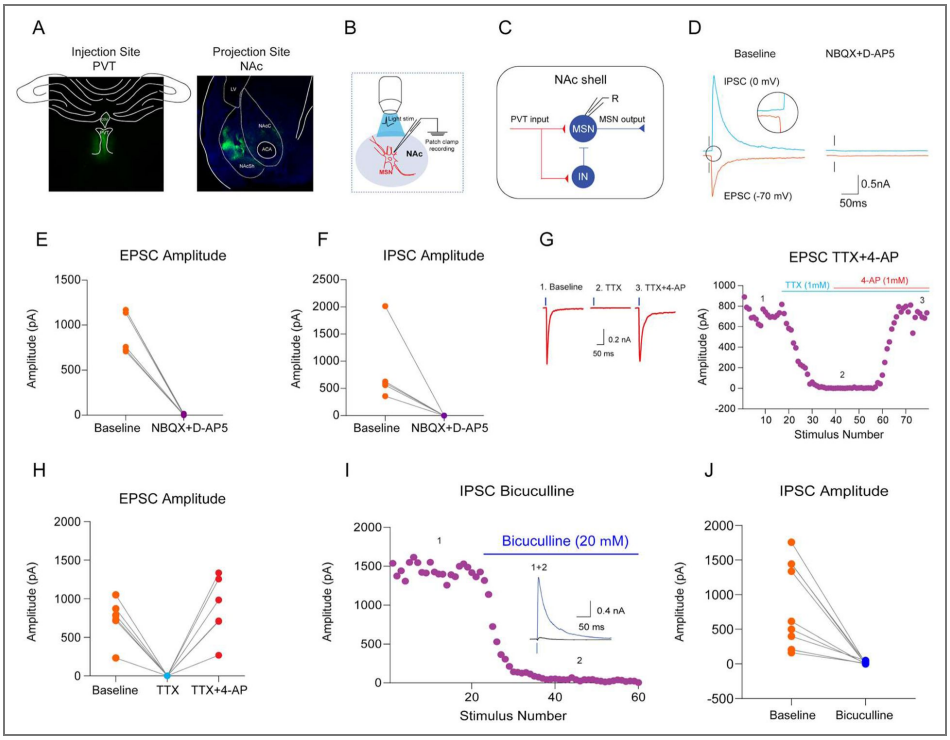


Figure 2. Synaptic Properties in PVT-to-NAcSh Projections.

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/mm2; 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 GABAA receptor antagonist bicuculline (20 μM). **J:** IPSC amplitudes recorded from NAcSh under control conditions (baseline) and after application of bicuculline.

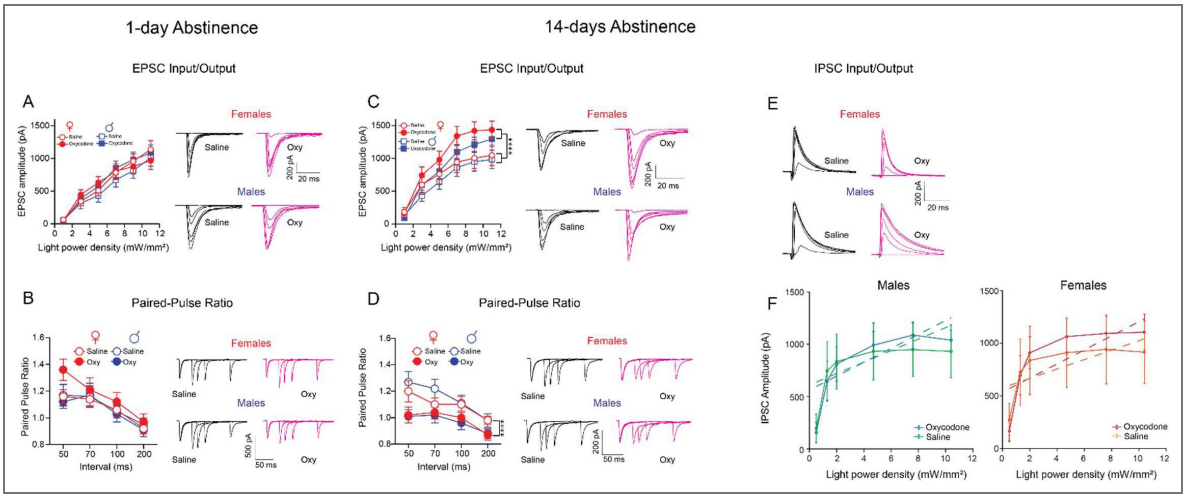


Figure 3. Prolonged abstinence from oxycodone self-administration is associated with increased synaptic strength in glutamatergic PVT projections to MSNs in NAcSh in male and female rats.

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:** 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. **F: Left (males),** linear mixed-effects modeling revealed a significant main effect of light intensity on IPSC amplitude ($F_{(1, 158)}$ = 43.49, p = 6.1 × 10^{-10}), indicating robust recruitment of inhibitory synaptic responses with increasing stimulation. No significant main effect of condition was observed in males ($F_{(1, 158)}$ = 0.00023, p = 0.988), and no Light × Condition interaction was detected ($F_{(1, 158)}$ = 0.543, p = 0.462), indicating that prolonged abstinence from oxycodone does not alter baseline inhibitory strength or recruitment dynamics in male NAcSh MSNs. **Right (females)**, linear mixed-effects modeling revealed a significant main effect of light intensity on IPSC amplitude ($F_{(1, 164)}$ = 21.26, p = 8.0 × 10^{-6}), indicating robust recruitment of inhibitory synaptic responses with increasing stimulation. No significant main effect of condition was observed in females ($F_{(1, 164)}$ = 0.025, p = 0.875), and no Light × Condition interaction was detected ($F_{(1, 164)}$ = 1.298, p = 0.256), indicating that prolonged abstinence from oxycodone does not alter baseline inhibitory strength or recruitment dynamics in female NAcSh MSNs. Together, these data indicate that the efficacy of feed-forward inhibition in the PVT–NAcSh pathway, as assessed by input-output curves for light-induced IPSCs, is unaffected by prolonged oxycodone abstinence in either sex. Saline (n = males: 5 rats, 11 cells; females: 7 rats, 14 cells) and oxycodone (n = males: 7 rats, 16 cells; females: 5 rats, 14 cells).

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].

To assess whether prolonged abstinence from oxycodone self-administration alters feed-forward inhibition within the PVT-NAcSh pathway (Fig. 3E-F), light-evoked IPSC amplitudes were analyzed using linear mixed-effects modeling. In both males and females, IPSC amplitude increased robustly with light intensity, indicating preserved recruitment of inhibitory synaptic responses with increasing stimulation (males: $F_{(1, 158)} = 43.49$, $p = 6.1 \times 10^{-10}$; females: $F_{(1, 164)} = 21.26$, $p = 8.0 \times 10^{-6}$). No significant main effect of treatment condition was detected in either sex (males: $F_{(1, 158)} = 0.00023$, $p = 0.988$; females: $F_{(1, 164)} = 0.025$, $p = 0.875$), indicating that prolonged abstinence from oxycodone did not alter baseline inhibitory synaptic strength within the PVT-NAcSh pathway in males or females. No significant Light × Condition interactions were observed in either sex (males: $F_{(1, 158)} = 0.543$, $p = 0.462$; females: $F_{(1, 164)} = 1.298$, $p = 0.256$), indicating that the scaling of inhibitory responses with increasing stimulation intensity was not altered by oxycodone exposure in either sex. Together, these results demonstrate that feed-forward inhibition within the PVT-NAcSh pathway is preserved following prolonged abstinence from oxycodone, with no significant changes in either the magnitude or the input-output scaling of inhibitory synaptic responses in males or females.

Taken together with the observed strengthening of excitatory synaptic transmission in PVT–NAcSh projections after prolonged abstinence (Fig. 3C), these results suggest that prolonged abstinence from oxycodone selectively potentiates excitatory but not inhibitory synaptic transmission within this pathway. This relative shift in the excitation–inhibition balance may facilitate MSN output within relapse-related circuitry and contribute to the behavioral effects observed following prolonged abstinence.

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 [38] 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 [38]. 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: $F_{(1,68)} = 0.35$, $p = 0.6$).

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

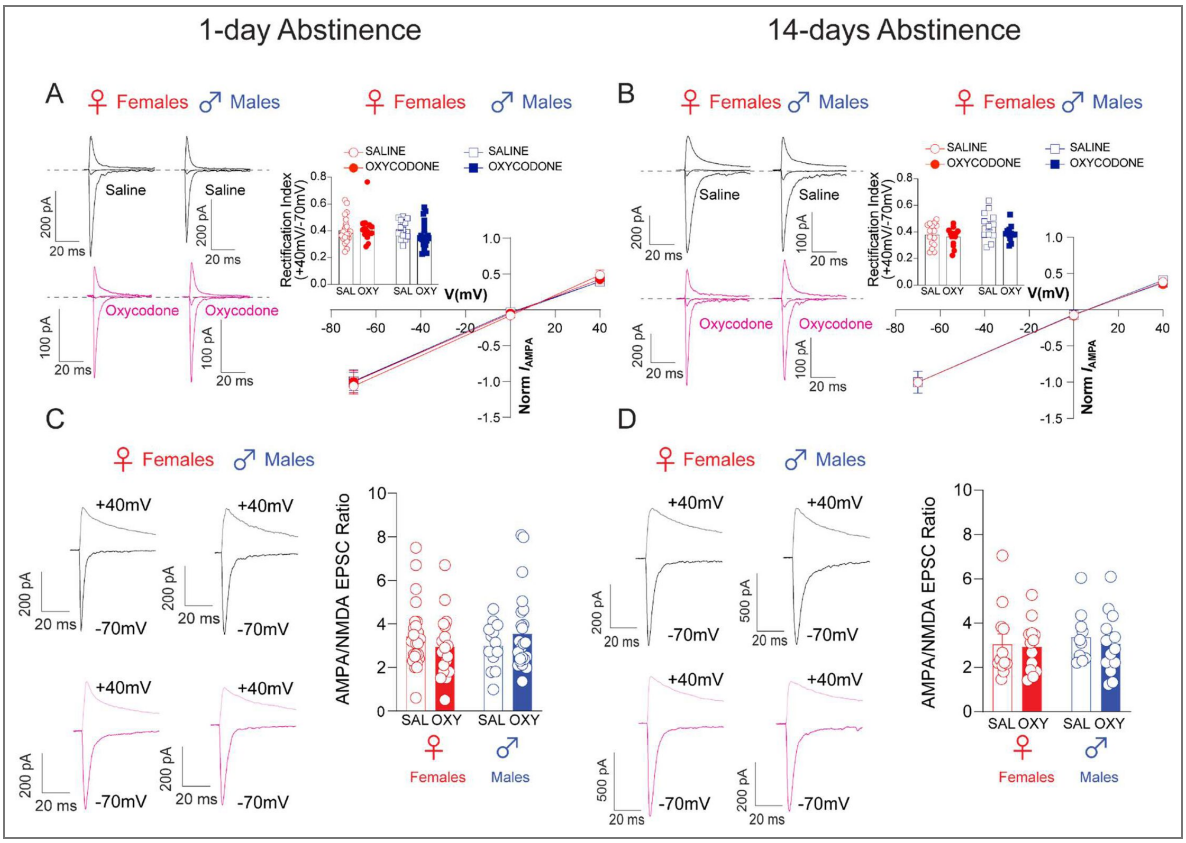


Figure 4. Abstinence from oxycodone self-administration does not change AMPAR subunit composition or AMPAR/NMDAR EPSC amplitude ratio in glutamatergic PVT projections to MSNs in NAcSh.

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.

synapses formed by PVT projecting fibers on MSNs in NAcSh. Thus, synaptic potentiation in PVT-NAcSh projections, observed after 14 days of abstinence (see Fig. 3C), was unlikely due to increased GluR1 trafficking at the synapses studied.

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 as assessed by these synaptic measures in PVT-NAcSh projections.in PVT-NAcSh projections.

Prolonged but not acute abstinence from oxycodone self-administration shows a trend toward increased NAcSh MSN intrinsic excitability in males but not females

To determine whether abstinence from oxycodone self-administration alters intrinsic excitability of NAcSh medium spiny neurons (MSNs), we performed whole-cell current-clamp recordings from NAcSh MSNs in slices from saline- (males: 7 rats, 16 cells; females: 4 rats, 7 cells) and oxycodone-treated rats (males: 6 rats, 18 cells; females: 8 rats, 15 cells) following acute abstinence (1 day; Fig. 5A, C–E), as well as from saline- (males: 3 rats, 8 cells; females: 6 rats, 14 cells) and oxycodone-treated rats (males: 4 rats, 14 cells; females: 6 rats, 15 cells) following prolonged abstinence (14 days; Fig. 5B, F–H). Spike output was quantified as the number of action potentials fired during depolarizing current steps. Across all conditions, injected current robustly increased spike output, confirming intact spike count–current relationships in all groups (acute abstinence males: $F_{(1, 370)} = 50.03$, $p = 7.6 \times 10^{-12}$; acute abstinence females: $F_{(1, 230)} = 71.63$, $p = 3.1 \times 10^{-15}$; prolonged abstinence males: $F_{(1, 225)} = 19.23$, $p = 1.8 \times 10^{-5}$; prolonged abstinence females: $F_{(1, 306)} = 30.14$, $p = 8.4 \times 10^{-8}$).

Following acute abstinence, oxycodone exposure did not significantly alter baseline spike output or spike output gain in males (Fig. 5D: condition, $F_{(1, 370)} = 0.454$, $p = 0.501$; current × condition, $F_{(1, 370)} = 0.236$, $p = 0.628$) or females (Fig. 5E: condition, $F_{(1, 230)} = 0.571$, $p = 0.451$; current × condition, $F_{(1, 230)} = 0.776$, $p = 0.379$), indicating that acute oxycodone abstinence does not detectably alter MSN intrinsic excitability in either sex.

Following prolonged abstinence, no significant effect of oxycodone exposure on baseline spike output or spike output gain was detected in females (Fig. 5H: condition, $F_{(1, 306)} = 0.025$, $p = 0.875$; current × condition, $F_{(1, 306)} = 0.366$, $p = 0.546$). In males, oxycodone-treated animals showed numerically higher spike output compared to saline controls (mean spikes per step: oxycodone 7.46 ± SD 2.29 vs saline 4.43 ± SD 2.82), corresponding to a large effect size (Cohen's d = 1.18); however, this difference did not reach statistical significance (Fig. 5G: condition, $F_{(1, 225)} = 2.419$, $p = 0.121$; current × condition, $F_{(1, 225)} = 0.008$, $p = 0.930$). A post-hoc power analysis indicated that the current sample size (3 saline rats, 4 oxycodone rats) provided only approximately 20% power to detect this effect, and that 13 rats per group would be required to achieve 80% power. These results should therefore be interpreted with caution, and the possibility of a biologically meaningful increase in MSN excitability in males following prolonged oxycodone abstinence cannot be excluded.

No significant difference in baseline spike output or spike output gain was observed between males and females at either 1 day (Fig. 5C: condition, $F_{(1, 351)} = 0.063$, $p = 0.802$; current × condition, $F_{(1, 351)} = 0.105$, $p = 0.746$) or 14 days of abstinence (Fig. 5F: condition, $F_{(1, 300)} = 0.176$, $p = 0.675$; current × condition, $F_{(1, 300)} = 0.036$, $p = 0.849$).

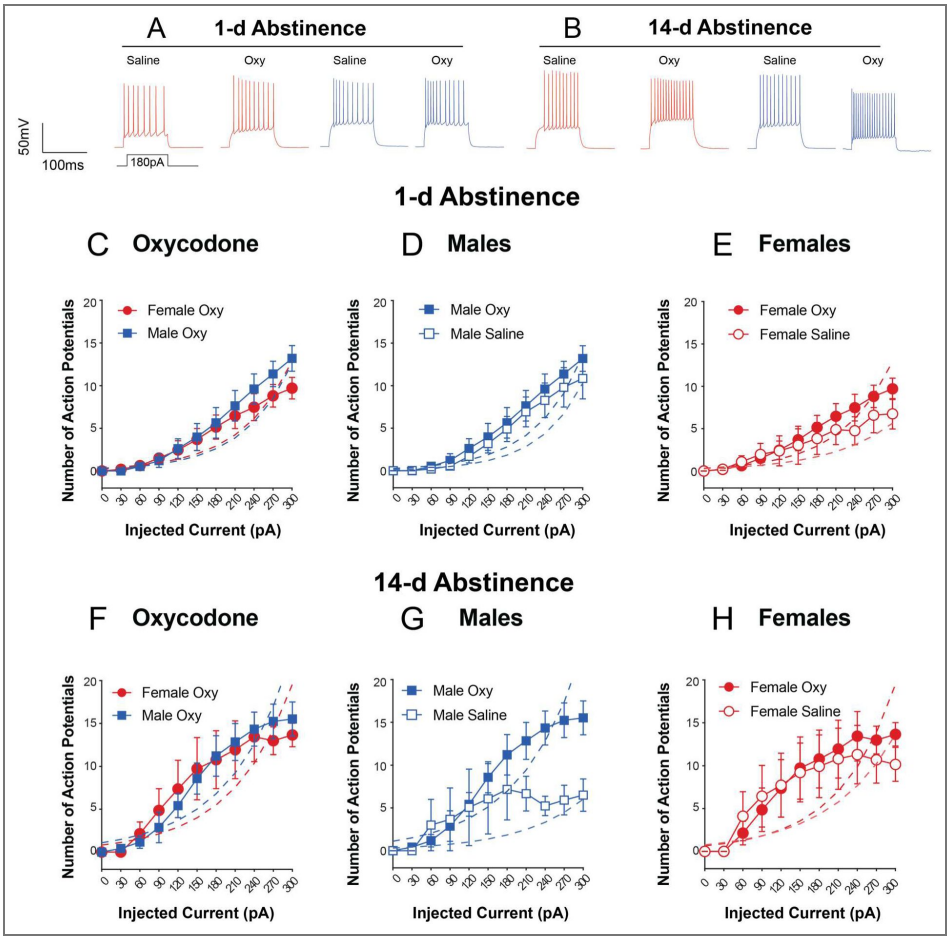


Figure 5. Intrinsic excitability of NAcSh medium spiny neurons is largely unaltered following oxycodone abstinence, with a trend toward increased spike output in males after prolonged abstinence.

A–B: Example current-clamp traces from NAcSh MSNs from saline- and oxycodone-treated rats after 1 day (**A**) and 14 days (**B**) of abstinence. Sample sizes: 1-day saline (males: 7 rats, 16 cells; females: 4 rats, 7 cells), 1-day oxycodone (males: 6 rats, 18 cells; females: 8 rats, 15 cells), 14-day saline (males: 3 rats, 8 cells; females: 6 rats, 14 cells), 14-day oxycodone (males: 4 rats, 14 cells; females: 6 rats, 15 cells). **C, F**: Spike count–current relationships in oxycodone-treated animals revealed no significant sex differences in baseline spike output or spike output gain at 1 day (**C**: condition $F(1, 351) = 0.063$, $p = 0.802$; interaction $F(1, 351) = 0.105$, $p = 0.746$) or 14 days of abstinence (**F:** condition $F(1, 300) = 0.176$, $p = 0.675$; interaction $F(1, 300) = 0.036$, $p = 0.849$). **D–E**: No significant effect of oxycodone on spike output was detected in males or females after 1 day of abstinence (males: condition $F(1, 370) = 0.454$, $p = 0.501$; females: condition $F(1, 230) = 0.571$, $p = 0.451$). **G:** After 14 days of abstinence, oxycodone-treated males showed numerically higher spike output than saline controls (7.46 vs. 4.43 spikes/step; Cohen's $d = 1.18$), but this did not reach statistical significance (condition: $F(1, 225) = 2.419$, $p = 0.121$; interaction: $F(1, 225) = 0.008$, $p = 0.930$); post-hoc power analysis indicated only ~20% power with the current sample, and 13 rats per group would be required for 80% power. **H**: No significant effect of oxycodone on spike output was detected in females after 14 days of abstinence (condition: $F(1, 306) = 0.025$, $p = 0.875$; interaction: $F(1, 306) = 0.366$, $p = 0.546$). Spike count data were analyzed using nested hierarchical Poisson GLMMs fitted separately for each sex and abstinence duration. Data are shown as mean ± SEM.

Together, these results indicate that intrinsic excitability of NAcSh MSNs is largely unaltered following oxycodone abstinence in both sexes. The exception is a trend toward increased spike output in males following prolonged abstinence, which, despite a large observed effect size, did not reach statistical significance due to limited sample size in this group.

Dendritic morphology of NAcSh-MSNs remained unchanged regardless of abstinence duration from oxycodone self-administration

More than 85% of Neurobiotin-stained cells obtained from the medial division of NAcSh in male or female rats are medium spiny neurons (MSNs) characterized by dense spines on a second or higher order of dendritic branches (Supplementary Figure 1A-C). The number of MSNs primary dendrites (3.3-3.6 on average for male rats, 2.8-4.1 in average for female rats) and elongated dendritic trees (~1.5 on average ratios of long axis over short axis of dendritic field among treatment groups) are consistent with the previously described morphological features of NAcSh-MSNs [32]. Axons arising from soma or proximal dendrites can be observed in the majority of reconstructed MSNs (86.8% or 85.7% in male or female rats, respectively). Some of the axons (21% or 37% in male or female rats) were found to give rise to axonal collateral ramifications with terminal-like varicosities in the vicinity of their soma. This observation suggests that some MSNs may contribute to feedforward inhibition in studied projections as manifested by polysynaptic IPSC activated by photostimulation of PVT afferents (Supplementary Figure 1A, left and Figure 2C,D,F,I and J [39,40]). In addition, less than 15% of Neurobiotin-stained neurons were identified either as sparsely spined neurons (SSNs) characterized by a few spines on thread-like dendrites, or aspiny neurons (ASNs) without visible dendritic spine (Supplementary Figure 1A-C). These two types of neurons observed in both female and male rats were not included in the subsequent quantitative analysis.

To examine the effects of abstinence from oxycodone self-administration on neuronal structure in NAcSh, we analyzed 45 reconstructed MSNs from 23 female rats (8-14 cells from 5-7 rats among treatment groups) and 41 reconstructed MSNs from 21 male rats (6-16 cells from 3-8 rats among treatment groups). We found that soma sizes of the MSNs, estimated by maximal projection area at a single optical plane, vary in a fivefold range (~40-200 μm2) in both male and female rats with identical cumulative frequency distributions (p = 0.954, Mann-Whitney test; Supplementary Figure 1D). The varied soma sizes are correlated with dendritic size (dendritic length or volume), and, to a lesser degree, with the number of branch points (Supplementary Figure 1E, F). This observation indicates a wide variation in the somatodendritic morphology of NAcSh-MSNs in both male and female rats. We then examined cumulative frequency distributions of soma size among four treatment groups, including male and female rats, and found no differences at 1 day of abstinence (p = 0.2703) or 14 days of abstinence (p = 0.5982, Kruskal-Wallis test, Supplementary Figure 2B and F). We did not detect significant effect of oxycodone abstinence on any parameter assayed in dendritic morphology (Two-way ANOVA, variables: treatment x sex) either at 1-day abstinence ($F(1,40) = 0.455$, $p = 0.504$ for dendritic length; $F_{(1,40)} = 0.465$, $p = 0.499$ for branch points), or at 14-days abstinence ($F_{(1,38)} = 0.001$, $p = 0.972$ for dendritic length; $F_{(1,38)} = 0.114$, $p = 0.738$ for branch points, left panels in Supplementary Figure 2C,D,G, H). Consistent with these observations, Sholl analyses (right panels in Supplementary Figure 2C,D,G,H; three-way ANOVA, variables: treatment x sex x radial distance) indicate no changes after 1 day abstinence ($F_{(1, 40)} = 0.4568$, $p = 0.5030$ for dendritic length; $F_{(1, 40)} = 1.547$ $p = 0.2208$ for branch points), or at 14 days abstinence ($F(_{1, 38)} = 0.001$, $p = 0.9693$ for dendritic length; $F_{(1, 38)} = 0.1138$ $p = 0.7378$ for branch points). On the other hand, we observed small sex differences in the number of branch points ($F_{(1,40)} = 4.444$, $p = 0.041$; Bonferroni's multiple comparisons test, $p = 0.546$ for male versus female saline rats) in NAcSh-MSNs at 1 day abstinence (whereas the effect on dendritic length was not significant, $F_{(1,40)} = 3.396$, $p = 0.073$; left panels in Supplementary Figure 2C, D). At 14 days of abstinence (left panels in Supplementary Figure 2G,H), there were no differences between male and female rats ($F_{(1,38)} = 1.675$, $p = 0.203$ for dendritic length; $F_{(1,38)} = 1.964$, $p = 0.169$ for branch points). No significant interaction between treatment and sex was found on dendritic length and

branch points. The Sholl analyses of same data as above showed no sex differences both at 1 day abstinence ($F_{(1, 40)}$ = 3.396, p = 0.0728 for dendritic length; $F_{(1, 40)}$ = 1.848, p = 0.1816 for branch point) and at 14 days abstinence ($F_{(1, 38)}$ = 1.670, p =0.2041 for dendritic length; $F_{(1, 38)}$ = 1.964, p = 0.1692 for branch point). Taken together, our quantitative analyses demonstrate the lack of effect on somatodendritic morphology of NAcSh-MSNs at either 1 day or 14 days of abstinence from oxycodone self-administration in both female and male rats.

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 [28,35]. However, it contrasts with other studies that have found that female rats orally self-administered more oxycodone than males in a short-access (1-hr) FR-1 schedule self-administration paradigm [41], 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 [25]. 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 [26]. 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 [10,42,43]. 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 [10,23,44–48]

In addition, a recent study [49] 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 [50–54]. Several studies have shown that the PVT is involved in retrieving and consolidating drug-associated memories [55,56]. 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 [55]. 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 [52], which seems to be dependent on the type of reinstatement model and individual differences [57]. 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.

Lack of changes in intrinsic excitability of MSNs in NAcSh after prolonged abstinence

The NAc plays an important role in regulating motivated behaviors such as reward-seeking and cravings [58,59]. 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 using nested hierarchical Poisson GLMMs to appropriately account for the hierarchical structure of the electrophysiological data.

One goal of this study was to examine the interplay between PVT-NAcSh glutamatergic transmission and NAcSh MSN intrinsic excitability following oxycodone abstinence. The standing theory suggests that MSN excitability decreases as a homeostatic response to increased glutamatergic input [23,45,60]. Our data do not support a compensatory decrease in excitability in either sex at either abstinence time point. Instead, the trend toward increased excitability in males after prolonged abstinence, if confirmed with adequate sample sizes, would suggest that prolonged oxycodone abstinence may produce maladaptive neuroadaptations that enhance rather than suppress MSN excitability. Such an increase, acting in concert with the strengthening of PVT-NAcSh synaptic transmission observed in the current study, could facilitate signal flow through this circuit and enhance MSN output to downstream targets, potentially contributing to relapse-related behaviors.

Future studies with larger cohorts will be necessary to determine whether this trend in males represents a reproducible and sex-specific neuroadaptation following prolonged oxycodone abstinence.

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 [13,16,21]. The anterior/posterior PVT have been shown to have different subcortical targets [61] and increasing evidence demonstrates substantial functional differences in anterior vs posterior PVT outputs [62]. Posterior projections to the NAc are selectively tuned to aversive stimuli, while the anterior PVT is more involved in reward-seeking behaviors [63]. 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, PVT-to-D2 MSN, and PVT-to-Interneurons in mediating drug-seeking or withdrawal after either acute or prolonged abstinence from opioid self-administration [13,21]. Thus, it would be interesting to explore, in future studies, the effects of our oxycodone administration regimens on neurotransmission in PVT projections to D1- or D2-MSNs, using *ex vivo* optogenetic studies analogous to those described in the present work.

Previous work has demonstrated cell-type-specific plasticity at PVT-NAc circuit following heroin self-administration and extinction. It includes strengthening PVT inputs to D2 MSNs and reducing synaptic strength from PVT to parvalbumin-expressing interneurons (PV-INs) [21]. In more detail, stimulation of PVT terminals in the NAcSh has been shown to increase opioid-seeking prior to, but not after, extinction of drug availability[13]. Consistent with this behavioral shift, extinction training can occlude the increase in synaptic strength at PVT to NAc D2 MSNs observed following heroin self-administration in mice [21]. This increase in synaptic strength was reflected by enhanced opto-evoked EPSC amplitude, an increased AMPA/NMDA ratio, and a decreased paired-pulse ratio (PPR), all consistent with potentiated excitatory transmission. PVT projections also synapse onto parvalbumin-expressing interneurons in the NAc (NAc PV-INs; decreased opto-evoked EPSCs and AMPA/NMDA ratios) [21], where an overall decrease in synaptic strength has

been reported after both self-administration and extinction [21]. In contrast, PVT-to-NAc D1 MSNs do not show changes in synaptic strength during self-administration or extinction [21]. However, a shift in rectification index following heroin self-administration suggests increased calcium-permeable AMPA receptor (CP-AMPAR) function at these synapses [21]. Importantly, restoring normal synaptic transmission in the PVT-to-NAc PV-IN pathway was sufficient to prevent relapse-like behavior, measured as active lever pressing for heroin [21]. Furthermore, a recent study found that prolonged abstinence period increased CP-AMPAR expression in D1 and D2 MSNs in the NAc of male and female rats [64].

In a morphine conditioned place preference (CPP) model, transient inhibition of the PVT-NAc pathway prevented morphine-primed relapse after both short (4 days) and prolonged (14 days) abstinence without affecting morphine-induced locomotor activity. Importantly, inhibition of this pathway disrupted relapse only when animals were re-exposed to the drug-associated context, indicating that PVT-NAc activity is required for the retrieval and maintenance of opiate-associated memories [55]. Consistent with a circuit architecture in which PVT inputs both directly excite MSNs and recruit local inhibitory microcircuits, our results show that stimulation of glutamatergic PVT terminals in the NAcSh evokes monosynaptic excitation alongside disynaptic inhibition mediated by local GABAergic interneurons. Following prolonged abstinence from oxycodone, excitatory synaptic transmission at PVT-NAcSh projections was strengthened, suggesting enhanced excitatory drive within this pathway. In contrast, inhibitory synaptic transmission was not significantly altered in either males or females, indicating that feedforward inhibitory recruitment and overall inhibitory tone within the PVT-NAcSh circuit remain intact following prolonged abstinence from oxycodone self-administration. Together, these findings suggest that prolonged oxycodone abstinence selectively potentiates excitatory rather than inhibitory synaptic transmission within this pathway, potentially shifting the excitation-inhibition balance in a manner that may contribute to relapse-related behaviors.

In our study, morphological analyses revealed no detectable changes in dendritic length or branching complexity in NAcSh MSNs at either 1 day or 14 days of abstinence following oxycodone self-administration. Additionally, no sex differences were observed in the somatodendritic morphology of NAcSh MSNs between control male and female rats. These findings suggest that the abstinence-dependent adaptations previously reported in this circuit may occur primarily through synaptic or molecular mechanisms, rather than through large-scale structural remodeling of MSN dendritic architecture.

Conclusion

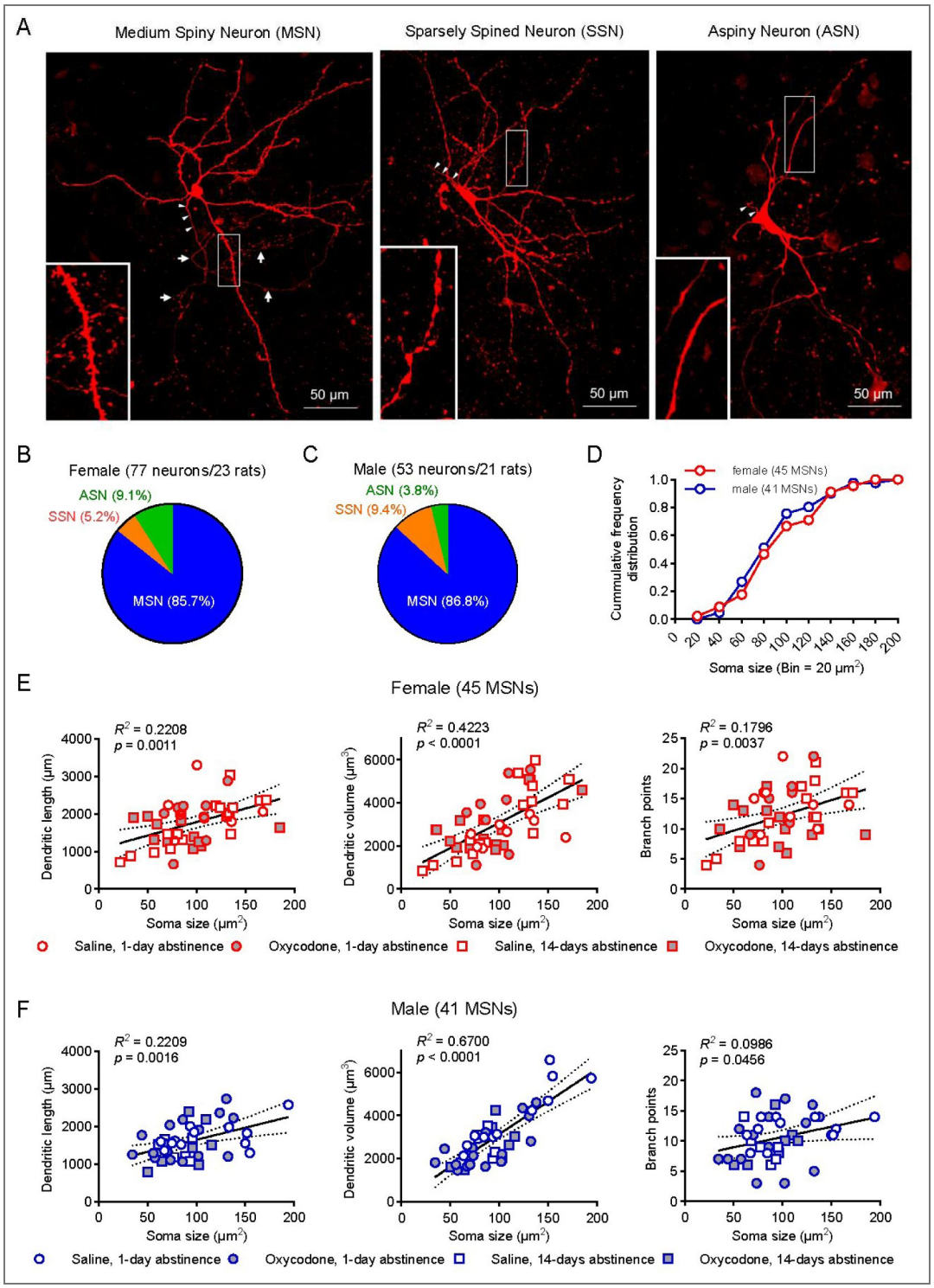
When considered alongside prior work, these results support a model in which the anatomical and cell-type-specific organization of PVT inputs to the NAcSh plays a critical role in relapse-related behaviors. Specifically, PVT projections onto D2 MSNs and PV interneurons may contribute to relapse vulnerability during early or acute abstinence, potentially through mechanisms linked to negative affective states. In contrast, adaptations involving D1 MSNs, including CP-AMPAR recruitment during prolonged abstinence, may underlie the incubation of craving that emerges after extended drug abstinence.

Overall, our findings provide further insight into the circuit mechanisms underlying oxycodone relapse and highlight the importance of considering cell-type specificity, abstinence duration, and behavioral context when interpreting plasticity within the PVT-NAc pathway. Importantly, differences across studies may also reflect variations in drug exposure paradigms, including contingent (self-administration) versus non-contingent drug delivery, as well as whether animals undergo extinction training or remain in forced abstinence. These experimental variables engage distinct learning processes and motivational states that can differentially shape circuit adaptations and relapse-related behaviors.

Future studies should aim to dissect the distinct contributions of anterior versus posterior PVT projections onto D1 and D2 MSNs in the NAcSh and determine how selective manipulation of these pathways influences relapse behaviors across different abstinence states and relapse triggers (e.g.,

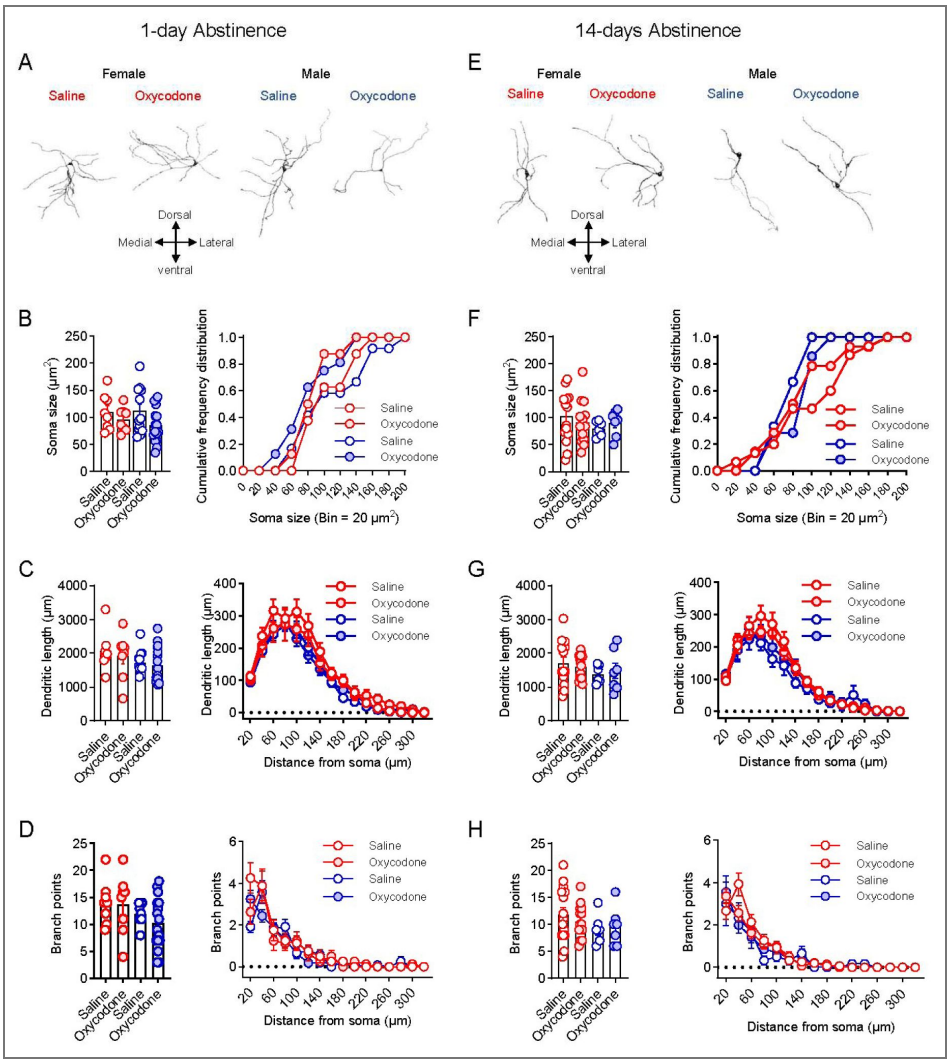
drug-, cue-, or stress-induced). In addition, understanding how thalamic inputs interact with cortical projections to ventral striatal circuits during abstinence and relapse represents an important avenue for future investigation.

Figure supplements



Supplementary Figure 1. Cell types in the NAcSh and variation in morphology of MSNs. A: Stacked confocal images show examples of a medium spiny neuron (**left**), a sparsely spine neuron (**middle**) and an aspiny neuron (**right**), respectively. Inset images show enlarged view of a segment of dendritic branch and its spines from the same neuron. Arrowheads in three panels indicate the initial segment of axons. Arrows in the left panel indicate

the axonal collaterals in the vicinity of its soma. **B-C**: Summary plot shows the percentages of three types of neurons observed in NAcSh in female and male rats. D: There is no difference in cumulative frequency distribution of soma size of examined MSNs between female (**red circles**) and male (**blue circles**) rats (p = 0.988, Kolmogorov-Smirnov test). **E-F**: Correlation analysis of soma sizes versus their dendritic length (**left**), dendritic volume (**middle**), or branch points (**right**) in female (**E**) and male rats (**F**). The values of the correlation coefficient (R2) and significance (p) are shown in each panel. The solid line in each panel represents the linear trendline (line of best fit) of the data, and dash lines represent 95% confidence interval.



Supplementary Figure 2. Dendritic morphology of NAcSh-MSNs remained unchanged at either 1-day or 14-days abstinence from oxycodone self-administration in both female and male rats.

A & E: Representative reconstructed NAcSh-MSNs from saline or oxycodone group in female (**left**, 8-14 cells from 5-7 rats among four treatment groups) and male (**right**, 6-16 cells from 3-8 rats among four treatment groups) rats at 1-day (**A**) or 14-days (**E**) abstinence, respectively. **B & F**: There is no difference in soma sizes (**left**, p = 0.077 or 0.352, respectively, two-way ANOVA) and their cumulative frequency distributions (**right**, p = 0.270 or 0.598, respectively, Kruskal-Wallis test) among treatment groups at 1 day (**B**) or 14 days (**F**) abstinence after oxycodone self-administration. **C & G**: No difference was observed in the total dendritic length (**left**, p = 0.504 or 0.972, respectively, two-way ANOVA) and Sholl analysis on dendritic length (**right**, p = 0.503 or 0.969, respectively, three-way ANOVA) between saline and oxycodone groups at 1-day or 14-days abstinence. **D & H**: No difference was observed in the total number of branch points (**left**, p = 0.499 or 0.738, respectively) and Sholl analysis on branch points (**right**, p = 0.221 or 0.738, respectively) between saline and oxycodone group at 1-day or 14-days abstinence. **Red and blue symbols** represent data sets from **female or male** rats, respectively. **Open or filled** symbols represent the data set of **saline or oxycodone**, respectively.

Data availability

All data is available upon request.

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Author Contributions

EHC, VYB, YAC and YL collaboratively designed, discussed, and planned all experiments. EHC secured funding for the studies. YAC, NJC, MAN, GSD and MM collected the behavioral data. YAC analyzed behavioral data. YAC and YL collected and analyzed electrophysiology data. YM and GKC analyzed morphological data. EHC and YAC wrote the manuscript. All authors contributed to the studies and approved the submitted version.

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Peer reviews

Reviewer #1 (Public review):

Summary:

This manuscript by Alonso-Caraballo et al, is a novel piece of work that examines the impact of oxycodone self-administration on neural plasticity within paraventricular thalamic (PVT) to nucleus accumbens shell (Shell) pathway - two regions shown to play a key role in cue-induced drug seeking on their own, and whether this plasticity varies based on abstinence period and biological sex.

Strengths:

The authors show using a clinically relevant long-access model of opioid self-administration promotes dependence and acute withdrawal in both male and female rats. During subsequent cue-induced relapse tests at 1 or 14-days following the conclusion of self-administration, data show that while both male and females demonstrate drug-seeking behavior at both time points, females show a further elevation in responding on day 14 versus day 1 that is not observed in the males. When accounting for past work showing elevations in drug seeking in males after 30 days, these data indicate that craving-induced

relapse for opioids may develop faster and may be more pronounced in females compared to males.

These behavioral findings were paralleled by use of ex vivo acute slice electrophysiology and circuit-specific ex vivo optogenetics to examine the impact of oxycodone self-administration on synaptic strength within the paraventricular thalamus (PVT) to nucleus accumbens shell (NAcSh) pathway(s). Data support a time-dependent but sex independent strengthening of glutamatergic signaling at PVT-to-NAcSh medium spiny neurons (MSNs) that is only present following a relapse test at 14 days post abstinence in males versus females, providing the first evidence that opioid self-administration and/or cue-induced drug-seeking augments this pathway. Using an extensive set of physiological measures, the authors show that this increased synaptic strength reflects a upregulation of presynaptic release probability. Further, this upregulation of excitatory signaling aligned temporally with an increase in MSN excitability, as assessed by increases in action potential firing frequency. Finally, the authors provide the first evidence that similar to other inputs to the NAcSh, PVT projections innervate both MSN as well as local interneurons, promoting a GABA-A specific feedforward inhibitory circuit. Interestingly, unlike direct excitatory inputs to MSNs, no changes were observed ostensibly within this feedforward circuit, highlighting a selective enhancement of excitatory drive and output of MSNs with protracted abstinence.

Overall, these data highlight a potential role for heightened synaptic strength within the PVT-NAcSh pathway in cue-induced relapse behavior during protracted abstinence and identify a potential therapeutic target during abstinence to reduce relapse risk in abstaining individuals.

Weaknesses:

Overall, the experimental approach and data provided appear rigorous and support their overall conclusions and achieve their goal of understanding how opioid self-administration impacts synaptic strength within the PVT-NAcSh pathway. Although not undermining these data, there are a few potential weaknesses that reduce the impact of the work. For example, the inability to directly assess whether cue-induced drug-seeking is in fact augmented compared to daily intake during self-administration in the maintenance face only permits the authors to denote that reexposure to cues and the context is sufficient to promote active lever pressing without demonstrating whether seeking behavior is in fact elevated further during a cue test. This is notably understandable as drug available sessions were 6-hours versus a 1hour relapse test. Importantly, it is clearly demonstrated that drug seeking is higher on average in female mice after 14 days versus 1 day.

With regard to interpretation of electrophysiology findings, the lack of inclusion of an abstinence only group does not permit interpretations to parse out whether observed increases in synaptic strength (or the lack of) reflect abstinence or an interaction between abstinence period and re-exposure to the operant chamber, as slices were taken 30-45 min post relapse test. While much literature has shown that drug induced adaptations in the NAc requires a post drug period for plasticity to measurably emerge, studies have also shown that re-exposure to heroin-associated cues following abstinence seemingly "reverses" increases in cell excitability in prelimbic-NAc pyramidal neurons (Kokane et al., 2023) and that depotentiation of morphine-induced increases in synaptic strength in the NAc shell can be depotentiated by drug re-exopsure -- an effect also observed with cocaine re-exposure (Madayag et al., 2019). Notably, the lack of effect at 14 but not 1 day supports the likelihood that the relapse test does not in fact influence the plasticity within the PVT-NAcSh circuit.

While the lack of effect on AMPAR:NMDAR ratio and rectification indices do support the notion that enhanced EPSC amplitudes in input-output curves do not reflect a change in AMPAR subunit expression (i.e., increased GluA2-lacking receptors that exhibit inward rectification at depolarized potential) nor a change in postsynaptic sensitivity to glutamate,

without direct assessment of AMPAR-specific and NMDAR-specific input-output curves, it doesn't definitively exclude the possibility that both AMPA and NMDA receptor currents are being upregulated, thus negating an observable change in postsynaptic strength.

Overall, these findings provide novel insight into how the PVT-NAcSh pathway is altered by opioid self-administration and whether this is unique based on abstinence period and sex. Importantly, these were the primary objectives stated by the author. Data highlight a potential role for the observed adaptations in relapse behavior and identify a potential therapeutic target during abstinence to reduce relapse risk in abstaining individuals. However, it should be noted that no causal link is demonstrated without experiments to reduce/prevent relapse.

Comments on revisions:

The authors addressed previous concerns brought up, specifically by clarifying data interpretation as well as text modifications related to potential caveats of these interpretations. However, I recommend that the title be changed to not focus on sex differences to avoid misunderstanding. The authors should also address the lack of difference physiologically compared to the behavior as a caveat more clearly in the discussion (i.e. likely suggests this isn't the pathway driving the difference).

https://doi.org/10.7554/eLife.102189.2.sa3

Reviewer #2 (Public review):

Summary:

This is an interesting paper from Alonso-Caraballo and colleagues that examines the influence of opioid use, acute and prolonged abstinence, and sex on cue-induced relapse and paraventricular thalamus (PVT) to nucleus accumbens shell (NAcSh) medium spiny neurons circuit physiology. The study presents a valuable finding that following prolonged, but not acute abstinence from oxycodone self-administration, female rodents exhibit higher relapse rates to drug paired cues. Additionally, the study presents the useful finding that prolonged abstinence increased PVT-NAcSh MSN synaptic strength in both sexes, an effect that is likely due to presynaptic adaptations. While the evidence to support these two findings is solid, further experiments are required to determine the functional role of the PVT-NAcSh MSN circuit in relapse following prolonged oxycodone abstinence, and the mechanism underlying the heightened relapse vulnerability in females in this model of opioid use disorder.

Strengths:

The paper is interesting, well written and presented, and the experiments are well designed and conducted. The revised analysis of spike count data that models the hierarchical structure of the data is appropriate to overcome low animal numbers and the potential for oversampling. The authors are transparent in reporting the results related to this analysis in figure 5 and acknowledge the study is underpowered to confirm the trend of increased intrinsic excitability in male MSNs following prolonged oxycodone analysis.

Weaknesses:

A major weakness of this study is the disconnect between the behavioral and neurophysiological data reported. While a striking sex difference in relapse-like behavior is observed, there are no statistically significant sex differences in any of the neurophysiological data reported. Moreover, without an experiment to functionally test the role of the PVT-NAc projection in relapse-like behavior following prolonged oxycodone these two arms of the study seem divorced.

While the authors don't directly conclude that the PVT-NAc MSN circuit is required for relapse following prolonged oxycodone abstinences, in the introduction the authors state they aim to test the hypothesis that increased synaptic strength in PVT-NAcSh projections are necessary for drug-seeking. This study does not include the required experiments to test this hypothesis.

Impact:

The topic is of interest to the field of substance use disorders and gives solid evidence for the need to consider targeted therapeutics aimed at relapse prevention in opioid use disorder.

https://doi.org/10.7554/eLife.102189.2.sa2

Reviewer #3 (Public review):

Summary:

Alonso-Caraballo et al. use behavioral testing and ex vivo patch-clamp electrophysiology combined with circuit-specific optogenetic stimulation of PVT terminals to examine how oxycodone self-administration and abstinence duration shape cue-induced relapse and PVT-NAcSh synaptic transmission in male and female rats. In the revision, the authors reanalyzed intrinsic excitability using nested hierarchical GLMMs, acknowledged the low power in the male prolonged-abstinence group, and expanded the discussion of relevant PVT-NAc literature. These changes improve the manuscript. That said, most of the revisions are textual and the main experimental gap remains. Both sexes show increased oxycodone seeking compared to saline at 14 days, but only females show a time-dependent incubation from 1 to 14 days, and the PVT-NAcSh synaptic strengthening is the same in both sexes. Nothing in the revision brings those two observations closer together. The excitability data also come from NAcSh MSNs with no confirmation of PVT connectivity, which limits what circuit-specific conclusions can be drawn. The study is a solid characterization of abstinence-related synaptic changes in this pathway, but some of the conclusions still go further than the data allow.

Strengths:

The behavioral characterization is thorough and well-executed, covering self-administration, somatic withdrawal, and cue-induced relapse across two abstinence durations in both sexes. The sex-specific escalation in oxycodone seeking from 1 to 14 days in females but not males is a clear and compelling finding. The use of circuit-specific ex vivo optogenetics to isolate PVT terminal inputs onto NAcSh neurons is a genuine methodological strength, and the demonstration of feedforward inhibitory recruitment through local GABAergic interneurons adds meaningful novelty to the circuit characterization. The reanalysis of intrinsic excitability using nested hierarchical GLMMs appropriately accounts for the non-independence of cells recorded within the same animal and is a real improvement over the original approach. The expanded discussion of prior PVT-NAc work, particularly the more accurate treatment of Keyes et al. (2020) and Paniccia et al. (2024), better situates the findings within the existing literature.

Weaknesses:

The core limitation of the study remains unchanged after revision. The PVT-NAcSh synaptic strengthening after prolonged abstinence is statistically indistinguishable between sexes, while females but not males show a time-dependent escalation in oxycodone seeking from 1 to 14 days of abstinence. The Discussion proposes hormonal modulation or differences in upstream inputs as possible explanations, but none of these are tested and the gap is left unresolved. The intrinsic excitability recordings come from NAcSh MSNs with no confirmation that those neurons receive direct PVT input, which was raised in the original

review, acknowledged in the revision, and not experimentally addressed. The male prolonged-abstinence excitability trend has approximately 20% statistical power and is non-significant, yet the Discussion interprets it as a potential neuroadaptation that could facilitate signal flow through the PVT-NAcSh circuit and contribute to relapse, which goes well beyond what the data support. The failure to distinguish between D1 and D2 MSNs remains a significant limitation given that cell-type-specific plasticity at PVT-NAc synapses has been shown to be directly relevant to opioid seeking in prior work. Finally, the Conclusion builds a mechanistic framework around D2 MSNs, PV interneurons, and D1 MSNs that is drawn from studies using different drugs or experimental designs, and none of these cell-type-specific mechanisms are tested in the present experiments.

https://doi.org/10.7554/eLife.102189.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *(1) Although not undermining these data, there are a few potential weaknesses that reduce the impact of the work. For example, the inability to directly assess whether cue-induced drug-seeking is in fact augmented compared to daily intake during self-administration in the maintenance face only permits the authors to denote that re-exposure to cues and the context is sufficient to promote active lever pressing without demonstrating whether seeking behavior is in fact elevated further during a cue test. This is notably understandable as drug available sessions were 6-hours versus a 1-hour relapse test. Importantly, it is clearly demonstrated that drug seeking is higher on average in female mice after 14 days versus 1 day.*

We agree that the current design does not allow us to directly assess whether cue induced drug-seeking is augmented relative to the average self-administration intake. However, this comparison was not a question examined in the manuscript and was not an intended interpretation of the data. Our analyses and interpretations focused on comparisons between saline and oxycodone groups tested under identical cue-induced relapse conditions. While it does not change or contradict the reviewer's point, we would also like to clarify that the relapse test was 2 hours long.

> *(2) With regard to the interpretation of electrophysiology findings, the lack of inclusion of an abstinence-only group does not permit interpretations to parse out whether observed increases in synaptic strength (or the lack of) reflect abstinence or an interaction between abstinence period and re-exposure to the operant chamber, as slices were taken 30-45 min post relapse test.*

The inclusion of an abstinence-only control group would have been required to definitively dissociate synaptic changes driven by abstinence alone from those arising from an interaction between abstinence and re-exposure to the operant context during the relapse test. In the present study, electrophysiological recordings were intentionally performed 30 to 45 minutes following the relapse test to capture synaptic modifications associated with cue-induced drug-seeking after abstinence. Accordingly, we interpret these findings as reflecting the neural state following relapse rather than abstinence alone, and we have revised the text accordingly to clarify this point.

> *(3) With regard to the interpretation of electrophysiology findings, the lack of inclusion of an abstinence-only group does not permit interpretations to parse out whether observed*

> *increases in synaptic strength (or the lack of) reflect abstinence or an interaction between abstinence period and re-exposure to the operant chamber, as slices were taken 30-45 min post relapse test. While much literature has shown that drug-induced adaptations in the NAc require a post-drug period for plasticity to measurably emerge, studies have also shown that re-exposure to heroin-associated cues following abstinence seemingly "reverses" increases in cell excitability in prelimbic-NAc pyramidal neurons (Kokane et al., 2023) and that depotentiation of morphine-induced increases in synaptic strength in the NAc shell can be depotentiated by drug re-exposure - an effect also observed with cocaine re-exposure (Madayag et al., 2019). Notably, the lack of effect at 14 but not 1 day supports the likelihood that the relapse test does not in fact influence the plasticity within the PVT-NAcSh circuit.*

We thank the reviewer for highlighting relevant literature showing that drug or cue re exposure can modify or reverse drug-induced plasticity in NAc-related circuits. We want to clarify that, in our dataset, synaptic changes in the PVT-NAcSh pathway are seen after 14 days of abstinence, but not after 1 day. Therefore, the lack of effect at the earlier time point and its appearance after extended abstinence support the idea of time-dependent plasticity. Although electrophysiological recordings were taken soon after the relapse test, this temporal pattern argues against relapse testing alone as the primary driver of the observed synaptic changes. We have updated the text to clarify this point.

> *(4) While the lack of effect on AMPAR:NMDAR ratio and rectification indices do support the notion that enhanced EPSC amplitudes in input-output curves do not reflect a change in AMPAR subunit expression (i.e., increased GluA2-lacking receptors that exhibit inward rectification at depolarized potential) nor a change in postsynaptic sensitivity to glutamate, without direct assessment of AMPAR-specific and NMDAR-specific input output curves, it doesn't definitively exclude the possibility that both AMPA and NMDA receptor currents are being upregulated, thus negating an observable change in postsynaptic strength.*

We agree that unchanged AMPAR/NMDAR ratios and rectification index suggest against altered AMPAR subunit composition or simple postsynaptic sensitivity changes. Although receptor-specific input-output analyses would be necessary to definitively rule out proportional increases in both AMPA and NMDA receptor currents, we have updated the manuscript to clarify that our conclusions are limited to the synaptic measures we obtained. The revised text now states that acute or prolonged abstinence “might have no detectable postsynaptic effects as assessed by these synaptic measures” at PVT-NAcSh synapses.

> ***Reviewer #2 (Public review):***
>
> *(5) While this paper is certainly interesting, and well-written, and the experiments seem to be well performed, the behavioral and physiological effects observed are somewhat divorced. Specifically, what accounts for the heightened relapse in females? Since no opioid-related sex differences were observed in PVT-NAcSh neurophysiology, it is unclear how the behavioral and neurophysiological data fit together. Furthermore, the lack of functional manipulation of PVT-NAcSh circuitry leaves one to wonder if this circuit is even important for the behavior that the authors are measuring. I would be more positive about this study if the authors were able to resolve either of the two issues noted above.*

A key challenge in circuit-based studies of motivated behavior is connecting circuit-level plasticity to complex, sex-dependent behavioral phenotypes. In this study, we do not mean to imply that synaptic plasticity within the PVT-NAcSh projection alone explains the increased relapse seen in females. Instead, our electrophysiological data indicate that this projection experiences time-dependent, abstinence-dependent changes in synaptic strength, offering important insights into when and where circuit-level adaptations may occur. We also believe that the lack of obvious sex differences in PVT-NAcSh synaptic strength does not rule out this

circuit's role in sex-specific behavior. Growing evidence suggests that sex differences in relapse and motivated behaviors may stem from different modulation of shared circuits (for example, via ovarian hormones, neuromodulatory tone, or upstream inputs), rather than from significant differences in baseline synaptic properties within a given projection. Regarding circuit relevance, extensive previous research has identified the PVTNAcSh pathway as a critical regulator of cue-induced reward seeking and relapse. Our findings expand on this by showing that this projection displays abstinence-dependent synaptic strengthening after oxycodone self-administration. Although functional manipulation of this circuit is needed to confirm its causal role, such experiments were beyond the scope of this study.

> *(6) There are insufficient animals in some cases. For example, in Figure 4, the Male Saline 14-day abstinence group (n = 3 rats) has less than half of the excitability as compared to the Male Saline 1-day abstinence group (n = 7 rats). This is likely due to variance between animals and, possibly, oversampling. Thus, more rats need to be added to the 14-day abstinence group. Additionally, the range of n neurons/rat should be reported for each experiment to ensure readers that oversampling from single animals is not occurring.*

We appreciate the reviewer's concern regarding the number of animals and the potential for oversampling. We take this concern seriously and have substantially revised our statistical approach in response.

All spike count data were reanalyzed using nested hierarchical Poisson generalized linear mixed-effects models (GLMMs), fitted separately for each sex and abstinence duration. Each model included injected current (mean-centered), drug condition, and their interaction as fixed effects, with random intercepts and slopes for injected current at the animal level, and random intercepts for cells nested within animals. Importantly, this reanalysis changed several of our original conclusions. Effects that appeared significant under the conventional cell-level analysis were no longer statistically significant once the hierarchical structure of the data was properly modeled. We report these corrected results transparently throughout the revised manuscript.

However, in males after prolonged abstinence, oxycodone-treated animals showed a higher spike output than controls, with a large effect size. Post-hoc analysis showed only 20% power with current sample (3 saline, 4 oxycodone rats). To reach 80% power, 13 rats per group are needed. We report this as a trend that warrants further study and have revised related sections to reflect this. The data suggest a possible neuroadaptation in males that the study is underpowered to confirm, not a null effect.

In response to this comment, we have updated Figure 5, the Results and Discussion sections, and the Statistics/Methods section to clearly describe the nested hierarchical modeling approach, report corrected statistical values, and acknowledge the power limitation for the male prolonged abstinence group. The figure legend now reports the number of neurons recorded per rat, showing the distribution across animals rather than individual subjects.

> *(7) The IPSC data, for example in Figure 4, is one of the more novel experiments in the manuscript. However, it is quite challenging to see the difference between males and females, saline and oxycodone, at low stimulation intensities within the graph. Authors should expand this so that reviewers/readers can see those data, especially considering other work suggesting that PVT synaptic input onto select NAc interneurons is disrupted following opioid self-administration. Additional comment: It's also interesting that the IPSC amplitude seems to be maximal at ~2mW of light, whereas ~11 mW is required to evoke maximal EPSC amplitude. It would be interesting to know the authors' thoughts on why this may be.*

While visual separation between conditions at low light levels is subtle, we addressed this directly using linear mixed-effects modeling, which evaluates IPSC amplitudes across the full range of stimulation intensities while accounting for repeated measurements from cells nested within animals. This approach provides greater sensitivity than visual inspection alone and avoids over interpretation of noise at individual stimulation levels.

Using this framework, we observed robust main effects of light intensity in both males and females, indicating preserved recruitment of inhibitory synaptic responses as stimulation increased. Importantly, no significant Light × Condition interactions were detected in either sex, indicating that the scaling of IPSC amplitudes with light intensity was not altered by oxycodone exposure.

With respect to the observation that IPSC amplitudes appear to reach near-maximal levels at lower light intensities (~2 mW) compared to EPSCs (~11 mW), we agree that this distinction is intriguing. One possible explanation is that the depend on the recruitment of local interneurons. However, the number of interneurons activated by PVT interneurons is limited and inhibitory responses may reach a plateau at relatively low light intensities once these interneurons are fully recruited.

On the other hand, the increased intensity of photostimulation would result in an increase of monosynaptic EPSC amplitude over a wider range of stimulation (light) intensities, as increased intensity of light would recruit more ChR2-expressing PVT fibers, resulting in larger EPSCs.

> *(8) There is an inadequate description of what has been done to date on the PVT-NAc projection regarding opioid withdrawal, seeking, disinhibition, and the effects on synaptic physiology therein. For example, a critical paper, Keyes et al., 2020 Neuron, is not cited. Additionally, Paniccia et al., 2024 Neuron is inaccurately cited and insufficiently described. Both manuscripts should be described in some detail within the introduction, and the findings should be accurately contextualized within the broader circuit within the discussion.*

In the revised manuscript, we expanded the Discussion to give a more thorough overview of previous research on the PVT-NAc pathway in relation to opioid-related behaviors and synaptic changes. Specifically, we added more detail about Keyes et al., 2020 and Paniccia et al., 2024, clarifying their findings and placing them within the context of the circuit mechanisms studied in our work. We also revised the text to ensure the descriptions of these studies are accurate and that their conclusions are properly related to our findings.

> *(9) Related to the above, the authors should provide a more comprehensive description of how PVT synapses onto cell-type specific neurons in the NAc which expand beyond MSNs, especially considering that PVT has been shown to influence drug/opioid seeking through the innervation of NAc neurons that are not MSNs. For example, see PMIDs 33947849, 36369508, 28973852, 38141605.*

In the revised manuscript, we expanded the Discussion to describe the diversity of PVT projections within the NAc and the potential role of non-MSN neuronal populations in drug-related behaviors. We added discussion on the broader circuit context and other cell types where relevant to the focus on synaptic transmission onto MSNs. Since our experiments specifically examined synaptic physiology in MSNs, we focused the literature discussion on studies most directly related to MSNtargeted PVT inputs and opioid-related behaviors.

> ***Reviewer #3 (Public review):***
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> *(10) Additional experiments could strengthen the results and help clarify synaptic mechanisms underpinning behavioral sex differences.*

We agree that additional experiments focused on identifying cell-type-specific mechanisms within the PVT-NAcSh circuit would further enhance understanding of the neural substrates behind the observed behavioral sex differences. In the revised manuscript, we have expanded the Discussion to explicitly acknowledge these limitations and clarify the scope of our current study. Specifically, we discuss the possibility that sex-specific adaptations might occur in particular neuronal subpopulations or circuit components that were not resolved in the present experiments. We also mention that future research using cell-type–specific approaches will be necessary to determine if such mechanisms contribute to the increased oxycodone seeking seen in females after prolonged abstinence. We appreciate the reviewer's suggestions and have incorporated this perspective into the revised manuscript to better contextualize our findings and outline future directions.

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