Received: 31 August 2021 | Revised: 11 December 2021 | Accepted: 10 January 2022

DOI: 10.1111/adb.13148

ORIGINAL ARTICLE

Addiction Biology SSA WILEY

Fos-expressing neuronal ensembles in rat infralimbic cortex encode initial and maintained oxycodone seeking in rats

Christina Gobin | Bo Sortman | Samantha Rakela | Richard Quintana-Feliciano | Brandon L. Warren

Department of Pharmacodynamics, University of Florida, Gainesville, Florida, USA

Correspondence
Brandon Warren, Department of Pharmacodynamics, University of Florida, Gainesville, FL 32610, USA.
Email: brandon.warren@ufl.edu

Funding information
National Institute on Drug Abuse, Grant/Award Numbers: DA042102, 4R00DA042102-02; Brain and Behavior Research Foundation

Abstract

Neuronal ensembles within the infralimbic cortex (IL) and their projections to the nucleus accumbens (NAc) mediate opiate seeking in well-trained rats. However, it is unclear how early this circuitry is recruited during oxycodone self-administration. Here, we used retrograde labelling (CTb) and immunohistochemistry to identify NAc-projecting neurons in the IL that were activated during initial oxycodone seeking. Next, we sought to determine the role of IL neuronal ensembles in initial oxycodone self-administration. We used the Daun02 procedure in male and female *Fos-LacZ* rats to chemogenetically inactivate IL Fos-expressing neurons at different time points in oxycodone self-administration training: immediately after meeting criteria for acquisition of behaviour and following nine daily sessions with increasing schedules of reinforcement (FR1, FR2 and FR3) in which rats demonstrated stable oxycodone intake under increasing effort to self-administer. We found that Daun02 infusions attenuated oxycodone seeking at both the initial learning and well-trained time points. These results suggest that IL neuronal ensembles are formed during initial learning of oxycodone self-administration and required for the maintenance and expression of oxycodone seeking.

KEYWORDS
acquisition, chemogenetics, opiates

1 | INTRODUCTION

Oxycodone is an addictive opioid analgesic that has contributed to the ongoing opioid epidemic.[1] In humans and preclinical animal models, drug-paired contexts and cues become powerful indicators of drug availability that can induce craving and drug seeking.[2] Preclinical models of substance use commonly rely on intravenous drug self-administration, wherein discrete cues are paired with delivery of the drug reinforcer.[3,4] This procedure models the behavioural stages of substance use including initiation of drug taking (acquisition), stable drug intake (maintenance), increased drug intake (escalation) and reinstatement of drug seeking (relapse). Studies employing drug self-administration have shown that projections from the medial prefrontal cortex (mPFC) to the nucleus accumbens (NAc) drive drug-seeking behaviours in response to drug-paired stimuli.[5–15] Pharmacological inactivation studies have implicated the prelimbic cortex (PL) and infralimbic cortex (IL) regions of the mPFC as well as their projections to the NAc core and NAc shell in heroin-primed, cue-induced and context-induced reinstatement of heroin seeking in well-trained rats.[5,16–18] It is currently unknown whether this same circuitry is implicated in initial oxycodone seeking.

A popular hypothesis suggests that drug rewards and associated cues activate sparsely distributed neurons that form neuronal ensembles to mediate drug associated behavioural responses.[19] Pharmacogenetic manipulations of neuronal ensembles find that IL neuronal ensembles control context-induced reinstatement of heroin seeking in well-trained rats.[20] However, it is currently unknown at what stage these neuronal ensembles are recruited and if neuronal ensembles

recruited early on during oxycodone self-administration (initial neuronal ensembles) within the mPFC mediate initial oxycodone seeking. Here, we hypothesize that neurons from the IL-NAc circuitry are rapidly recruited during initial oxycodone self-administration and that these neuronal ensembles continue to mediate oxycodone seeking in well-trained rats. We designed the following experiments to determine if initial oxycodone-paired neuronal ensembles are activated within the IL after initial oxycodone seeking and proceed to test the role of IL ensembles in mediating initial and maintained oxycodone seeking.

In Experiment 1, we sought to determine whether IL-NAc-projecting neurons are activated during initial oxycodone seeking. Using wildtype rats, we conducted a retrograde tracing experiment with fluorescent cholera toxin subunit B (CTb) as previously described.[21–24] We microinjected CTb-594 unilaterally into the NAc shell and CTb-488 unilaterally into the NAc core. Following microinjection, CTb is retrogradely transported along axons to the soma of projecting neurons.[25,26] We trained rats to lever press for intravenous oxycodone until they reached acquisition criteria. We found that after initial acquisition of oxycodone self-administration, rats had greater Fos labelling in neurons projecting from the IL to the NAc core and shell subregions compared with experimentally matched home-cage controls.

Next, we applied the Daun02 inactivation procedure (which silences Fos-expressing neurons) to determine the necessity of IL neuronal ensembles in driving oxycodone seeking after initiation of oxycodone self-administration (Experiment 2) as well as in oxycodone seeking after prolonged self-administration (Experiment 3 and 4). We demonstrate that after initial learning as well as after maintenance of oxycodone self-administration, IL neuronal ensembles are necessary for oxycodone seeking.

2 | METHODS

2.1 | Subjects

We used male and female Sprague Dawley wildtype rats ($n = 20$) as well as male and female *Fos-lacZ* transgenic rats ($n = 64$), weighing 175–400 g at the beginning of the experiments. Rats were individually housed, maintained on a reverse 12-h light/dark cycle (lights off at 10:00 AM), and given ad libitum access to water and food. All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee. A total of two wildtypes and eight *Fos-LacZ* transgenic rats failed to meet acquisition criteria during training and were excluded. Eight *Fos-LacZ* transgenic rats were excluded for misplaced cannulas and two *Fos-LacZ* transgenic rats were excluded from Experiment 4 as significant outliers (greater than 3 SD from the mean).

2.2 | Surgery

We anesthetized rats with isoflurane (5% induction and 3% maintenance) during all surgical procedures and administered buprenorphine (0.03 mg/kg s.c.) and meloxicam (5 mg/kg s.c.) for 3 days as post-operative analgesia. We allowed rats to recover for 5 days prior to initiating behavioural testing.

2.3 | Intravenous catheterization surgery

In all experiments (1–4), we implanted SILASTIC catheters into the jugular vein as described previously.[24,27] We constructed catheters such that 3 cm of tubing entered the jugular vein, whereas the other end of the tubing was affixed to a modified 22-gauge cannula adhered to a custom-made backmount. We threaded the proximal end of the tubing subcutaneously between the shoulder blades and ported the modified cannula through the midscapular region of the back. We flushed the catheters daily with gentamicin in sterile saline (4.25 mg/ml; APP Pharmaceuticals).

2.4 | Intracranial CTb injections

In Experiment 1, we contralaterally microinfused (0.3 μl per side) fluorescent CTb-488 (50 μg/μl; Thermo Fisher, Ref: C22841, Lot: 2155272) into the NAc core [anteroposterior: +1.6, mediolateral: ±2.5, and dorsoventral: −6.5 (10° angle)] and CTb-594 (50 μg/μl; Thermo Fisher, Ref: C22842, Lot: 2160063) into the NAc shell [anteroposterior: +1.6, mediolateral: ±2.5 and dorsoventral: −7.3 (10° angle)] using a Micro4 Microsyringe Pump Controller (World Precision Instruments) and 10-μl Hamilton syringes. We infused over 1 min and left the injectors in place for at least 1 min before removal. We chose the concentration and volume based on previous studies.[24]

2.5 | Intracranial cannula implantation

In Experiments 2 and 4, we implanted permanent guide cannulas (23-gauge, Plastics One) bilaterally 1 mm above the IL. The nose bar was set at −3.3 mm, and the coordinates for the IL were anteroposterior: +3.0, mediolateral: ±1.5 and dorsoventral: −3.8 (10° angle). We fixed cannulas to the rat's skull with dental cement and jeweller's screws. We selected these coordinates based on pilot and previous studies.[20,24,28]

2.6 | Intracranial infusions

In Experiments 2 and 4, we performed intracranial infusions using a syringe pump (Kent Scientific) and 10-μl Hamilton syringes that were attached via polyethylene tubing to 30-gauge injectors (RWD) that extended 1 mm beyond the guide cannula. We infused 0.5 μl of Daun02 or vehicle over 1 min and left the injectors in place for an additional 1 min before removal, as previously described.[20,24,28]

2.7 | Drugs

We received oxycodone hydrochloride from the National Institute on Drug Abuse Drug Supply Program. We selected a dose of 0.1 mg/kg per infusion with a volume of 0.1 ml per infusion for self-administration based on pilot testing. We obtained Daun02 from MedChemExpress and dissolved 2 μg/0.5 μl per side in vehicle solution containing 5% DMSO, 6% Tween 80, and 89% 0.01-M PBS. We chose the dose of Daun02 based on previous studies.[20,23,27–30]

2.8 | Self-administration apparatus

We habituated, trained and tested rats in Med Associates self-administration chambers, each equipped with a house light, fan, retractable active and inactive levers and a cue light positioned above the active lever. The house light and fan remained on throughout the session. Pressing the active lever resulted in activation of the cue light directly above the active lever, initiation of the tone cue and delivery of a 0.1 mg/kg per infusion of oxycodone over a period of 7 s with a 10-s timeout during which active lever presses did not result in additional infusions. Pressing the inactive lever had no programmed consequences. Induction and test sessions were identical to self-administration sessions described above but were shortened to 30 min and were nonreinforced.

2.9 | Intracranial Daun02 injection

We performed intracranial injections using a syringe pump (Harvard Apparatus) and 10-μl Hamilton syringes that were attached via polyethylene-50 tubing to 30-gauge injectors (Plastics One) that extended 1 mm beyond the guide cannula. We infused 0.5 μl over 1 min and left the injectors in place for 1 min before removal.

2.10 | Fos immunofluorescence and CTb imaging and analysis

We used immunohistochemistry to visualize Fos protein expression in brain tissue. We washed coronal brain sections (40 μm) in 1X PBS, blocked with 2.5% normal goat serum (NGS) in 1X PBS with 0.02% Triton X-100 (PBS-Tx), and incubated 48 h at 4°C with Phospho-c-Fos (Ser32) antibody (1:2000 dilution; Cell Signaling Technology Cat# 5348S, RRID:AB_10557109) in blocking solution. We then washed sections in 1X PBS, and incubated them with Alexa Fluor 647 conjugated goat anti-rabbit secondary antibody (1:500 dilution; Thermo Fisher Scientific Cat# A-11008, RRID:AB143165). Next, we washed the sections in 1X PBS, mounted them onto polysine precoated microscope slides and coverslipped them with DAPI Fluoromount-G (Southern Biotech).

Fluorescent images were captured using a Keyence BZ-X810 microscope at 200X magnification with BZ-X Analyzer software. We used ImageJ to manually quantify fluorescently labelled cells in the IL. Two brain sections (four hemispheres) were quantified per rat, and these counts were averaged to return a representative count for each individual rat. For Experiment 1, we captured fluorescent images of immunoreactive cells, CTb-488 and CTb-594 in the IL at 200× magnification. Observers (SR and CG) blind to the test conditions used ImageJ to manually count total number of cells labelled with CTb-488, CTb-594, and Fos, as well as cells colabelled with CTb-488 + Fos and CTb-594 + Fos (bilateral) per rat (four images per rat) and averaged the counts from individual rats.

2.11 | Cresyl violet staining

We performed cresyl violet staining to check placement for CTb injection in the NAc (Experiment 1) and cannula placement in the IL (Experiments 2 and 4). We dehydrated 40 μm coronal sections containing NAc (Experiment 1) and IL (Experiments 2 and 4) in graded concentrations of ethanol (100%, 95% and 70%), rehydrated sections in distilled water, stained with Cresyl Violet Acetate solution, rehydrated in distilled water, dehydrated with ethanol (70%, 95% and 100%), cleared with Citrasolv (Fisher Scientific), and coverslipped with Permount (Sigma Aldrich). We captured images using a Keyence BZ-X810 microscope at 40× magnification with BZ-X Analyzer software.

2.12 | Experimental design and statistical analysis

2.12.1 | Experiment 1: CTb labelling of IL to NAc projections after initial oxycodone learning

The purpose of Experiment 1 was to capture the time point of initial learning of oxycodone self-administration and determine whether initial oxycodone-seeking behaviour would preferentially activate subsets of IL neurons that project to the NAc core or shell. Experiment 1 was a single-factor between-subjects design with two groups (test vs. no test). The experimental timeline for Experiment 1 is shown in Figure 1. We first injected CTb-488 and CTb-594 unilaterally into the NAc core or shell, respectively. We then trained rats to self-administer oxycodone (0.1 mg/kg/inf) under an FR1 schedule of reinforcement in operant chambers (described above) for 3 h/day with a 10-s timeout until rats met an acquisition criterion of at least 30 active lever presses and ≥70% responding on the active lever compared with the inactive lever. We used this criterion to operationalize initial learning of oxycodone self-administration. Once a rat reached criterion, we discontinued further training sessions and matched each rat to a test or no test group based on a similar number of active lever presses on the day oxycodone self-administration was acquired. Twenty-four hours after the training session in which a rat met acquisition criteria, rats in the test group were re-exposed to the operant chamber for 30 min and given access to both levers under nonreinforced conditions, wherein presses on the active lever resulted in the light and tone cues but no drug infusion. Rats in the no test group remained in

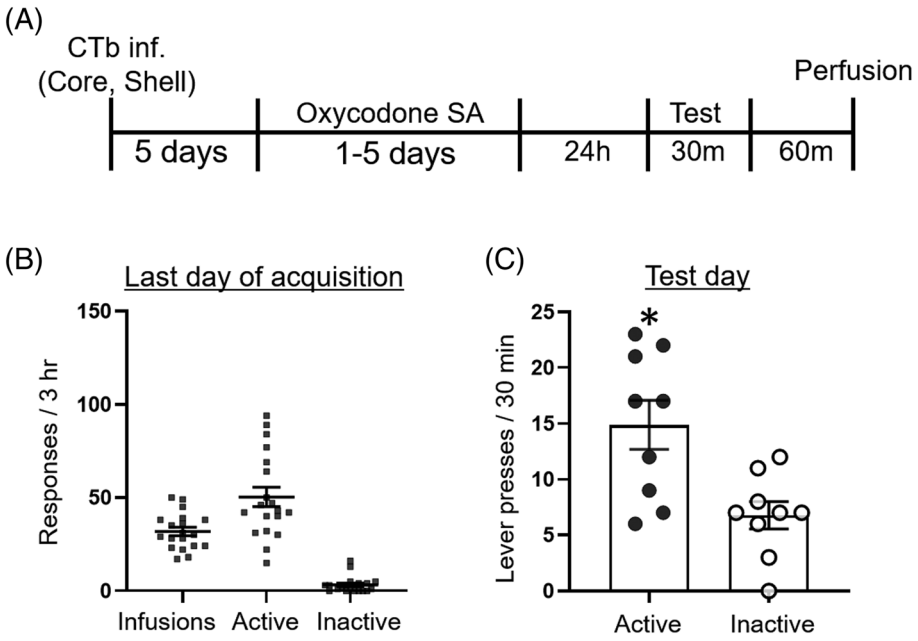

FIGURE 1 Experiment 1: CTb labelling of IL-NAc projections after initial oxycodone learning. (A) Timeline showing the behavioural procedure. We microinfused CTb into the NAc core and shell subregions of wildtype rats. We trained rats to lever press for infusions of oxycodone for one to five 3-h daily sessions until they reached acquisition criteria of at least 30 active lever presses with ≥70% discrimination from the inactive lever. We tested rats 24 h later under extinction. (B) Number of infusions, active lever presses and inactive lever presses on the day acquisition criterion was met. (C) Average number of active versus inactive lever presses during the 30-min test day session. Data are presented as mean ± SEM ($n = 9$). *$p < 0.05$ compared with inactive lever presses

their home cages. We reasoned that re-exposure to the drug context and cues without reward delivery would reactivate the initial oxycodone-paired neuronal ensemble (ensemble formed after initial learning of oxycodone self-administration) for rats in the test group. On the other hand, rats in the no test group should exhibit baseline Fos expression unrelated to initial oxycodone seeking. We transcardially perfused rats in the no test group directly from their home cages and rats in the test group 90 min after the start of the test. We then harvested all brains for Fos immunofluorescence and CTb quantification. We quantified Fos and CTb colabelling to assess activity-dependent labelling in IL projections to the NAc following reactivation of the initial oxycodone-paired ensemble in order to identify a neural substrate for the initial oxycodone-seeking memory.

2.12.2 | Experiment 2: Role of IL Fos-expressing ensembles in initial oxycodone seeking

The purpose of Experiment 2 was to determine whether neuronal ensembles in the IL play a causal role in initial oxycodone self-administration. This experiment was a single-factor between-subjects design with two groups (vehicle vs. Daun02). We trained *Fos-lacZ* rats to self-administer oxycodone (0.1 mg/kg/inf) under an FR1 schedule of reinforcement in operant chambers (described above) for 3 h/day with a 10-s timeout until rats met an acquisition criterion for initial oxycodone self-administration of at least 30 active lever presses and ≥70% responding on the active lever compared with the inactive lever. Once a rat reached criteria, we discontinued further training sessions and matched each rat to either the vehicle or Daun02 group based on a similar number of active lever presses on the day oxycodone self-administration was acquired. On induction day, we re-exposed rats to the self-administration operant chamber for 30 min and gave them access to both levers under nonreinforced conditions, wherein presses on the active lever resulted in the light and tone cues, but no drug infusion delivery. The purpose of the induction session was to induce Fos expression associated with reactivation of the initial oxycodone-paired neuronal ensemble (ensemble formed after initial learning of oxycodone self-administration).

Ninety minutes after the start of the induction session, we microinfused vehicle or Daun02 into the IL. Two days later, to allow for Daun02 apoptotic effects, we tested the rats under identical nonreinforced conditions in a recall test to compare active lever pressing between vehicle and Daun02 groups. Ninety minutes after the start of the recall test, we transcardially perfused rats with 4% PFA, extracted their brains, and processed their brains to check placement and to quantify Fos immunofluorescence.

2.12.3 | Experiment 3: Fos induction after maintenance of oxycodone seeking

The purpose of Experiment 3 was to determine whether Fos expression is induced following recall of oxycodone self-administration in well-trained rats. Experiment 3 was a single-factor between-subjects design with two groups (test vs. no test). All rats underwent 9 days of oxycodone self-administration, wherein training occurred under increasing schedules of reinforcement (3 days at FR1, 3 days at FR2 and 3 days at FR3). We selected a longer training period under increased FR schedules of reinforcement to demonstrate stable oxycodone intake under increased effort to self-administer. We reasoned that increased active lever pressing commensurate with increased FR demand would denote a point past initial learning of oxycodone self-administration when oxycodone taking is well-trained and maintained. We then forced rats to undergo 1 week of home-cage abstinence prior to the Fos induction test in order to allow for oxycodone washout and avoid acute withdrawal effects during behavioural testing.

We matched rats to test and no test groups based on a similar number of active lever presses on the last day of oxycodone self-administration prior to the induction test. On induction day, we re-exposed rats in the test group to the self-administration operant chamber for 30 min with access to both levers under nonreinforced conditions, wherein presses on the active lever resulted in the light and tone cues but no drug infusion. Rats in the no test group remained in their home cages. We reasoned that re-exposure to the drug context and cues without reward delivery would reactivate the well-trained oxycodone-paired neuronal ensemble (ensemble formed after well-trained and maintained oxycodone self-administration) for rats in the test group. On the other hand, rats in the no test group should exhibit baseline Fos expression unrelated to maintained oxycodone seeking. We then transcardially perfused rats in the no test group from their home-cages and rats from the test group 90 min after the start of the induction session. We harvested and later processed all brains for Fos immunofluorescence.

2.12.4 | Experiment 4: Role of IL Fos-expressing ensembles in the maintenance of oxycodone seeking

The purpose of Experiment 4 was to determine whether IL neuronal ensembles associated with maintained oxycodone self-administration mediate oxycodone seeking. Experiment 4 was a single-factor between-subjects design with two groups (vehicle vs. Daun02). In Experiment 4, all rats underwent 9 days of oxycodone self-administration, wherein training occurred under increasing schedules of reinforcement (3 days at FR1, 3 days at FR2 and 3 days at FR3). We selected a longer training period under increased FR schedules of reinforcement to demonstrate stable oxycodone intake under increased effort to self-administer. We reasoned that increased active lever pressing commensurate with increased FR demand would denote a point past initial learning of oxycodone self-administration when oxycodone taking is well-trained and maintained. We then forced rats to undergo 1 week of home-cage abstinence prior to the induction test to avoid acute withdrawal effects on behavioural responding. We matched rats to vehicle and Daun02 groups based on a similar number of active lever presses on the last day of self-administration prior to the induction test. On induction day, we re-exposed rats to the operant chamber for 30 min with access to both levers under nonreinforced conditions, wherein presses on the active lever resulted in the light and tone cues but no drug infusion. The purpose of this induction test was to induce Fos expression associated with reactivation of the oxycodone-paired neuronal ensemble formed after maintained oxycodone self-administration. Ninety minutes after the start of the induction test, we microinfused vehicle or Daun02 into the IL. Two days later, to allow for Daun02 apoptotic effects, we tested the rats under identical nonreinforced conditions in a recall test to compare active lever pressing between vehicle and Daun02 groups. Ninety minutes after the start of the recall test, we transcardially perfused rats and harvested their brains to check cannula placement and to quantify Fos immunofluorescence.

2.13 | Statistical analysis

We analysed all data using GraphPad Prism (Version 9.2.0) software, setting the alpha level at 0.05 for all statistical analyses. We used paired and unpaired Student's *t* tests to analyse behavioural and immunohistochemical data when appropriate. We also used two-way repeated measures analysis of variance (ANOVAs) followed by Holm–Sidak's post hoc tests where appropriate. We found no sex differences in test-day lever pressing in any experiment ($p > 0.05$) and so collapsed data obtained from male and female rats for all analyses.

3 | RESULTS

3.1 | Experiment 1: Fos and CTb colabelling of IL-NAc projections after initial oxycodone seeking

Figure 1A shows the experimental timeline for Experiment 1. Figure 1B shows the total number of infusions, active lever presses and inactive lever presses on the day rats met acquisition criteria for oxycodone seeking, prior to group assignment to the test or no test conditions. Figure 1C shows the number of active versus inactive presses on test day. Rats pressed significantly more on the active versus inactive lever on test day ($t_{(8)} = 6.2$, $p = 0.0003$). Figure 2A shows the placement for CTb injections in the NAc core and shell subregions. Figure 2B shows representative images of Fos, CTb-594 and CTb-488 labelling as well as Fos + CTb-594 + CTb-488 colabelling in the IL of rats killed 90 min (a time point associated with peak Fos and β-gal expression) after the start of a 30 min nonreinforced oxycodone-seeking test (test) and experimentally matched rats killed from the home cage (no test). Figure 2C shows total labelled Fos nuclei per mm^2 in the IL of rats from the no test versus test condition. Rats from the test condition showed a significantly greater number of Fos positive nuclei per mm^2 ($t_{(17)} = 5.3$, $p = 0.0005$). Figure 2D shows the percentage of CTb-488 cells colabelled with Fos in the IL between the no test and test groups. Rats from the test condition showed significantly greater Fos positive cells colabelled with CTb-488 cells ($t_{(17)} = 4.3$, $p = 0.0005$). Figure 2E shows the percentage of CTb-594 cells colabelled with Fos in the IL between the no test and test groups. Rats from the test condition showed significantly greater Fos+ cells colabelled with CTb-594 cells ($t_{(17)} = 2.2$, $p = 0.04$).

3.2 | Experiment 2: Role of IL Fos-expressing ensembles in initial oxycodone seeking

Figure 3A shows the experimental timeline for Experiment 2. All rats took an average of 2.91 days (SEM = 0.29, $n = 44$) to reach acquisition criteria. Figure 3B shows the total number of infusions, active lever presses and inactive lever presses on the day each rat met acquisition criteria for initial oxycodone self-administration, prior to group assignment to the vehicle or Daun02 conditions. There were no significant differences in infusions earned ($t_{(23)} = 1.2$, $p = 0.3$), active lever

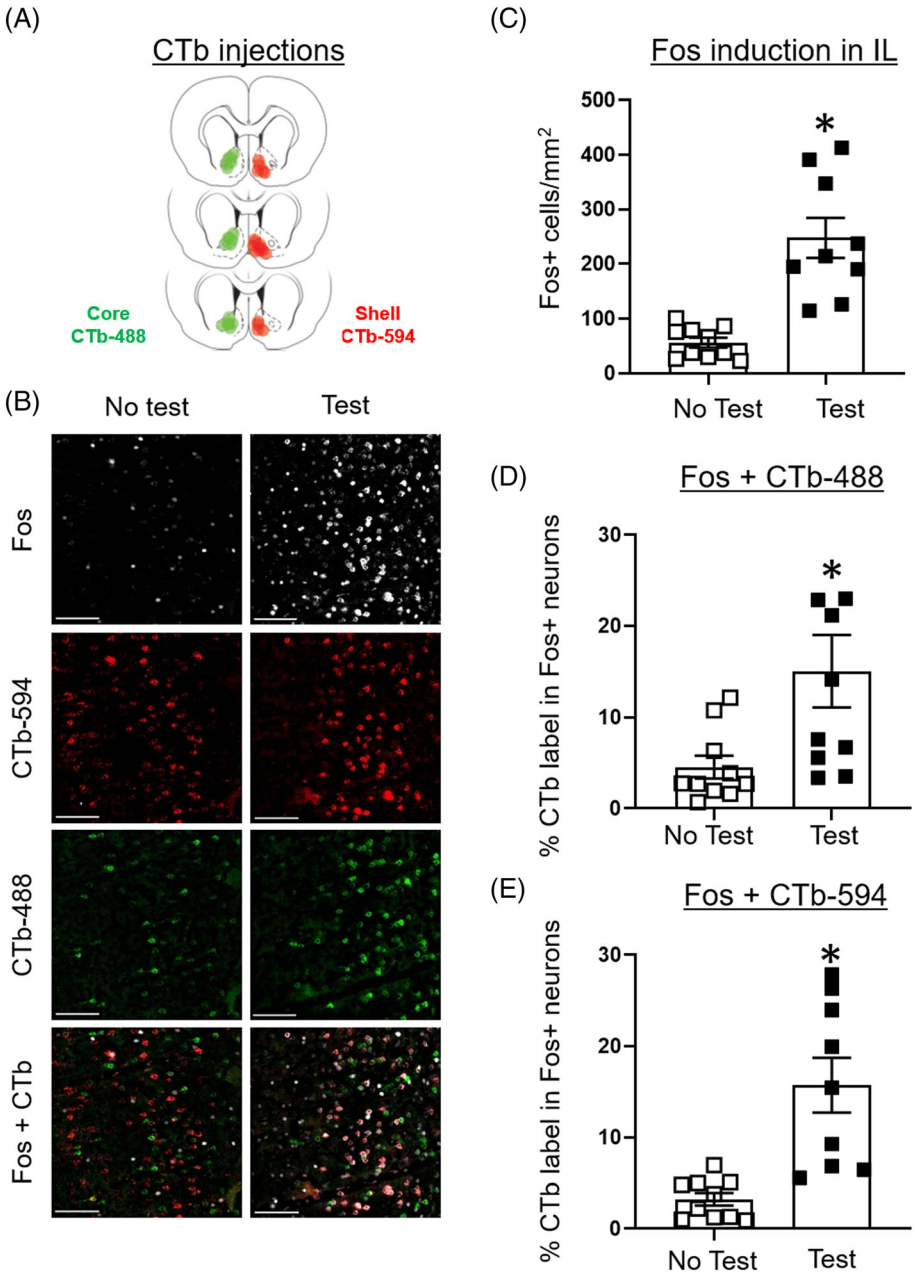

FIGURE 2 Experiment 1: CTb labelling of IL-NAc projections after initial oxycodone learning. (A) Depiction of the placement of CTb-488 and CTb-594 injections into the NAc core and shell subregions. (B) Representative images of Fos, CTb-594, CTb-488 and Fos + CTb-594 + CTb-488 fluorescence in the IL of rats killed from the home cage (no test) compared with rats killed following the oxycodone-seeking test (test). The scale bars represent 100 μm. (C) Average number of Fos+ cells/mm^2 (Fos expression) in the IL of rats killed from the home cage (no test) compared with rats killed after the oxycodone-seeking test (test). (D) Percentage of Fos+ cells colabelled with CTb-488 in the IL of rats killed from the home cage (no test) compared with rats killed after the oxycodone-seeking test (test). (E) Percentage of Fos+ cells colabelled with CTb-594 in the IL of rats killed from the home cage (no test) compared with rats killed after the oxycodone-seeking test (test). Data are presented as mean ± SEM (n = 9–10 per group). *p < 0.05 compared with no test controls

presses ($t_{(23)} = 1.3$, $p = 0.\ 2$) or inactive lever presses ($t_{(23)} = 0.26$, $p = 0.8$) between rats assigned to the vehicle and Daun02 groups. Prior to microinfusions of Daun02 or vehicle, rats did not differ in their number of active lever presses on induction day ($t_{(23)} = 0.22$, $p = 0.83$). Figure 3C shows the number of active lever presses on test day between the vehicle and Daun02 groups. Rats that received bilateral IL microinfusions of Daun02 following the induction session displayed significantly less active lever pressing on test day than vehicle-treated controls ($t_{(23)} = 2.4$, $p = 0.02$). Figure 3D shows cannula placements in the IL. Figure 3E shows the number of Fos+ cells per mm^2 between rats in the vehicle versus Daun02 groups after test day. We conducted an unpaired t test to assess Fos+ cells per mm^2 between vehicle and Daun02 groups and found that rats in the Daun02 groups exhibited significantly less Fos expression in the IL compared with rats in the vehicle group ($t_{(23)} = 2.71$, $p = 0.01$). Below Figure 3E shows representative images of Fos expression in vehicle and Daun02 groups 90 min after nonreinforced active lever pressing on test day.

3.3 | Experiment 3: Fos induction after prolonged oxycodone self-administration

Figure 4A shows the experimental timeline for Experiment 3. Figure 4B shows the number of active versus inactive lever presses across the nine self-administration training sessions at FR1, FR2 and FR3 schedules of reinforcement. We conducted separate two-way repeated measures ANOVAs for each FR schedule with lever presses

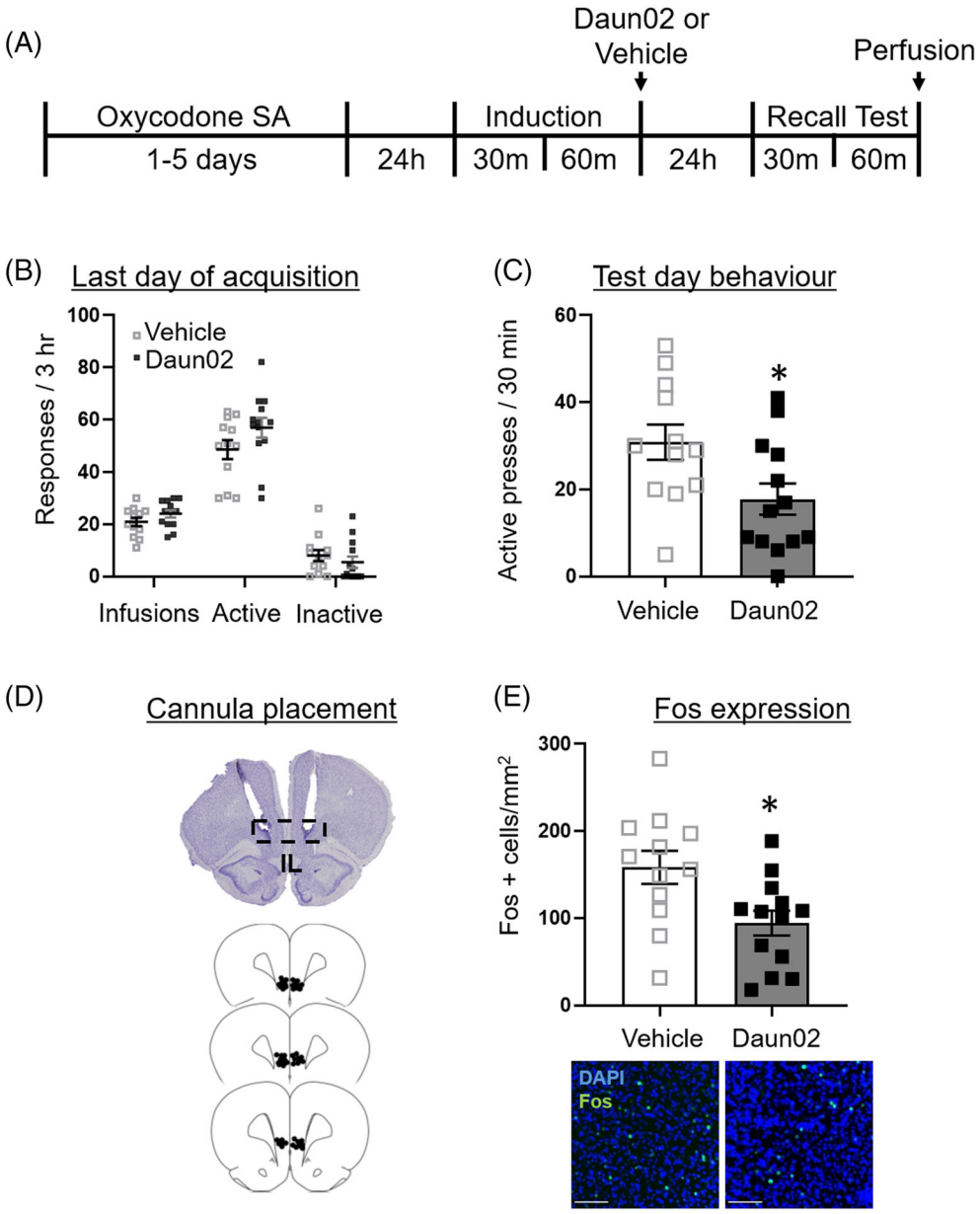

FIGURE 3 Experiment 2: Role of IL neuronal ensembles in initial learning of oxycodone self-administration. (A) Timeline showing the behavioural procedure. (B) Number of infusions, active lever presses and inactive lever presses on the day acquisition criterion was met for rats that would later receive vehicle or Daun02. (C) Average number of active lever presses during the 30-min test day session between rats that received vehicle or Daun02 on induction day. (D) Representative image of cannula placements in the IL. (E) Top panel: Average number of IL Fos+ cells/mm^2 (Fos expression) following the recall test in rats that received vehicle or Daun02 on induction day. Below: Representative images of Fos immunofluorescence in the IL of vehicle versus Daun02 rats following the recall test. The scale bars represent 100 μm. Data are presented as mean ± SEM ($n = 12–13$ per group). *$p < 0.05$ compared with vehicle-treated controls

(active vs. inactive) as the between-subjects factor and days (1–3) as the within-subjects factor. At FR1, we found main effects of lever ($F_{(1, 22)} = 10.59$, $p = 0.003$), but not day ($p = 0.7$) with no significant interaction ($p = 0.4$). At FR2, we found main effects of day ($F_{(2, 44)} = 5.7$, $p = 0.006$) and lever presses ($F_{(1, 22)} = 9.9$, $p = 0.005$), with no significant interaction ($p = 0.14$). At FR3, we found a main effect of lever presses ($F_{(1, 22)} = 10.7$, $p = 0.004$), but not day ($p = 0.63$), with no significant interaction ($p = 0.92$). Rats pressed significantly more on the active lever compared with the inactive lever averaged across all days of self-administration, indicating successful discrimination. Holm–Sidak's post hoc analysis revealed that rats pressed significantly more on the active lever than the inactive lever beginning on Day 1 of the FR1 schedule through Day 3 of FR3. Next, to assess increased active lever pressing at each FR block, we first averaged the number of active lever presses for each rat within each FR testing block. We then conducted a repeated measures ANOVA with Greenhouse–Geisser correction to compare average active lever presses within each FR block and found significantly greater active lever pressing with each increasing FR schedule block ($F_{(1.27, 13.98)} = 7.89$, $p = 0.01$). Holm–Sidak's multiple comparison test revealed that rats pressed the active lever more at FR2 ($p = 0.04$) and FR3 ($p = 0.03$) than at FR1. Active lever pressing at FR3 was also significantly greater than active lever pressing at FR2 ($p = 0.04$).

Figure 4C shows the number of infusions across each FR block. To assess oxycodone intake despite increased effort to self-administer at increasing FR schedules of reinforcement, we first averaged the number of infusions for each rat within each FR testing block. We conducted a repeated measures ANOVA with Greenhouse–Geisser correction to compare average infusions within each FR block and did not find a significant effect ($F_{(1.36, 14.94)} = 0.67$, $p = 0.48$). There was

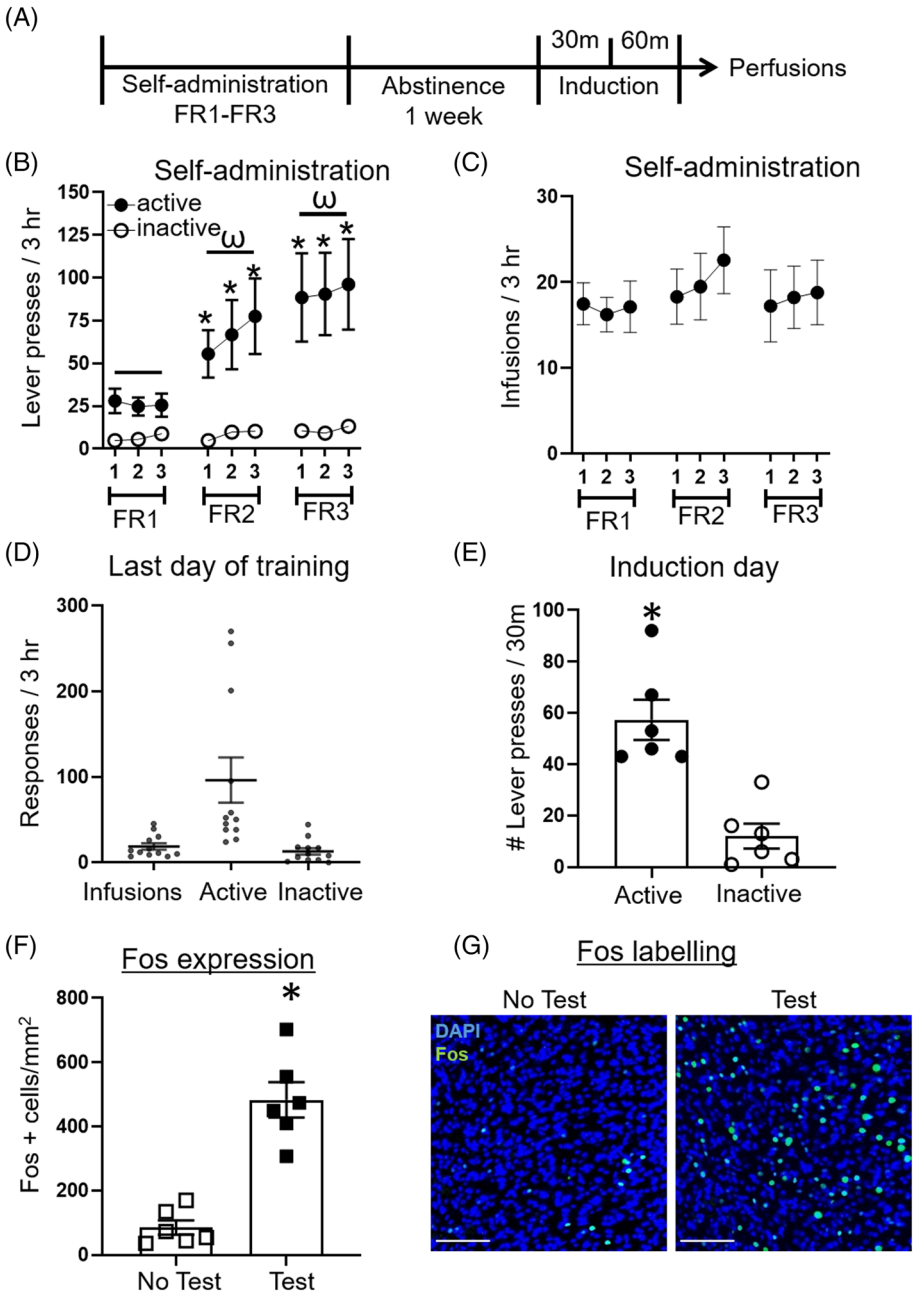

FIGURE 4 Experiment 3: Fos induction after maintenance of oxycodone seeking. (A) Timeline showing the behavioural procedure. (B) Average number of active versus inactive lever presses in rats across the oxycodone self-administration period from FR1 to FR3. (C) Average number of infusions across the oxycodone self-administration period from FR1 to FR3. (D) Number of infusions, active lever presses and inactive lever presses during the last day of training. (E) Average number of active versus inactive lever presses during the 30-min induction test. (F) Average number of IL Fos+ cells/mm^2 (Fos expression) in rats killed from the home cage (no test) compared with rats killed following the induction test (test). (G) Representative images of Fos immunofluorescence in the IL of rats from the no test and test conditions. The scale bars represent 100 μm. Data are presented as mean ± SEM ($n = 6$ per group). *$p < 0.05$ compared with inactive lever presses, or no test controls. ω $p < 0.05$ compared with FR1 active lever pressing

no difference in the number of infusions across any FR block despite significantly greater active lever pressing between these blocks, commensurate with the increasing FR demand, indicating stable maintenance of oxycodone self-administration.

Figure 4D shows the total number of infusions, active lever presses and inactive lever presses on the last day of self-administration training prior to group assignment into the no test or test conditions. There were no significant differences in infusions ($t_{(10)} = 0.47$, $p = 0.65$), active lever presses ($t_{(10)}$=0.17, $p = 0.87$), nor inactive lever presses ($t_{(10)}$=0.25, $p = 0.81$) between rats assigned to no test compared with test groups. Figure 4E shows the number of active lever presses compared with inactive lever presses on induction day. We conducted a paired samples *t* test to assess active versus inactive lever presses on induction day and found that rats pressed significantly more on the active lever compared with the inactive lever on induction day ($t_{(5)} = 7.7$, $p = 0.0006$).

Figure 4F shows the number of Fos+ cells per mm^2 in the no test and test groups. We conducted an unpaired *t* test to assess Fos+ cells per mm^2 between no test and test groups and found that rats in the test group showed significantly greater Fos expression compared with rats in the no test group ($t_{(10)} = 6.7$, $p < 0.0001$). Figure 4G shows representative images of Fos expression in the IL between the no test and test groups on induction day.

Incubation of heroin craving has been previously demonstrated after 1 week of abstinence compared with 1 day of abstinence.[31] To determine whether incubation occurred in this experiment, we conducted a *t* test to assess number of active lever presses on induction day after 1 week of abstinence (Experiment 3) compared with 1 day

of abstinence (Experiment 1). We found significantly greater active lever presses on induction day after 1 week of abstinence compared with 1 day of abstinence ($t_{(14)} = 4.77$, $p = 0.0003$). Additionally, because incubated drug responses coincide with time-dependent increases in Fos expression,[32] we also conducted a *t* test to compare induction day Fos expression after 1 week of abstinence (Experiment 3) to 1 day of abstinence (Experiment 1). We found significantly greater Fos expression in the IL of rats undergoing the cue-induced oxycodone test after 1 week of abstinence compared with 1 day of abstinence ($t_{(14)} = 3.88$, $p < 0.01$).

3.4 | Experiment 4: Role of IL Fos-expressing ensembles in the maintenance of oxycodone seeking

Figure 5A shows the experimental timeline for Experiment 4. Figure 5B shows the number of active versus inactive lever presses across the nine self-administration training sessions at FR1, FR2 and FR3 schedules of reinforcement. We conducted separate two-way repeated measures ANOVAs for each FR schedule with lever presses (active vs. inactive) as the between-subjects factor and days (1–3) as the within-subjects factor. At FR1, we found main effects of lever ($F_{(1,\ 36)} = 11.6$, $p = 0.002$), but not day ($p = 0.11$) with no significant interaction ($p = 0.4$). At FR2, we found a main effect of lever ($F_{(1,\ 36)} = 40.9$, $p < 0.0001$), with no effect of day ($p = 0.24$) and no significant interaction ($p = 0.38$). At FR3, we found a main effect of lever presses ($F_{(1,\ 36)} = 36.7$, $p < 0.0001$), but not day ($p = 0.36$), with no significant interaction ($p = 0.4$). Holm–Sidak's post hoc analysis revealed that rats pressed significantly more on the active lever than the inactive lever beginning on Day 1 of the FR1 schedule through Day 3 of FR3. Next, to assess increased active lever pressing at each FR block, we first averaged the number of active lever presses for each rat within each FR testing block. We then conducted a repeated measures ANOVA with Greenhouse–Geisser correction to compare average active lever presses within each FR block and found significantly greater active lever pressing with each increasing FR schedule block ($F_{(1.22,\ 21.91)} = 38.59$, $p < 0.0001$). Holm–Sidak's multiple comparison test revealed that rats pressed the active lever more at FR2 and FR3 than at FR1 ($p < 0.0001$). Active lever pressing at FR3 was also significantly greater than active lever pressing at FR2 ($p = 0.0002$).

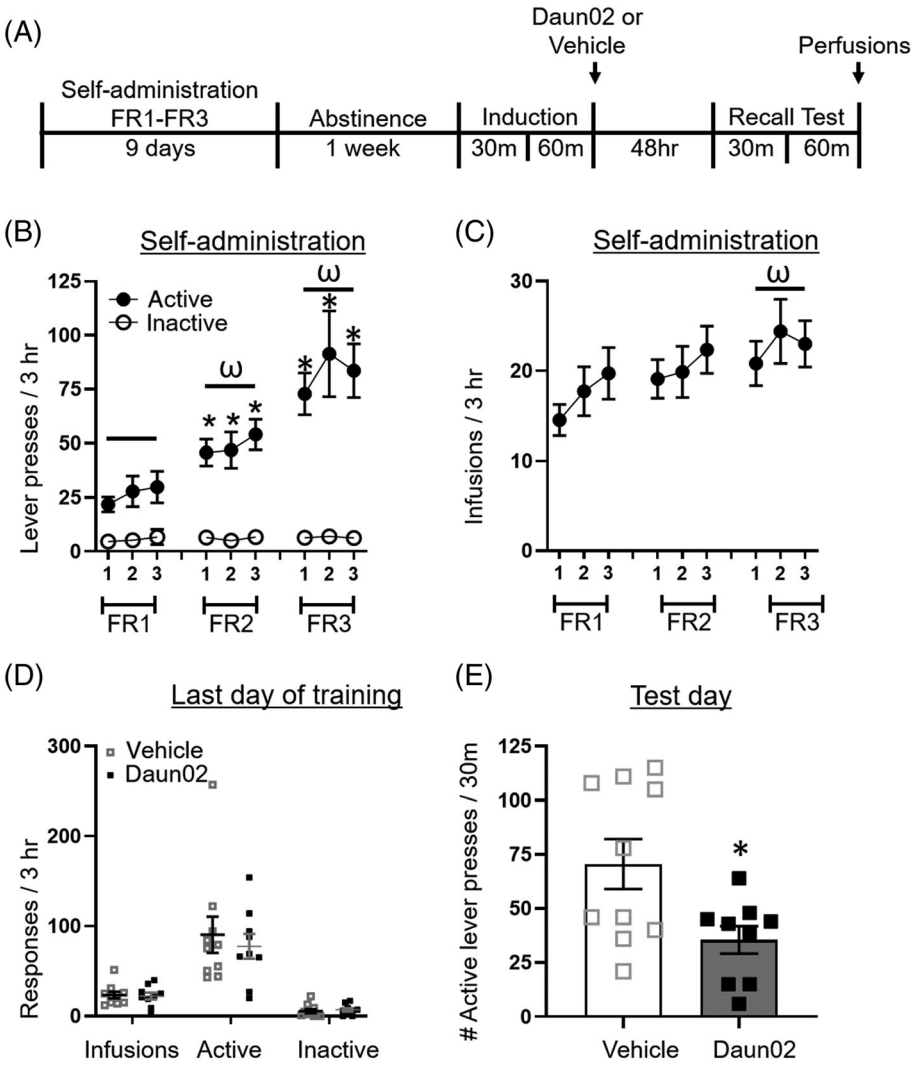

FIGURE 5 Experiment 4: Role of IL neuronal ensembles in the maintenance of oxycodone seeking. (A) Timeline showing the behavioural procedure. (B) Average number of active versus inactive lever presses in rats across the oxycodone self-administration period from FR1 to FR3. (C) Average number of infusions across the oxycodone self-administration period from FR1 to FR3. (D) Number of infusions, active lever presses and inactive lever presses during the last day of training between rats that would later receive vehicle or Daun02 microinfusions. (E) Average number of active lever presses during the 30-min test day session between rats that received vehicle or Daun02 on induction day. Data are presented as mean ± SEM. ($n = 9$–10 per group). *$p < 0.05$ compared with inactive lever presses, or no test controls. ω $p < 0.05$ compared with FR1 responding

Figure 5C shows the number of infusions across each FR block. To assess stable oxycodone intake despite the increased effort to self-administer at increasing FR schedules of reinforcement, we first averaged the number of infusions for each rat within each FR testing block. We conducted a repeated measures ANOVA with Greenhouse–Geisser correction to compare average infusions within each FR block and found a significant effect ($F_{(1.90,\ 34.20)} = 6.58$, $p = 0.004$). Holm–Sidak's multiple comparisons test showed greater infusions only in the FR3 block compared with the FR1 block ($p = 0.01$). There was no difference in the number of infusions from FR1 to FR2 ($p = 0.06$) and FR2 to FR3 ($p = 0.17$) despite significantly greater active lever pressing between these blocks, commensurate with the increasing FR demand, indicating stable maintenance of oxycodone self-administration.

Figure 5D shows the total number of infusions, active lever presses and inactive lever presses on the last day of self-administration training prior to group assignment into the vehicle or Daun02 conditions. There were no significant differences in infusions ($t_{(17)} = 0.20$, $p = 0.85$), active lever presses ($t_{(17)}$=0.51, $p = 0.61$), nor inactive lever presses ($t_{(17)} = 0.43$, $p = 0.67$) between rats assigned to vehicle compared with Daun02 groups. We also conducted an unpaired *t* test on the number of active lever presses between the vehicle versus Daun02 groups on induction day and found no differences between groups prior to microinfusions and subsequent test day ($t_{(17)} = 1.31$, $p = 0.21$). Figure 5E shows the number of active lever presses on test day between the vehicle and Daun02 groups. Rats that received IL microinfusions of Daun02 following the induction session displayed significantly less active lever pressing on test day than vehicle-treated controls ($t_{(17)} = 2.58$, $p = 0.02$).

Figure 6A shows cannula placements in the IL. Figure 6B shows the number of Fos+ cells per mm^2 in rats in the vehicle versus Daun02 groups. We conducted an unpaired *t* test to assess Fos+ cells per mm^2 between vehicle and Daun02 groups and found that rats in the Daun02 groups exhibited significantly less Fos expression compared with rats in the vehicle group ($t_{(17)} = 2.7$, $p = 0.015$). Figure 6B shows representative images of Fos expression in rats in the vehicle and Daun02 groups 90 min after nonreinforced active lever pressing on test day.

4 | DISCUSSION

We used CTb retrograde labelling combined with a novel behavioural procedure to capture initial learning of oxycodone self-administration. We found that IL neurons projecting to the NAc core and shell subregions were activated by initial oxycodone seeking. Using the Daun02 inactivation procedure, we further investigated the role of IL neuronal ensembles in initial oxycodone self-administration and showed that IL neuronal ensembles are necessary for the expression of oxycodone seeking after initial oxycodone self-administration. Additionally, we used an increasing FR schedule to demonstrate increased effort to lever press for oxycodone while maintaining stable infusions to represent well-trained and maintained responding for oxycodone compared with initial learning. We used the Daun02 inactivation procedure to test the specificity of IL neuronal ensembles in the maintenance of oxycodone self-administration and found that IL neuronal ensembles are also necessary for oxycodone seeking in well-trained rats. These findings are consistent with studies demonstrating a role of the IL in

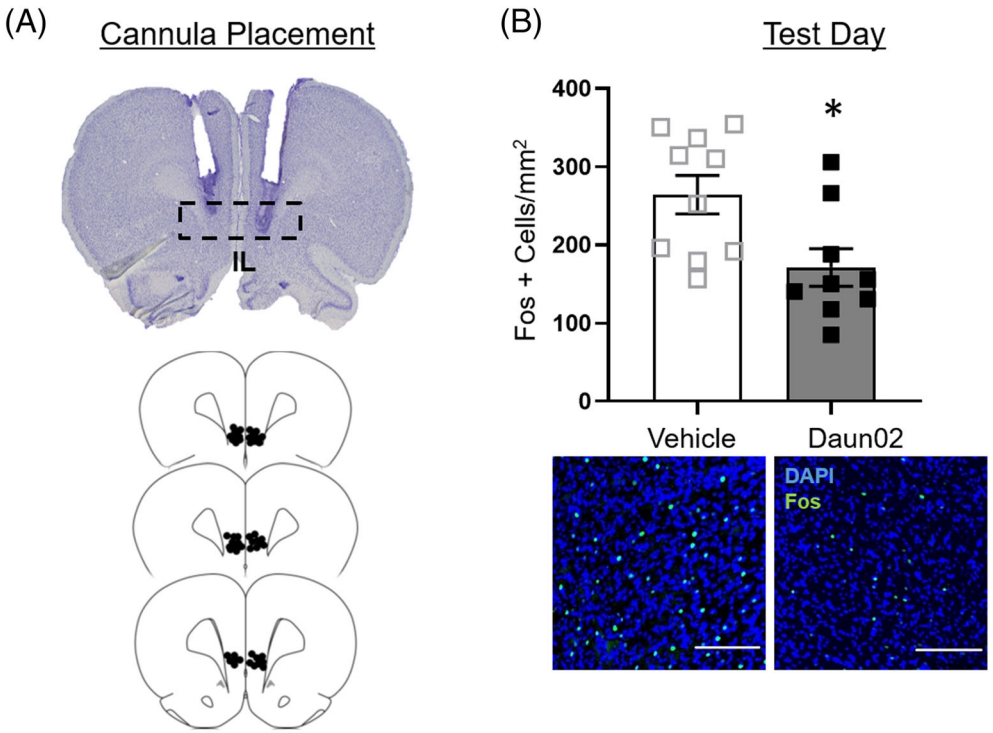

FIGURE 6 Experiment 4: Fos expression following Daun02 ablation of well-trained oxycodone-seeking ensembles. (A) Representative image of cannula placements in the IL. (B) above: Average number of IL Fos + cells/mm^2 (Fos expression) following the recall test in rats that received vehicle or Daun02 on induction day. Images were taken at 200× magnification. The scale bars represent 100 μm. Data are presented as mean ± SEM (n = 9–10 per group). *p < 0.05 compared with vehicle controls

the self-administration and seeking of other opiates such as heroin.[5,18,20,33,34] Here, we are the first to utilize a model to capture initial learning after oxycodone self-administration and show that IL neuronal ensembles mediate oxycodone seeking both after initial learning and maintenance of oxycodone self-administration.

4.1 | The role of the IL in opiate seeking

The IL plays a unique role in opiate seeking compared with psychostimulant seeking. Whereas the IL suppresses psychostimulant seeking and promotes extinction learning, it has been demonstrated to facilitate opiate seeking.[5,34,35] In support of this, lesioning the IL blocks morphine-induced conditioned place preference.[36] In studies using self-administration, Fos expression has been identified within the IL following context-induced heroin seeking.[33] Pharmacological inactivation of the IL attenuates drug- and/or cue-induced heroin reinstatement[5] and pharmacogenetic selective disruption of IL neuronal ensembles prevents context-induced heroin reinstatement.[20] Collectively, these results highlight the IL as important in the expression of opiate seeking.

Consistent with these previous findings, we identified Fos expression within the IL during cue-induced oxycodone seeking after initial learning and maintenance of oxycodone self-administration. Re-exposure to the drug-paired context and cues reactivates the memory for oxycodone self-administration and is behaviourally expressed as oxycodone seeking. The accompanying Fos expression we found in the IL indicates that the memory for oxycodone self-administration may be housed in the IL as early as after initial learning and persists after maintenance and forced abstinence (1 week). After showing behaviourally relevant Fos expression within the IL at these time points, we used the Daun02 inactivation procedure in subsequent experiments to selectively ablate these Fos-expressing ensembles. In *Fos-LacZ* transgenic rats, β-galactosidase (β-gal) and Fos protein are coexpressed only in strongly activated neurons following the drug-seeking recall test. Microinfusing Daun02 during the time point of maximal protein coexpression (90 min after the start of the test) enables β-gal to hydrolyse Daun02 into Daunorubicin, which precipitates apoptosis exclusively in behaviourally relevant neuronal ensembles.[37–39] Rats that received Daun02 versus vehicle microinfusions demonstrated attenuated drug seeking (less active lever pressing) during the recall test following initial learning as well as after maintained oxycodone self-administration. We also found that rats in the Daun02 versus vehicle group exhibited reduced Fos expression during the recall test following initial learning as well as after maintained oxycodone self-administration. This reduction in Fos expression indicates that the Daun02 microinjections received on induction day induced apoptosis in those behaviourally relevant Fos-expressing neurons implicated in initial and well-trained oxycodone seeking on test day.[39] Collectively, these results indicate that Fos-expressing ensembles in the IL mediate the expression of initial oxycodone seeking as well as well-trained oxycodone seeking.

It is important to note that in these studies, we did not include a novel context control group. The novel context control has been used to show the specificity of a neuronal ensemble in mediating a behaviour.[40] Several studies have now shown that Daun02 silencing of unrelated neuronal ensembles does not disrupt drug-seeking behaviour.[24,27,28,41] Therefore, the decrease in oxycodone seeking following our Daun02 manipulation is likely specific to oxycodone seeking, rather than a general effect of inactivating IL neurons. It is also important to note that maintained oxycodone seeking was assessed after 1 week of abstinence, a time point when incubation of heroin craving has been detected as compared with 1 day of abstinence.[31] Typically, preclinical models of incubation of drug craving assess drug seeking at multiple time points within extended abstinence.[42–44] Though, we did not employ the traditional incubation of drug craving paradigm, our results may implicate the IL in early incubation of oxycodone craving and should be further investigated. In support of this, we found significantly greater active lever presses on induction day after 1 week of abstinence (Experiment 3) compared with 1 day of abstinence. We also found significantly greater Fos expression in the IL of rats undergoing the cue-induced oxycodone test following 1 week of abstinence compared with 1 day of abstinence. Indeed, time-dependent increases in Fos expression have been shown to accompany incubated drug responses.[32] Although increased IL Fos expression noted after 1-week compared with 1 day of abstinence may indicate a role of the IL in early incubated oxycodone seeking, it is important to note that the differences in Fos expression may instead represent different expression patterns in well-trained responding versus initial learning of oxycodone reward. However, the opposite has been previously shown wherein increased Fos expression coincided with early learning compared with well-trained responding.[45] Additionally, the differences in Fos expression may be attributed to different FR schedules between the test after initial oxycodone seeking (FR1) and the test 1 week following maintained oxycodone seeking (FR3), though this seems unlikely, as differences in Fos expression were not detected in well-trained rats pressing for a reward under an FR5 compared with an FR1 schedule of reinforcement.[45] Furthermore, we did not see a correlation between Fos labelling and lever pressing or expected rewards in either test (data not shown). This indicates that the distinct Fos expression pattern in the IL between 1 week and 1 day of abstinence may be attributed to a more robust memory formation after stable self-administration and incubation of craving compared with initial learning.

4.2 | The role of IL projections in opiate seeking

The NAc is a critical projection from the IL that has been heavily implicated in reward seeking.[46] Here, we microinfused retrograde CTb into the NAc core and shell and assessed Fos colabelling with CTb within the IL after initial, but not extended oxycodone seeking. This enabled us to label activated IL neurons relevant for initial oxycodone seeking that project to the NAc subregions. We showed that initial oxycodone seeking activated IL cells that projected to both the NAc core and

shell subregions, suggesting that the IL-NAc core and IL-NAc shell projections may be involved in initial oxycodone seeking. Although we did not assess the role of these projections in well-trained rats, the NAc shell, rather than the NAc core has been implicated in in context-induced heroin reinstatement after prolonged training, whereas the NAc core is critical for cue-induced reinstatement of heroin seeking.[16] It is possible that this circuitry shifts activity following prolonged oxycodone self-administration. Our finding that initial oxycodone seeking activated both the IL-NAc core and IL-NAc shell is unsurprising because our initial oxycodone-seeking test exposed the rats to both the drug-paired context and cues. In studies using a context + cue-induced heroin seeking test (following well-trained heroin self-administration), inactivation of the NAc shell with an $mGluR_{2/3}$ agonist dose-dependently attenuated context + cue-induced heroin seeking, whereas inactivation of the NAc core attenuated this reinstatement only at the highest tested mGluR agonist dose.[17] This finding highlights the role of glutamatergic transmission in the NAc shell and to a lesser extent, the NAc core, during heroin seeking. A subsequent study extended these findings to specifically test the IL-NAc shell during heroin seeking after well-trained heroin self-administration and identified increased Fos expression in IL neurons projecting to the NAc shell during context + cue-induced heroin reinstatement.[18] Furthermore, inactivating the IL in one hemisphere (muscimol + baclofen) combined with inactivating the contralateral or ipsilateral shell (D1 agonist) decreased context + cue-induced heroin seeking.[18] These studies to date suggest that opiate seeking after well-trained opiate self-administration is mediated by glutamatergic projections from the IL to the NAc shell as well as local dopamine D1 postsynaptic receptors.[17,18] Although this circuit is critical for well-trained opiate-seeking behaviour, less is known about the role of IL to NAc projections at the earlier time point tested in this study. It is also important to note that other projections have been shown to mediate opiate seeking. Glutamatergic projections from the PL to the NAc core are necessary for cue- and drug-primed reinstatement of heroin seeking after well-trained heroin self-administration.[14] Additionally, hippocampal glutamatergic projections to the NAc shell[47] and the IL[48] have been shown to mediate heroin seeking after well-trained heroin self-administration. Moreover, it is crucial to mention that we assessed behaviourally relevant Fos expression after initial oxycodone learning only from the IL-NAc, whereas the IL projects to several other regions (including the PL, anterior cingulate, thalamus, hypothalamus, amygdala and bed nucleus of the stria terminalis).[49] By utilizing the Daun02 inactivation procedure to ablate Fos-expressing neuronal ensembles within the IL, it is probable that we are ablating IL neurons projecting to the NAc core and shell. However, it is also likely that some of the ablated IL neurons project to the aforementioned regions and may also mediate oxycodone seeking; this should be further investigated.

5 | CONCLUSIONS

The current study found that activation within IL neurons projecting to both NAc core and NAc shell regions are implicated in initial oxycodone seeking. Neuronal-ensemble-specific inactivation of the IL decreased both initial oxycodone seeking and maintained oxycodone seeking in well-trained rats during a recall test. Taken together, these findings suggest that IL neuronal ensembles are initially formed and maintained during oxycodone self-administration and that these ensembles are necessary for the expression of oxycodone-seeking behaviour. These findings may help uncover the neurobiological underpinnings of oxycodone memories as they are formed and subsequently maintained to control the expression of oxycodone seeking.

ACKNOWLEDGEMENTS

BLW was supported by a grant from the National Institute on Drug Abuse (NIDA: Grant 4R00DA042102-02) and a NARSAD Young Investigator Grant from the Brain and Behavior Research Foundation. Oxycodone was supplied by the NIH Drug Supply Program.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

B.W. and C.G. designed the behavioural experiments. B.W., C.G., B.S. and S.R. performed surgeries or intracranial infusions on the rats and were responsible for collecting behavioural and histochemical data. C.G. and B.W. wrote the manuscript. All authors provided critical reviews of the content and approved the final version for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Christina Gobin https://orcid.org/0000-0001-7780-0397
Brandon L. Warren https://orcid.org/0000-0002-5922-9382

REFERENCES

1. Kibaly C, Alderete JA, Liu SH, et al. Oxycodone in the opioid epidemic: high 'liking', 'wanting', and abuse liability. *Cell Mol Neurobiol*. 2021;41(5):899-926.
2. Stewart J, de Wit H, Eikelboom R. Role of unconditioned and conditioned drug effects in the self-administration of opiates and stimulants. *Psychol Rev*. 1984;91(2):251-268.
3. Weeks JR, Collins RJ. Factors affecting voluntary morphine intake in self-maintained addicted rats. *Psychopharmacologia*. 1964;6(4):267-279.
4. Deneau G, Yanagita T, Seevers MH. Self-administration of psychoactive substances by the monkey. *Psychopharmacologia*. 1969;16(1):30-48.
5. Rogers JL, Ghee S, See RE. The neural circuitry underlying reinstatement of heroin-seeking behavior in an animal model of relapse. *Neuroscience*. 2008;151(2):579-588.
6. McFarland K, Lapish CC, Kalivas PW. Prefrontal glutamate release into the core of the nucleus accumbens mediates cocaine-induced reinstatement of drug-seeking behavior. *J Neurosci*. 2003;23(8):3531-3537.
7. Kalivas PW, Nakamura M. Neural systems for behavioral activation and reward. *Curr Opin Neurobiol*. 1999;9(2):223-227.

8. Chang JY, Janak PH, Woodward DJ. Comparison of mesocorticolimbic neuronal responses during cocaine and heroin self-administration in freely moving rats. *J Neurosci*. 1998;18(8):3098-3115.
9. Chang JY, Zhang L, Janak PH, Woodward DJ. Neuronal responses in prefrontal cortex and nucleus accumbens during heroin self-administration in freely moving rats. *Brain Res*. 1997;754(1–2):12-20.
10. Ikegami A, Olsen CM, D'Souza MS, Duvauchelle CL. Experience-dependent effects of cocaine self-administration/conditioning on prefrontal and accumbens dopamine responses. *Behav Neurosci*. 2007;121(2):389-400.
11. Suska A, Lee BR, Huang YH, Dong Y, Schluter OM. Selective presynaptic enhancement of the prefrontal cortex to nucleus accumbens pathway by cocaine. *Proc Natl Acad Sci U S a*. 2013;110(2):713-718.
12. Rebec GV, Sun W. Neuronal substrates of relapse to cocaine-seeking behavior: role of prefrontal cortex. *J Exp Anal Behav*. 2005;84(3):653-666.
13. Shen H, Kalivas PW. Reduced LTP and LTD in prefrontal cortex synapses in the nucleus accumbens after heroin self-administration. *Int J Neuropsychopharmacol*. 2013;16(5):1165-1167.
14. LaLumiere RT, Kalivas PW. Glutamate release in the nucleus accumbens core is necessary for heroin seeking. *J Neurosci*. 2008;28(12):3170-3177.
15. Kalivas PW. The glutamate homeostasis hypothesis of addiction. *Nat Rev Neurosci*. 2009;10(8):561-572.
16. Bossert JM, Poles GC, Wihbey KA, Koya E, Shaham Y. Differential effects of blockade of dopamine D1-family receptors in nucleus accumbens core or shell on reinstatement of heroin seeking induced by contextual and discrete cues. *J Neurosci*. 2007;27(46):12655-12663.
17. Bossert JM, Gray SM, Lu L, Shaham Y. Activation of group II metabotropic glutamate receptors in the nucleus accumbens shell attenuates context-induced relapse to heroin seeking. *Neuropsychopharmacology*. 2006;31(10):2197-2209.
18. Bossert JM, Stern AL, Theberge FR, et al. Role of projections from ventral medial prefrontal cortex to nucleus accumbens shell in context-induced reinstatement of heroin seeking. *J Neurosci*. 2012;32(14):4982-4991.
19. Hebb D. *The Organization of Behavior: A Neuropsychological Theory*. New York: Wiley; 1949.
20. Bossert JM, Stern AL, Theberge FR, et al. Ventral medial prefrontal cortex neuronal ensembles mediate context-induced relapse to heroin. *Nat Neurosci*. 2011;14(4):420-422.
21. Bohland JW, Wu C, Barbas H, et al. A proposal for a coordinated effort for the determination of brainwide neuroanatomical connectivity in model organisms at a mesoscopic scale. *PLoS Comput Biol*. 2009;5(3):e1000334.
22. Luppi PH, Fort P, Jouvet M. Iontophoretic application of unconjugated cholera toxin B subunit (CTb) combined with immunohistochemistry of neurochemical substances: a method for transmitter identification of retrogradely labeled neurons. *Brain Res*. 1990;534(1–2):209-224.
23. Quintana-Feliciano R, Gobin C, Kane L, et al. Food-seeking behavior is mediated by Fos-expressing neuronal ensembles formed at first learning in rats. *eNeuro*. 2021;8(2):ENEURO.0373-ENEU20.2021.
24. Warren BL, Kane L, Venniro M, et al. Separate vmPFC ensembles control cocaine self-administration versus extinction in rats. *J Neurosci*. 2019;39(37):7394-7407.
25. Coolen LM, Jansen HT, Goodman RL, Wood RI, Lehman MN. A new method for simultaneous demonstration of anterograde and retrograde connections in the brain: co-injections of biotinylated dextran amine and the beta subunit of cholera toxin. *J Neurosci Methods*. 1999;91(1–2):1-8.
26. Lanciego JL, Luquin MR, Guillén J, Giménez-Amaya JM. Multiple neuroanatomical tracing in primates. *Brain Res Brain Res Protoc*. 1998;2(4):323-332.
27. Kane L, Venniro M, Quintana-Feliciano R, et al. Fos-expressing neuronal ensemble in rat ventromedial prefrontal cortex encodes cocaine seeking but not food seeking in rats. *Addict Biol*. 2021;26(3):e12943.
28. Warren BL, Mendoza MP, Cruz FC, et al. Distinct Fos-expressing neuronal ensembles in the ventromedial prefrontal cortex mediate food reward and extinction memories. *J Neurosci*. 2016;36(25):6691-6703.
29. Cruz F, Babin KR, Leao RM, et al. Role of nucleus accumbens shell neuronal ensembles in context-induced reinstatement of cocaine-seeking. *J Neurosci*. 2014;34(22):7437-7446.
30. Koya E, Golden SA, Harvey BK, et al. Targeted disruption of cocaine-activated nucleus accumbens neurons prevents context-specific sensitization. *Nat Neurosci*. 2009;12(8):1069-1073.
31. Pickens CL, Airavaara M, Theberge F, Fanous S, Hope BT, Shaham Y. Neurobiology of the incubation of drug craving. *Trends Neurosci*. 2011;34(8):411-420.
32. Caprioli D, Venniro M, Zhang M, et al. Role of dorsomedial striatum neuronal ensembles in incubation of methamphetamine craving after voluntary abstinence. *J Neurosci*. 2017;37(4):1014-1027.
33. Doherty JM, Cooke BM, Frantz KJ. A role for the prefrontal cortex in heroin-seeking after forced abstinence by adult male rats but not adolescents. *Neuropsychopharmacology*. 2013;38(3):446-454.
34. Peters J, Pattij T, De Vries TJ. Targeting cocaine versus heroin memories: divergent roles within ventromedial prefrontal cortex. *Trends Pharmacol Sci*. 2013;34(12):689-695.
35. Peters J, LaLumiere RT, Kalivas PW. Infralimbic prefrontal cortex is responsible for inhibiting cocaine seeking in extinguished rats. *J Neurosci*. 2008;28(23):6046-6053.
36. Tzschentke TM, Schmidt WJ. Functional heterogeneity of the rat medial prefrontal cortex: effects of discrete subarea-specific lesions on drug-induced conditioned place preference and behavioural sensitization. *Eur J Neurosci*. 1999;11(11):4099-4109.
37. Koya E, Margetts-Smith G, Hope BT. Daun02 inactivation of behaviorally activated Fos-expressing neuronal ensembles. *Curr Protoc Neurosci*. 2016;76:8-36.
38. Cruz FC, Koya E, Guez-Barber DH, et al. New technologies for examining the role of neuronal ensembles in drug addiction and fear. *Nat Rev Neurosci*. 2013;14(11):743-754.
39. Pfarr S, Meinhardt MW, Klee ML, et al. Losing control: excessive alcohol seeking after selective inactivation of cue-responsive neurons in the infralimbic cortex. *J Neurosci*. 2015;35(30):10750-10761.
40. Fanous S, Goldart EM, Theberge FR, Bossert JM, Shaham Y, Hope BT. Role of orbitofrontal cortex neuronal ensembles in the expression of incubation of heroin craving. *J Neurosci*. 2012;32(34):11600-11609.
41. Suto N, Laque A, De Ness GL, et al. Distinct memory engrams in the infralimbic cortex of rats control opposing environmental actions on a learned behavior. *Elife*. 2016;5:e21920.
42. Grimm JW, Hope BT, Wise RA, Shaham Y. Incubation of cocaine craving after withdrawal. *Nature*. 2001;412(6843):141-142.
43. Lu L, Grimm JW, Shaham Y, Hope BT. Molecular neuroadaptations in the accumbens and ventral tegmental area during the first 90 days of forced abstinence from cocaine self-administration in rats. *J Neurochem*. 2003;85(6):1604-1613.
44. Freeman WM, Patel KM, Brucklacher RM, et al. Persistent alterations in mesolimbic gene expression with abstinence from cocaine self-administration. *Neuropsychopharmacology*. 2008;33(8):1807-1817.
45. Segovia KN, Correa M, Lennington JB, Conover JC, Salamone JD. Changes in nucleus accumbens and neostriatal c-Fos and DARPP-32

immunoreactivity during different stages of food-reinforced instrumental training. *Eur J Neurosci*. 2012;35(8):1354-1367.
46. Sesack SR, Grace AA. Cortico-basal ganglia reward network: microcircuitry. *Neuropsychopharmacology*. 2010;35(1):27-47.
47. Bossert JM, Adhikary S, St Laurent R, et al. Role of projections from ventral subiculum to nucleus accumbens shell in context-induced reinstatement of heroin seeking in rats. *Psychopharmacology (Berl)*. 2016;233(10):1991-2004.
48. Wang N, Ge F, Cui C, et al. Role of glutamatergic projections from the ventral CA1 to infralimbic cortex in context-induced reinstatement of heroin seeking. *Neuropsychopharmacology*. 2018;43(6):1373-1384.
49. Hurley KM, Herbert H, Moga MM, Saper CB. Efferent projections of the infralimbic cortex of the rat. *J Comp Neurol*. 1991;308(2):249-276.

How to cite this article: Gobin C, Sortman B, Rakela S, Quintana-Feliciano R, Warren BL. Fos-expressing neuronal ensembles in rat infralimbic cortex encode initial and maintained oxycodone seeking in rats. *Addiction Biology*. 2022; 27(2):e13148. doi:10.1111/adb.13148