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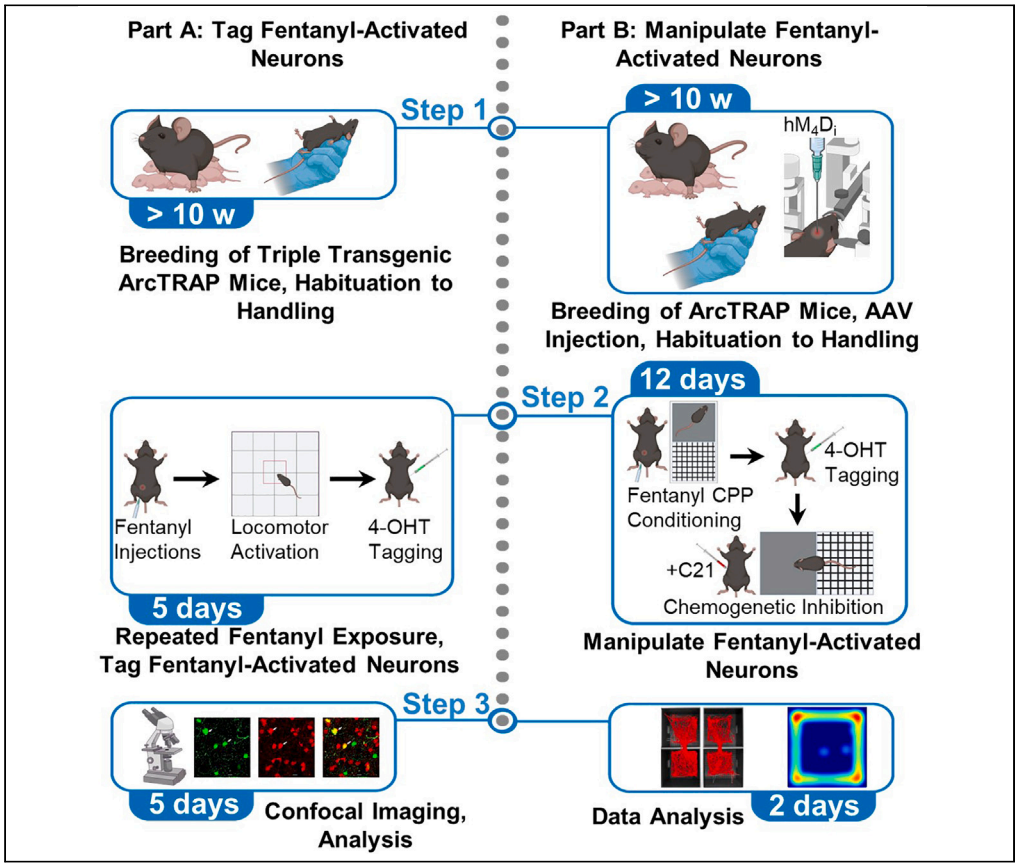
Activity-dependent labeling and manipulation of fentanyl-recruited striatal ensembles using ArcTRAP approach



Understanding the memory substrates underlying substance abuse requires the permanent tagging and manipulation of drug-recruited neural ensembles. Here, we present a protocol for activity-dependent labeling and chemogenetic manipulation of fentanyl-activated striatal ensembles using the ArcTRAP approach. We outline the necessary steps to breed ArcTRAP mice, prepare drugs/reagents, conduct behavioral training, and perform tagging and manipulation. This approach can be adapted to investigate drug-recruited ensembles in other brain regions, providing a versatile tool for exploring the neural mechanisms underlying addiction.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A protocol for tagging and manipulating fentanyl-activated neurons

Detailed requirements for breeding and drug preparation

Procedures for fentanyl-induced hyperlocomotion and place preference

Adaptable for tagging active ensembles by other drugs in different behaviors

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Activity-dependent labeling and manipulation of fentanyl-recruited striatal ensembles using ArcTRAP approach

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SUMMARY

Understanding the memory substrates underlying substance abuse requires the permanent tagging and manipulation of drug-recruited neural ensembles. Here, we present a protocol for activity-dependent labeling and chemogenetic manipulation of fentanyl-activated striatal ensembles using the ArcTRAP approach. We outline the necessary steps to breed ArcTRAP mice, prepare drugs and reagents, conduct behavioral training, and perform tagging and manipulation. This approach can be adapted to investigate drug-recruited ensembles in other brain regions, providing a versatile tool for exploring the neural mechanisms underlying addiction.

For complete details on the use and execution of this protocol, please refer to Wang et al.[1]

BEFORE YOU BEGIN

Engrams are the physical or chemical changes induced by learning and encode newly formed memories, with the neurons that hold these engrams being referred to as engram cells.[2,3] Since the first proposal of this idea by Richard Simon,[4] engram has always been illusional but not physical due to technical limitations to confirm the existence of the engrams in the brain. However, taking advantage of immediate early genes (e.g., *cfos*, *Arc*, etc.) and neuronal activity marker calcium, the outburst of new tools such as FosTRAP and ArcTRAP (targeted recombination in active populations),[5,6] CaLight,[7] and FLiCRE (fast light and calcium-regulated expression)[8,9] enable scientists to permanently label and even manipulate the active ensembles during a specific learning experience. This has greatly advanced our understanding of how memory is formed, consolidated, and changed.[2,3,10–15]

While engram studies have historically focused on hippocampal-, prefrontal- and amygdala-related learning such as fear memory,[3,12,16–23] the importance of engram cells in substance use disorder has recently come to light. Substance use disorder, which is often considered a maladaptive form of learning and memory,[24] critically depends on engram cells in various brain regions.[10,11,13,25–30] The dorsal striatum, as the relay point between the cortical activity and the basal ganglia, controls the action selection and has been implicated in numerous drug-related behaviors.[31–33] However, striatal-related drug engrams have not been extensively studied. We seek to understand whether and how striatal neurons are activated by fentanyl and contribute to fentanyl-related learning. Here, we present a protocol for permanent tagging together with chemogenetic manipulation of fentanyl-recruited neurons in the dorsal striatum using the ArcTRAP approach to study fentanyl-recruited striatal engrams.

This protocol outlines the procedure for tagging neurons that are active during a drug experience using triple transgenic ArcTRAP;Ai140-GFP;D1-tdTomato (D1-tdT) mice. This particular mouse line also allows us to analyze the component of tagged neurons within the dorsal striatum, given the fact that major striatal neurons are direct-pathway medium spiny neurons (dMSNs) expressing dopamine D1 receptors and indirect-pathway MSNs (iMSNs) expressing dopamine D2 receptors.[34,35] Using a conditioned place preference (CPP) paradigm, we establish a basic fentanyl pavlovian conditioning in ArcTRAP mice and induce DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) expression specifically in fentanyl-activated ensembles. The procedures for functionally examining the role of fentanyl-recruited ensembles in CPP memory retrieval are also outlined. While we mainly used ArcTRAP lines in our study, this protocol can also be adapted to other transgenic lines, such as FosTRAP, which may be better suited for specific brain regions where ArcTRAP has non-specific expression. This streamlined protocol will enable researchers to investigate drug engrams in various brain regions and improve our understanding of the neural mechanisms underlying addiction.

Institutional permissions

All animal care and experimental procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee and were conducted in agreement with the National Research Council Guide for the Care and Use of Laboratory Animals. Readers should acquire animal-use permissions from the corresponding institutions before starting this protocol.

Breeding of ArcTRAP mice

Timing: >10 weeks

1. Purchase breeders from the Jackson Laboratory.

Note: The use of Cre-dependent reporter lines (e.g., Ai140) or cell-type-specific reporter lines (e.g., D1-tdTomato) are selected based on the needs.

   a. For this protocol, our breeders include:
      i. ArcTRAP (Arc^{CreER}),[5] which expresses Cre recombinase in active neurons under the control of 4-hydroxytamoxifen (4-OHT).
      ii. Ai140 (TIT2L-GFP-ICL-tTA2),[36] which is a Cre-dependent GFP reporter to tag captured neurons.
      iii. D1-tdT (Drd1a-tdTomato),[37] which labels striatal dMSNs with the red fluorescent protein, tdTomator. This will be switched with reporter lines of your choice. The color should be different from the reporter for Arc-tagged neurons, e.g., eGFP from the Ai140 mice.
      iv. Wild-type (WT) C57BL/6J mice.

Note: Some strains may only have frozen embryos available. Please refer to published protocols for the cryo recovery procedure.[38–40]

Note: For breeding triple transgenic mice, like ArcTRAP;Ai140-GFP;D1-tdT, it is important to consider the inheritance of each genotype. To label Arc-tagged neurons with Cre-dependent green reporter, we can choose from Ai140-GFP and Snap25-GFP.[41] Since Ai140-GFP is homozygous yet Snap25-GFP is heterozygous, the theoretical yield of triple transgenic mice is expected to be twice as high when using Ai140-GFP mice (See table below).

Breeders	Triple transgenic offspring	Expected inheritance
$ArcTRAP^{+/-}$;$D1\text{-}tdT^{+/-}$ × $Ai140\text{-}GFP^{+/+}$	$ArcTRAP^{+/-}$;$D1\text{-}tdT^{+/-}$;$Ai140\text{-}GFP^{+/+}$	1/4
$ArcTRAP^{+/-}$;$D1\text{-}tdT^{+/-}$ × $Snap25\text{-}GFP^{+/-}$	$ArcTRAP^{+/-}$;$D1\text{-}tdT^{+/-}$;$Snap25\text{-}GFP^{+/-}$	1/8

2. Breed single transgenic lines to expand the repository by establishing the mating pairs. Our mating strategies are listed below, with each pair residing in one housing cage with suitable enrichments.
   a. For ArcTRAP mating pairs: one heterozygous ArcTRAP$^{+/-}$ male mouse with two WT female mice.
   b. For Ai140-GFP mating pairs: one homozygous Ai140-GFP$^{+/+}$ male mouse with two Ai140-GFP$^{+/+}$ female mice.
   c. For D1-tdT mating pairs: one heterozygous D1-tdT$^{+/-}$ male mouse with two WT female mice.

   Note: To increase the number of pups carrying the desired heterozygous gene in a shorter amount of time, we have established mating pairs consisting of one transgenic male and two WT females. Alternatively, mating pairs with one WT male and two transgenic females are also feasible, and the exact pairing is determined by the availability of breeders at the moment. This approach differs from the traditional method of using one transgenic male and one WT female in each mating pair. For more information, please refer to troubleshooting.

   Note: Genotype is determined by polymerase chain reaction (PCR) of tail DNA samples extracted around postnatal day 10–14. Litters with undesired genotypes (e.g., −/−) are sacrificed immediately after their genotypes are identified. Litters will be separated from parents around postnatal day 21 and group housed at a maximum number of 5 mice per cage.

3. Breed double transgenic ArcTRAP;D1-tdT line by creating mating pairs composed of one ArcTRAP$^{+/-}$ male and one D1-tdT$^{+/-}$ female mouse.
4. Breed triple transgenic ArcTRAP;Ai140-GFP;D1-tdT line by creating at least 4 groups of mating pairs to maximize the yielding. Our mating pairs are listed as below:
   a. Pair 1&2: one Ai140-GFP$^{+/+}$ male mouse with two ArcTRAP$^{+/-}$;D1-tdT$^{+/-}$ female mice.
   b. Pair 3&4: one ArcTRAP$^{+/-}$;D1-tdT$^{+/-}$ male mouse with two Ai140-GFP$^{+/+}$ female mice.

   Note: The exact arrangement of mating pairs is determined by the availability of your breeders. While our suggested pairs essentially yield the same result, we do recommend using both sexes for each genotype to ensure optimal breeding outcomes in case a breeder of a specific sex has a low reproductive rate.

   Note: During the breeding of triple transgenic ArcTRAP;Ai140-GFP;D1-tdT lines, generated single or double lines that are ArcTRAP$^{+/-}$ can be used for chemogenetic manipulation. Therefore, breeding of ArcTRAP single line specifically may not be necessary. However, the exact animal number should be determined by the experiment design and actual yielding rate.

   Note: All mice are group housed initially in our experiment (maximum 5 mice per cage). However, some male mice may become irritable and aggressive after fentanyl exposure. If severe fighting occurs in the cage, separate the aggressor. In addition, single housing the mice has been shown to induce anhedonia and anxiety.[42] If your study is related to emotion, such as drug-withdrawal-induced negative emotions,[1] we suggest carefully considering the housing condition to avoid confounding factors from single housing. In addition, our protocol employs both male and female mice, and can accommodate studies requiring a specific sex.

Stereotaxic virus infusion

Timing: ~1 h per mouse

Our study includes two major parts: A) histological assessment of fentanyl-tagged neurons and B) functional manipulation of fentanyl-tagged neurons. Stereotaxic virus infusion is necessary only if

the study involves testing the functional roles of tagged neurons with DREADDs. If functional manipulation is not part of the study, this section may be skipped.

5. Prepare adeno-associated virues (AAVs).
   a. Purchase AAV8-hSyn-DIO-hM$_4$D$_i$-mCherry and AAV8-hSyn-DIO-mCherry (as a control) (titer $\geq 1\times10^{13}$ vg/mL) from the vector core at the University of North Carolina at Chapel Hill (UNC) or from Addgene.
   b. Thaw and aliquot the virus once received; store the aliquoted viruses at −80°C.

   Note: We recommend using the aliquot size of 5 μL. If using the coordinates exactly as ours, each mouse receives 0.5 μL/site × 4 injection sites = 2 μL of viruses. Each aliquot thus can accommodate 2 mice, with 1 μL left for adjustment.

6. Prepare ArcTRAP or ArcTRAP;Ai140 mice aged over 8 weeks for surgery.

   Note: In our case, we used 3- to 4-month-old mice. The surgery should be conducted at least 1 week before the day of pre-conditioning in the CPP experiment to allow sufficient time for AAV to infect cells and ensure the recovery of the animals.

7. Conduct the surgery.

   Note: The detailed procedures of the stereotaxic virus infusion have been provided by extensive protocols,[43–45] which we will not further elaborate here. Our coordinates (relative to the bregma) for the AAV infusion are listed below:

   a. Infusion sites 1 (bilateral): A/P 1: +0.74 mm, M/L 1: ±1.3 mm, D/V 1: −2.9 mm
   b. Infusion sites 2 (bilateral): A/P 2: +0.3 mm, M/L 2: ±1.7 mm, D/V 2: −2.9 mm

   Note: All ArcTRAP and ArcTRAP;Ai140-GFP mice that will be used in the CPP experiment will receive virus infusions at all four sites. We select four sites due to the large size of the striatum. The infusion rate is 0.12 μL/min. The volume is 0.5 μL per site to restrict the viruses at the dorsal striatum. At the end of the infusion, the injectors remained at the site for 10 min to allow virus diffusion. In addition, we use flat injectors to allow viruses to uniformly diffuse.

8. Return mice to their home cages after the surgery (group housed; maximum 5 mice per cage).

   ⚠ CRITICAL: Monitor the animal's activity for the following week. Mice with fully recovered incision will be used for later behavioral manipulation.

Habituate the mice to experimenter handling

Timing: ~15 min each day (depending on the group size) for 3 days

In this protocol, scruff handling is utilized to secure mice for intraperitoneal (i.p.) injection, but both the handling and injection can be stressful experiences,[46] which may lead to unwanted neuronal activation. To minimize the impact of stress, anxiety, and other uncomfortable factors during human handling, it is recommended to habituate the mice to experimenter handling at least 3 days prior to the experiment. We have provided our protocol for scruff handling the mice, although other less stressful restraint methods may also be used.[47] The crucial point is to acclimate the mice to the chosen method and maintain the restraint method throughout the experiment period to minimize any effects on ensemble tagging.

9. At least three days before the behavioral testing, habituate the mice to scruff handling (Figure 1).

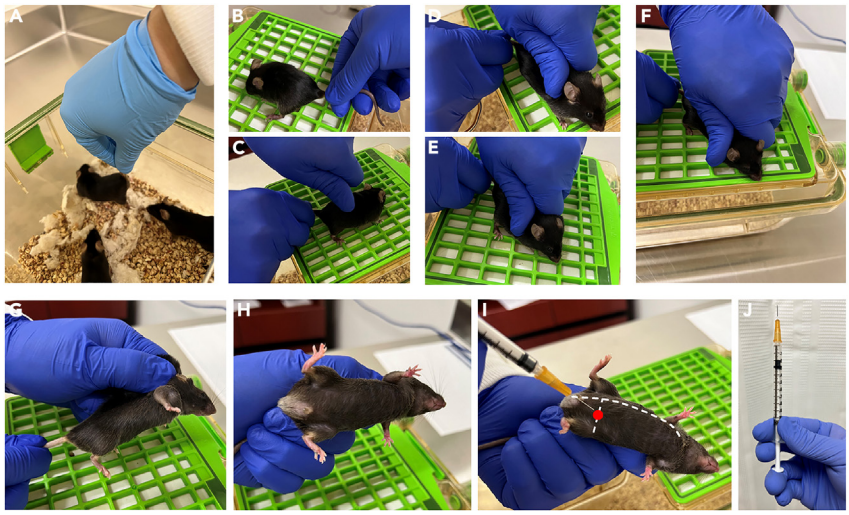

Figure 1. Handle the mouse

(A) Grab the mouse by the base of the tail.
(B) Place the mouse on a flat surface with grid (we use the cage cover).
(C) The thumb and index finger of the left hand form a "V" shape, and place right above the mouse back hump.
(D) Press down on the back hump. Exert pressure to restrict the mouse body that cannot move forward or backward.
(E) Push left hand forward until touching the shoulder.
(F) When feel the shoulder bone, squeeze the thumb and index finger on the skin.
(G) Lift the mouse up. The left hand holds the skin while the right hand holds the tail.
(H) Transfer the tail from the right hand to the ring and pinky finger of the left hand.
(I) The injection point (red dot) should be around the middle point between the base of the thigh and the body midline. Since the liver is located on the right side, in order to avoid the needle penetrating the liver, injection on the left side (red dot) is a safer option.
(J) Hold the syringe in this way so the ring finger can exert more pressure to push the 4-OHT/oil mix while holding the syringe still.

a. Gently lift the mouse from the cage by the tail, and immediately place the mouse on a surface with grid (Figures 1A and 1B). Use a curtain to cover the cage with other mice inside.
b. Gently but firmly place the thumb and forefinger on the back hump of the mouse (Figure 1C), push the mouse body down but do no exert too much pressure on the spine (Figure 1D). Move forward over the shoulders and quickly grasp the scruff of the neck close to the base of the skull (Figures 1E–1G). Right hand holds the tail to prevent hind body movement (Figure 1G).
c. Use the ring and pinky finger to restrain the tail (Figure 1H). Hold in position for 10 s and gently place the mouse back in the cage.
d. Repeat this handling practice every day until the behavior testing.

Measure the body weight

Timing: ~15 min (depends on the group size)

10. One day before starting the behavior test, measure the mouse's body weight.
    a. Put a clean 600 mL beaker on the scale. Zero the scale.
    b. Gently grab the mouse and place it in the beaker. Record the body weight.
    c. Calculate the required fentanyl volume based on body weight. If using the same fentanyl dose and working solution as ours, the required volume will be 0.1 mL/10 g of the body weight.

Note: The body weight will decrease after repeated fentanyl exposure (~90% of the baseline).[1] It is a good practice to monitor body weight on a daily basis. Adjust the injection volume when the body weight drops significantly (> 10% change).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
AAV8-hSyn-DIO-hM$_4$D$_i$-mCherry	UNC Vector Core (or available in Addgene)	UNC: AV4980 (Addgene: 44362-AAV8)
AAV8-hSyn-DIO-mCherry	UNC Vector Core (or available in Addgene)	UNC: AV4991 (Addgene: 50459-AAV8)
Chemicals, peptides, and recombinant proteins		
Fentanyl citrate	Sigma-Aldrich	Cat#:1270005
Compound 21 (C21)	NIMH Chemical Synthesis and Drug Supply Program	P-908
(Z)-4-hydroxytamoxifen (4-OHT)	NIMH Chemical Synthesis and Drug Supply Program	H-907
Castor oil	Sigma-Aldrich	259853
Sunflower oil	Sigma-Aldrich	S5007
Sterile saline solution 0.9%	Covetrus	069170
200-proof ethanol	VWR	89125-188
Antifade mounting medium with DAPI	Vectashield	Cat#: H-1500
Isoflurane	Covetrus	029404
Paraformaldehyde solution (PFA, 40%)	MACRON Chemicals	5016-08
Sodium chloride (NaCl)	Sigma-Aldrich	S7653
Potassium chloride (KCl)	Sigma-Aldrich	P3911
Disodium phosphate (Na_2HPO_4)	Sigma-Aldrich	S3264
Monopotassium phosphate (KH_2PO_4)	Sigma-Aldrich	P5379
Sucrose	Sigma-Aldrich	S1888
Tissue-Tek® O.C.T. Compound	Sakura	4583
Experimental models: Organisms/strains		
Mouse: C57BL/6J, male and female, aged 1.5–8 months (breeder)	The Jackson Laboratory	000664 RRID:IMSR_JAX:000664
Mouse: Ai140-GFP (TIT2L-GFP-ICL-tTA2), male and female, aged 1.5–8 months (breeder) (Genotyping information: https://www.jax.org/Protocol?stockNumber=030220&protocolID=30173)	The Jackson Laboratory	030220 RRID:IMSR_JAX:030220
Mouse: ArcTRAP (Arc^{CreER}), male and female, aged 1.5–8 months (breeder); aged 3–4 months (experiment) (Genotyping information: https://www.jax.org/Protocol?stockNumber=021881&protocolID=26031)	The Jackson Laboratory	021881 RRID:IMSR_JAX:021881
Mouse: D1-tdTomato, male and female, aged 1.5–8 months (breeder) (Genotyping information: https://www.jax.org/Protocol?stockNumber=016204&protocolID=25241)	The Jackson Laboratory	016204 RRID:IMSR_JAX:016204
Mouse: ArcTRAP;Ai140-GFP, male and female, aged 3–4 months (experiment)	This paper	N/A
Mouse: ArcTRAP;Ai140-GFP;D1-tdTomato, male and female, aged 3–4 months (experiment)	This paper	N/A
Software and algorithms		
IMARIS 8.3.1	Bitplane	https://imaris.oxinst.com/packages
EthoVision XT	Noldus	https://www.noldus.com/ethovision-xt
Med PC V	Med Associates Inc	https://med-associates.com/product/med-pc-v/
Fluoview 1200	Olympus	https://www.olympus-lifescience.com/en/laser-scanning/fv3000
MATLAB	MathWorks	https://www.mathworks.com/products/matlab.html
MATLAB code for making heatmap of locomotor activity	MathWorks	https://github.com/Xxie119/MATLAB.git
Other		
50 mL centrifuge tube	Fisher Scientific	14-955-239
15 mL centrifuge tube	Fisher Scientific	14-955-238
1.5 mL Microcentrifuge tube	Fisher Scientific	05-408-137
1 mL insulin syringe (29G × $^1/_2$")	Comfort point	26029

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
1 mL syringe with Luer Slip	Covetrus	060734
Pipette (1000 mL, Gilson™ PIPETMAN Classic™ Pipets)	Fisher Scientific	Gilson™ F123602
Pipette tip (1000 mL)	Fisher Scientific	02-707-124
Micro spatula	Fisher Scientific	13-820-086
26 Gauge × 1/2" needle	BD PrecisionGlide	305111
18 Gauge × 1″ needle	BD PrecisionGlide	305195
0.2 µm syringe filter	Gelman Laboratory	4454
600 mL beaker	Pyrex	CLS1000600
Scale	Mettler Toledo	AB204-S/FACT
Vortex Genie 2	Fisher Scientific	12-812
Vacuum centrifuge	Labconco	7810010
Hexagonal rotor (for vacuum centrifuge)	Labconco	7450700
Open field arena	Med Associates Inc	ENV-515S-A
Conditioned place preference chamber	Homemade	N/A
Recoding camera	Basler	1300-60
Laser scanning system	Olympus	FV1200
Microscope	Olympus	BX61WI
Anesthesia evaporator	VETEQUIP Inc	911104
Cryostat	Microm	HM505EVP
Tissue culture plate 24 wells	Fisher Scientific	FB012929
Microscope slides	Fisher Scientific	12-544-7
Microscope cover glass	Fisher Scientific	1254418
Black blanket for cage covering	Blue Zoca	ASIN: B09NS6LLR7

MATERIALS AND EQUIPMENT

Open field chamber overview

This protocol involves the monitoring of drug-induced locomotor activity. We use the open field chamber to record the distance traveled after the drug administration. Alternatively, any chambers with ample space for animals to move and equipped with a recoding camera or beam detector can be used (see alternatives below). Our open field chambers (Med Associates, 43 cm × 43 cm × 21 cm height) are each housed in a sound-attenuating box (Figure 2A), illuminated by two 45 lux bulbs (Figure 2B). All boxes are housed in a single room, with sound-attenuating doors. A table is used to house the animal cages after transferring the mice to the room (Figure 2A). All chambers are equipped with an infrared beam detector that connects to a computer running the Activity Monitor software (Med Associates). The software records movement and location information of the mice.

Conditioned place preference (CPP) chamber overview

We use the conditioned place preference (CPP) test to investigate pavlovian learning in animals, where they associate the rewarding effect of fentanyl with the drug-administering environment.[48] This type of learning recruits engram cells, which encode information about the learning experience.[49] By chemogenetically manipulating the engrams, we can test the role of fentanyl-activated neurons during this type of learning. Our CPP chambers are constructed in-house using plexiglass plates and are housed in a single room (Figure 2C). A three-layer rack is used to house the animal cages after transferring the mice to the room (Figure 2C). All CPP chambers are divided into two compartments that are illuminated by an adjustable room light. The two compartments had different floor textures (bar or grid) and wall colors (gray or white stripe) (Figures 2D and 2E). The two compartments are separated by a manual guillotine door. We have a total of six chambers, and every two chambers are equipped with an overhead camera to record the animals' locations. The camera system is connected to a computer, and EthoVision XT is used to monitor and evaluate the CPP (Figures 2F and 2G). Before starting the experiment, it is important to set the protocol and ensure

Figure 2. Open field and CPP setup

(A and B) Open field room setup. We have six sound-attenuating chambers (A, 1). A computer with Activity Monitor software controls all the open field chambers (A, 2). A table is used to house the cages after entering the room to allow mice to acclimate to the room environment (A, 3). Each open-field arena is housed in one sound-attenuating chamber (B).

(C–E) CPP room setup. We have six CPP chambers (C, 1). Three Basler cameras are mounted overhead to monitor the behavior (C, 2). Every two chambers are monitored by one camera. A computer equipped with EthoVision XT monitors the behavior (C, 3). A rack is used to house the cages after entering the room to allow mice to acclimate to the room environment (C, 4). Two chambers are placed side by side (D, E). The center guillotine door can be opened or closed by flipping the Plexiglas plate (Panel E shows the opening position).

(F) CPP protocol setup. Every two chambers are monitored by one camera. Each chamber is designated as one "Arena". Each Arena contains two compartments, which are separated by the center gate. In our case, we use the wall color to name the compartments as "Stripes" (showing as green in the software) and "Grey" (showing as purple in the software).

(G) CPP recording window. Red-dashed rectangle window shows the real-time video of the chambers. A red-dashed circle is the recording start button.

that the zone settings cover the entire region of the specific compartment (Figure 2F). Additionally, a spare mouse needs to be used to correct the subject detection settings.

Prepare fentanyl citrate solution

⚠ CRITICAL: Fentanyl is extremely skin-absorbable. Handle with extreme caution. Wear appropriate personal protective equipment (PPE) and seek medical assistance immediately if getting into contact with fentanyl.

- Make the stock solution at 1.2 mg/mL with 0.9% sterile saline (40× stock solution). Vortex until fully dissolved. Filter the stock solution through 0.2 μm syringe filter.

Note: The stock solution can be stored for at least three months under 4°C.[50] The amount and concentration of the stock solution should be determined by your group size and desired working concentration. Store the unused fentanyl powder under 4°C.

- Dilute the stock solution with 0.9% sterile saline to make the working solution at 0.03 mg/mL (1× working solution). We choose this concentration because the dose we use is 0.3 mg/kg for the injections. For 10 g of the mouse's body weight, we inject $\frac{0.3\ mg/kg}{0.03\ mg/mL}$ = 10 mL/kg = 0.1 mL/10 g. The concentration of the working solution is at user's discretion.

Note: Store the working solution under 4°C, which can be maintained for at least three months if not contaminated by used needles. When using, take only the volume that is sufficient for one injection day every time. If the working solution is contaminated by needles from the injection day, discard it according to institutional guidelines.

Prepare solutions for perfusion and sectioning

Stock 10× Phosphate-buffered saline (PBS), 1 L

Reagent	Final concentration	Amount
NaCl	1.54 M	90 g
KCl	27.83 mM	2 g
Na_2HPO_4	0.1 M	14.4 g
KH_2PO_4	17.64 mM	2.4 g
ddH_2O	N/A	~900 mL
Total	**N/A**	**1000 mL**

Note: After complete mixing, top up the final solution to 1 L. Filter the solution. The pH of 10× stock is approximately 6.8, but when diluted to 1× PBS, it should change to 7.4. Stock solution can be stored at room temperature (20°C–25°C /68–77°F) for at least 3 months.

4% PFA for tissue fixing

- For 1 L 4% PFA solution, dilute 40% PFA by adding 100 mL 40% PFA into 900 mL 1× PBS solution. Adjust pH to ~7. Solution is stored under room temperature (20°C–25°C /68–77°F) for at least 2 weeks.

30% sucrose solution for cryoprotection

- For 1 L 30% sucrose solution, add 300 g of sucrose into 1 L 1× PBS solution and mix well. Solution is stored under 4°C for at least 3 months.

Alternatives

- The open field and the CPP chambers utilized in this protocol can be substituted with either commercially available or home-made versions. However, when constructing a home-made chamber, certain features must be present to ensure a successful experiment:
  - For open field chambers, it is recommended to use a square, enclosed arena with a length and width of at least 27 cm and a height of at least 20 cm for mice. The walls can be either transparent or opaque, but if transparent, a curtain or cover must be placed between neighboring chambers to prevent between-subject interactions. A sound-attenuating box is recommended to house individual chambers. The floor must be flat and opaque, and locomotor activity can be

monitored by an infrared beam detector installed around the perimeter of the floor (e.g., Med Associates ENV-256, Panlab LE8815), or by the overhead camera with behavior tracking software such as EthoVision or ANY-maze.

- For CPP chambers, a rectangle-shaped, enclosed arena with a length of at least 40 cm, a width of at least 20 cm, and a height of at least 20 cm is recommended for mice. The chamber should be equally divided into two compartments with a manual guillotine door in the middle. The color of the walls and texture of the floor between the two compartments must be distinct, for example, black and white stripes versus pure grey color, and bar versus grid floor. Adding odor cues can also assist mice in distinguishing between the two compartments. For home--made CPP chambers, it is recommended to use an overhead camera with behavior tracking software such as EthoVision or ANY-maze since an infrared beam detector can be challenging to incorporate.

- Corn oil or peanut oil has been used to dissolve 4-OHT.[5,51,52] If the castor and sunflower oil are not readily available, corn or peanut oil may be used as a substitute.
- Depending on the neuronal type and brain region, AAV vectors containing Cre-dependent hM_4D_i with different fluorescence reporters and promoters can be used (e.g., AAV8-hSyn-DIO-hM_4D_i-mCitrine: 50455-AAV8 in Addgene).

STEP-BY-STEP METHOD DETAILS

Habituation and baseline locomotor activity in open field

Timing: 2 days

This step is performed to allow ArcTRAP;Ai140-GFP;D1-tdT mice (Figure 3A) habituate to the testing environment and prevent novelty-induced effects on neuronal activity. Each mouse will be assigned to one specific chamber and will be trained in that chamber for the entire experiment period. To track and record locomotor activity in the open field arena, we utilize the Activity Monitor 7 software (Med Associates), which is connected to an infrared beam detector on the floor. The default ambulatory detection is set to 7.62 cm of movement, while the default zone detection encompasses the entire arena without sub-regions.

1. Habituate the mice to the open field chamber and measure the baseline activity (Figure 3B).
   a. Prepare the testing room.
      i. Before bringing the mice to the room, turn the room light to the dimmest level (or use the red light).
      ii. Ensure the room is quiet and thoroughly cleaned.

   ⚠ CRITICAL: Exposure to bright room light has been demonstrated to induce anxiety-like behaviors in rodents, potentially affecting learning and memory.[53,54] To mitigate the impact of environmental stimuli on neuronal activation and behavior, it is highly recommended to use dim or red light during entire experimental procedures.

   b. Use clean, new cages to transfer the mice from the housing room to the testing room.
   c. Leave the mice undisturbed for at least 30 min before the behavioral testing.

   Note: Mice are placed with their original cage mates in the new cages to reduce unnecessary stress.

   d. Load the protocol in the Activity Monitor 7 software.

   Note: Default detection and zone settings are adequate. Set the session time to 30 min. The protocol will wait until the animal is detected in the box to start the recording.

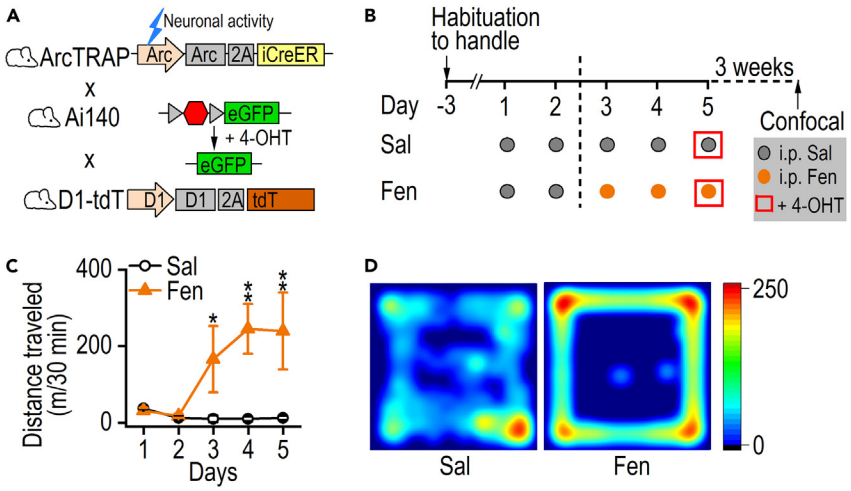

Figure 3. Fentanyl-induced hyperlocomotion in ArcTRAP;Ai140-GFP;D1-tdT mice and representative data

(A) Schematic of the ArcTRAP;Ai140-GFP;D1-tdT mouse illustrates that the expression of GFP is dependent on activity-induced Arc expression and 4-OHT injection.

(B) Protocol timeline; all mice will receive saline injections and habituate to the open field chamber for the first 2 days. Mice in the fentanyl group will receive fentanyl injects for days 3–5, whereas mice in the saline group will continue to receive saline injections. On day 5, all mice will receive 4-OHT injections after the open field measurement to tag active ensembles recruited by saline or fentanyl injections. Three weeks after the tagging, mice will be perfused, and their brains will be sliced for confocal imaging.

(C) Fentanyl injections significantly increase the locomotor activity as compared to the saline injections. *$p < 0.05$, **$p < 0.01$ as compared to the saline group; Two-way RM ANOVA followed by Tukey *post hoc* test.

(D) Representative heatmap during day 5 from one mouse received saline and one mouse received fentanyl injection. N = 3 mice (Sal) and 3 mice (Fen). Data points represented as mean ± SEM. Reprinted from "Striatal μ-opioid receptor activation triggers direct-pathway GABAergic plasticity and induces negative affect", Volume 42, Issue 2, 112089, Cell Reports, Copyright (2023), with permission from Elsevier.

e. Use 1-mL insulin syringe to prepare 0.9% sterile saline.

Note: Each mouse will have its own syringe. Based on the calculation of the required fentanyl volume for each mouse, withdraw the same volume of saline.

f. Administer 0.9% sterile saline intraperitoneally (i.p.) to the mouse (Figures 1I and 1J).
   i. Use the same way as during the scruffing habituation to grab the mouse (Figures 1A–1H).

Note: It is not necessary to sterilize the skin before the injection. The antiseptic agents could not penetrate the fur. This will simply prolong the injection time and make the animal stressful.

⚠ CRITICAL: For all the i.p. injections described in this protocol, after the needle enters the peritoneum, withdraw ~0.02 mL before injecting any substance to make sure the needle does not puncture the organs and no blood is withdrawn.

g. After injection, put the mouse back in the cage. Leave undisturbed for 15 min.
h. Hold the entire cage as close to the open field chamber as possible, gently grab the mouse by the tail and put it into the center of the open field arena.
i. Gently close the sound-attenuating door.

Note: The system will automatically start recording once it detects an object inside the arena.

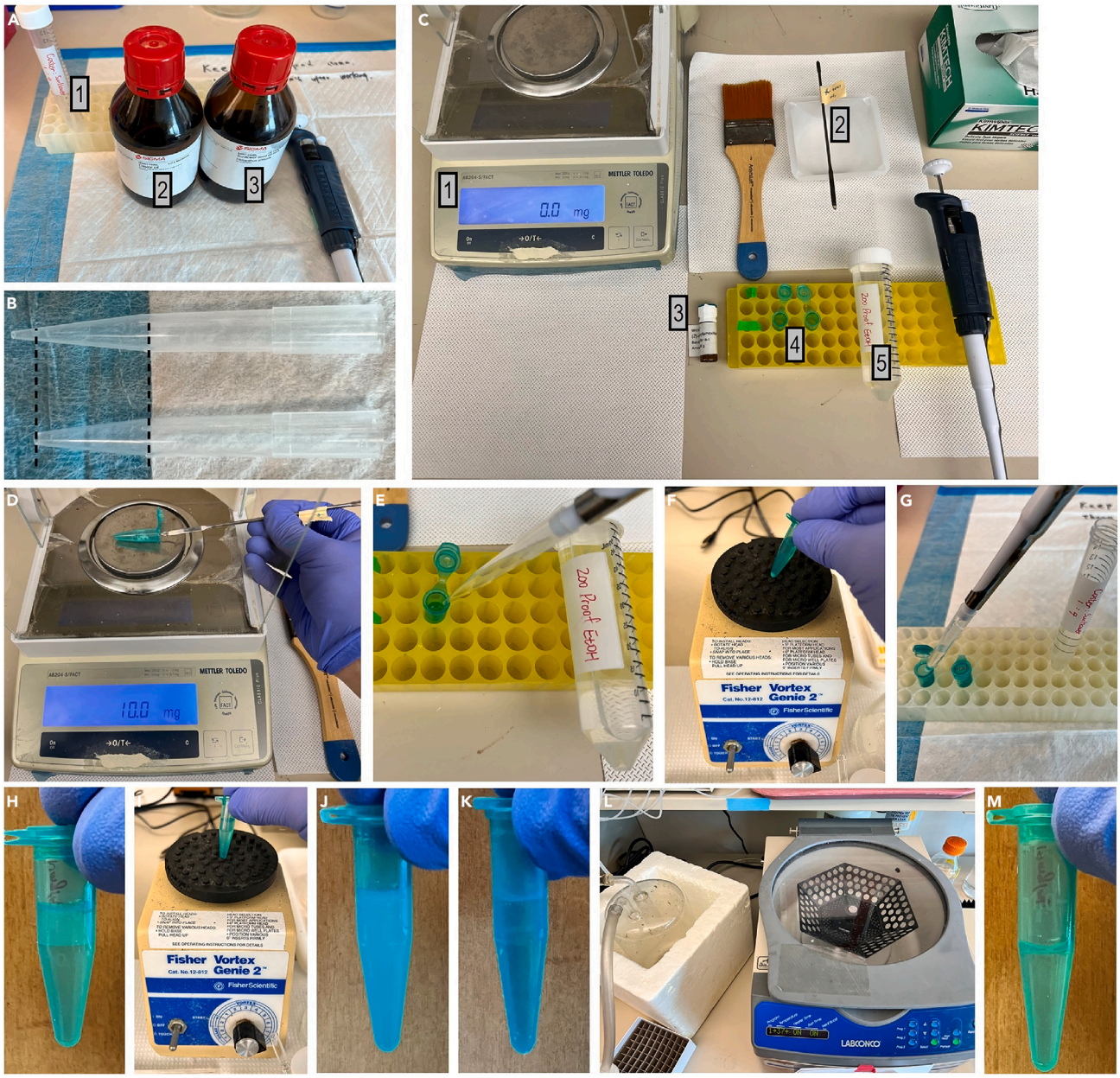

Figure 4. 4-OHT preparation

(A) Prepare the oil mix. In a 15-mL tube (1), add 2 mL castor oil (2) and 8 mL sunflower oil (3). Mix thoroughly.

(B) The pipette (1000 μL) that will be used to add oil mix to the 4-OHT needs to be trimmed for ~1/8″ to better withdraw oil.

(C) Setup the scale (1). A thin spatula (2) will be used to transfer 4-OHT powder (3). We will make two 1.5-mL microcentrifuge tubes (4) of 0.5 mL 4-OHT (10 mg/mL in 200-proof ethanol [5]).

(D) Zero the scale for the 1.5-mL microcentrifuge tube. Then slowly add 4-OHT powder. Here we take 10 mg of 4-OHT.

(E) Add 1 mL 200-proof ethanol to the tube with 10 mg 4-OHT.

(F) Vortex vigorously to allow 4-OHT completely dissolve in ethanol. After dissolving the 4-OHT, we will equally separate it into two 1.5-mL tubes.

(G) Equally separate 1 mL 4-OHT/ethanol solution into two tubes (0.5 mL each). Add 0.5 mL oil mix to each tube. Now each tube will have 0.5 mL oil and 0.5 mL 4-OHT/ethanol.

(H) The 4-OHT/ethanol solution and the oil mix will separate at the beginning.

(I) Vortex the mixture.

(J) The mixture may appear cloudy after vortexing.

Figure 4. *Continued*
(K) After sitting for a couple of seconds, the mixture will appear clear. Make sure no separation of the solution and oil before preceeding to the next step.
(L) Using the vacuum centrifuge, evaporate the ethanol. Set the temperature at 37°C and centrifuge for 45 min. If the volume is greater than 0.5 mL after 45 min, re-centrifuge for 15 min. Repeat until the final volume is 0.5 mL.
(M) The final volume is 0.5 mL after all ethanol is evaporated, and the solution is a clear 4-OHT/oil mixture.

j. Repeat this step for all the mice.
k. When the session is finished, hold the cage near the open field chamber and place the mouse back in the cage.
l. Leave all mice undisturbed for 30 min in the testing room before transferring them back to the housing room.

Note: If having multiple batches of mice, use 70% ethanol spray to clean the arena between batches.

Note: Repeat the above steps for the second day to record the baseline locomotor activity for two days.

⚠ CRITICAL: Ensure the behavioral testing is conducted around the same time of the day for each mouse to avoid circadian impact.

Fentanyl-induced hyperlocomotion in open field

Timing: 3 days

This step is to repeatedly expose the mice to fentanyl. At the same time, we will measure fentanyl-induced locomotor activation to confirm the drug effect (Figures 3C and 3D).

2. Follow all steps as steps 1a-l, except for the fentanyl group in which the injected solution is fentanyl.

Note: The saline group will receive saline injections for all three days. Conduct fentanyl/saline injections for three consecutive days (Figure 3B).

Note: See troubleshooting.

⚠ CRITICAL: On the third day of fentanyl/saline injections (Figure 3B, Day 5 of the procedure), which is also the tagging day, it is crucial to avoid introducing any salient events before or during testing (e.g., loud noise, sudden light, etc.). This is because the tagging window lasts approximately 6 hours and any events that trigger neuronal activity during this period will be labeled.[5]

Tagging of fentanyl-activated neurons in open field

Timing: 1.5 h

Timing: 1 h (for step 3)

Timing: 0.5 h (depends on the group size) (for step 4)

This step is to tag fentanyl (or saline)-activated neurons after the last fentanyl (or saline) injection.

3. Prepare 4-hydroxytamoxifen (4-OHT) (Figure 4).

⚠ CRITICAL: Make fresh 4-OHT only before the tagging session. We suggest preparing 4-OHT in the morning of the tagging day.

a. Make 1:4 mixture of castor oil: sunflower oil (Figures 4A and 4B).

Note: We suggest making 10 mL mixture (2 mL castor oil with 8 mL sunflower oil) and store in the vial wrapped with tin foil.

Note: This mixture can be stored under the room temperature (20°C–25°C or /68–77°F) in a cool, dark place, away from direct sunlight and heat sources for at least 3 months.

Caution: Wear appropriate PPE and face mask accordingly to institutional guidelines when handling 4-OHT.

b. Weigh and dissolve 4-OHT powder in 200-proof ethanol to make a 10 mg/mL solution (Figures 4C–4E).

Note: We suggest using a 1.5 mL microcentrifuge tube to make a small amount of 4-OHT each time. The volume of ethanol should not exceed 0.5 mL for each tube since we will add the same volume of oil mix in the next step. Therefore, the maximum amount of 4-OHT for each tube will be 5 mg (5 mg 4-OHT/0.5 mL ethanol = 10 mg/mL). Vortex until 4-OHT is fully dissolved (Figure 4F). This may take several minutes.

Note: A heated water bath can be used to facilitate the dissolving process.

Caution: 200-proof ethanol is flammable and may cause irritation if inhaled, handle it with caution.

Note: If the scale does not support weighing amount less than 10 mg, we suggest making stock solutions in 20 mg/mL in 200-proof ethanol then aliquot to the desired working solution. Stock solution in 20 mg/mL can be stored at −20°C for up to 3 weeks.

⚠ CRITICAL: Ensure no visible white 4-OHT solids are present in the ethanol. Double-check before proceeding to the next step.

c. In the same vial, add the oil mix to combine with the 4-OHT/ethanol solution at 1:1 ratio.

Note: For 0.5 mL 4-OHT/ethanol, it needs 0.5 mL of the oil mix (Figures 4G and 4H).

d. Vortex again to ensure 4-OHT/ethanol solution is fully mixed with the oil (Figure 4I).

Note: The solution may appear cloudy and then become clear (Figures 4J and 4K).

⚠ CRITICAL: The next step will evaporate the ethanol to "transfer" the 4-OHT from the ethanol to the oil. Therefore, it is critical to ensure that the 4-OHT/ethanol is fully mixed with the oil. Otherwise, the 4-OHT itself will not easily dissolve in the oil and the final concentration will be lower than desired.

e. Using the vacuum centrifuge, concentrate the 4-OHT/ethanol/oil mixture. Set the temperature at 37°C and time for 45 min (Figure 4L).

Note: If using the same centrifuge and rotor as ours, the speed is around 40 g.

⚠ CRITICAL: Check the volume after 45 min. If the volume is greater than the volume of the oil mix, the ethanol is not completely evaporated. Re-concentrate for another 15 min. Repeat this step until all the ethanol is evaporated (Figure 4M).

Note: If a vacuum centrifuge is not readily available, an alternative method is to directly mix 4-OHT into the oil mixture by shaking it overnight at 37°C.[52] However, this process may result in undissolved particles even after overnight shaking. Users should carefully consider the timing between 4-OHT preparation and the tagging experiment.

Note: The final 4-OHT/oil mix (10 mg/mL) can be stored under 4°C for a maximum of 12 h before use.

4. Tag fentanyl-activated neurons.

⚠ CRITICAL: We will inject 4-OHT when the mouse is under anesthesia. This is due to the fact that 4-OHT injection is very painful, and this experience will activate pain- or stress-related areas. By using anesthesia, we can minimize these confounding factors, especially for the control group that will not receive drug exposure. This approach will help ensure that our results accurately reflect the effects of the drug on the experimental group, while controlling for other variables that could affect neuronal activation.

a. Prepare a 1-mL regular syringe for 4-OHT/oil.

Note: The dose for 4-OHT is 50 mg/kg. Calculate the required volume based on the dose and the body weight.

i. Use a 18-gauge needle to withdraw the 4-OHT, then switch to a 26-gauge needle for injection.

Note: Each mouse should have its own syringe and needle.

ii. Place syringes next to the anesthesia chamber.

⚠ CRITICAL: Do not use a 1-mL insulin syringe in this step. Since the needle size is small and is fixed on the syringe, it will be difficult to withdraw the oil. Using needles with a size larger than 20-gauge (e.g., 18G as we used) is optimal to withdraw 4-OHT/oil mix.

b. After the locomotor measurements are completed for the third fentanyl-injection day, gently place the mice back in the cage.
c. Immediately transfer the mice to the anesthesia chamber table.
d. Turn on the oxygen flow and place the mice into the anesthesia chamber.
e. Set the isoflurane level to the maximum to quickly knock-down the mice.

Note: Once mice are under deep anesthesia, turn off the oxygen and isoflurane flow.

f. Hold the mice and inject 4-OHT (i.p.; Figures 1I and 1J).

⚠ CRITICAL: 4-OHT/oil mix is dense, so the injection will be difficult. Do not exert too much pressure on the syringe plunger, otherwise the needle may be pushed out by excessive pressure and harm the animal.

g. Immediately place the mice back in the cage after injection.

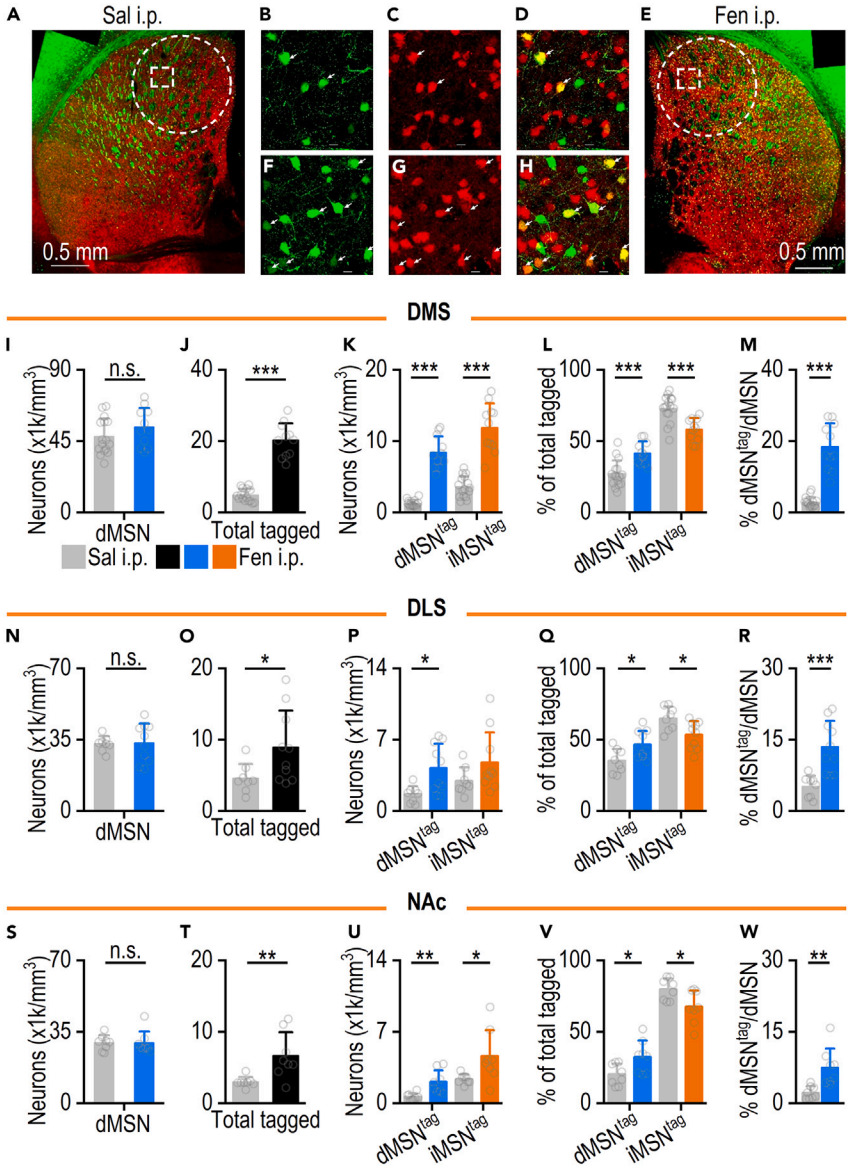

Figure 5. Fentanyl-induced neuronal activation and representative data in the striatum

(A–H) Tagged neurons following saline (A–D) or fentanyl (E–H) injection (showing in green channel) and dMSNs (showing in red channel) in the DMS. Tagged dMSNs expressed both GFP and tdT (yellow, indicated by arrows; B-D, F-H). B-D and F-H are enlarged images from dashed rectangle-enclosed regions.Scale bars: 10 μm (B-H).

(I–M) In the DMS, fentanyl did not alter dMSN density (I) but increased the density of total tagged neurons (J), tagged dMSNs (K, $dMSN^{tag}$) and tagged putative-iMSNs (K, $iMSN^{tag}$). However, the proportion of dMSNs in the tagged neurons is increased, but the proportion of putative-iMSNs is decreased (L). Consequently, the percentage of $dMSN^{tag}$ over total dMSNs is increased (M). n.s. not significant; ***$p < 0.001$ by unpaired *t* test.

(N–R) In the dorsolateral striatum (DLS), fentanyl did not alter dMSN density (N) but increased the density of total tagged neurons (O) and tagged dMSNs (P). The density of tagged putative-iMSNs marginally increases (P). However, the proportion of dMSNs in the tagged neurons is increased, but the proportion of iMSNs is decreased (Q). Consequently, the percentage of $dMSN^{tag}$ over total dMSNs is increased (R). n.s. not significant; *$p < 0.05$, ***$p < 0.001$ by unpaired *t* test.

(S–W) In the nucleus accumbens (NAc), fentanyl did not alter dMSN density (S) but increased the density of total tagged neurons (T), tagged dMSNs and tagged putative-iMSNs (U). However, the proportion of dMSNs in the tagged neurons is increased, but the proportion of putative-iMSNs is decreased (V). Consequently, the percentage of $dMSN^{tag}$ over total dMSNs is increased (W). n.s. not significant; *$p < 0.05$, **$p < 0.01$ by unpaired *t* test.

Figure 5. *Continued*
N = 16 sections/3 mice (Sal) and 11 sections/3 mice (Fen). Data points represent slices, with bars representing the mean ± SEM. Reprinted from "Striatal μ-opioid receptor activation triggers direct-pathway GABAergic plasticity and induces negative affect", Volume 42, Issue 2, 112089, Cell Reports, Copyright (2023), with permission from Elsevier.

⚠ CRITICAL: Leave undisturbed for the next 3 h before returning to the home cage.

Note: If the anesthesia chamber is far away from the behavioral testing room, consider the timing of transferring the animals to the anesthesia chamber. Minimize this time to ensure the best tagging.

5. House mice in the home cage for the next three weeks to allow the sufficient expresison of the target gene (e.g., GFP or hM4Di).
6. Perfuse the mice and section the brains for confocal imaging (Figures 3B and 5).

Note: We provide our perfusion and sectioning protocol here. For more detailed information, we recommend referring to other sources.[55,56]

a. Perfuse mice intracardially with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS).
b. Extract the brains and postfix them for 12 h or overnight in 4% PFA/PBS solution at 4°C.
c. Store the brains in 30% sucrose solution at 4°C until sectioning.
d. Cover each brain with embedding medium and serially section into 50-μm coronal slices using a cryostat at −25°C.
e. Store sections in PBS at 4°C for a maximum of one month.
f. Mount sections containing the striatum in DAPI-containing mounting media in an anterior to posterior orientation.
g. Obtain images using a confocal laser-scanning microscope.

Pre-conditioning in CPP

Timing: 1 day

ArcTRAP or ArcTRAP;Ai140-GFP mice that will be trained for CPP should have virus infused at least 1 week before the day of CPP pre-conditioning as described in "Before you begin: Stereotaxic virus infusion" and as Figure 6A. This step is to measure the baseline preference for each compartment in the CPP chamber. Each mouse will be assigned to one specific chamber and will be trained in that chamber for the entire experiment period.

7. CPP pre-condition with saline injections (Figure 6B).

⚠ CRITICAL: Make sure the conditioning time during the day for each mouse is roughly the same to reduce the circadian effect. See troubleshooting.

a. Prepare the testing room.

⚠ CRITICAL: Before bringing the mice to the room, turn the room light to the dimmest level. Ensure the room is quiet and thoroughly cleaned.

i. Open the manual guillotine door.

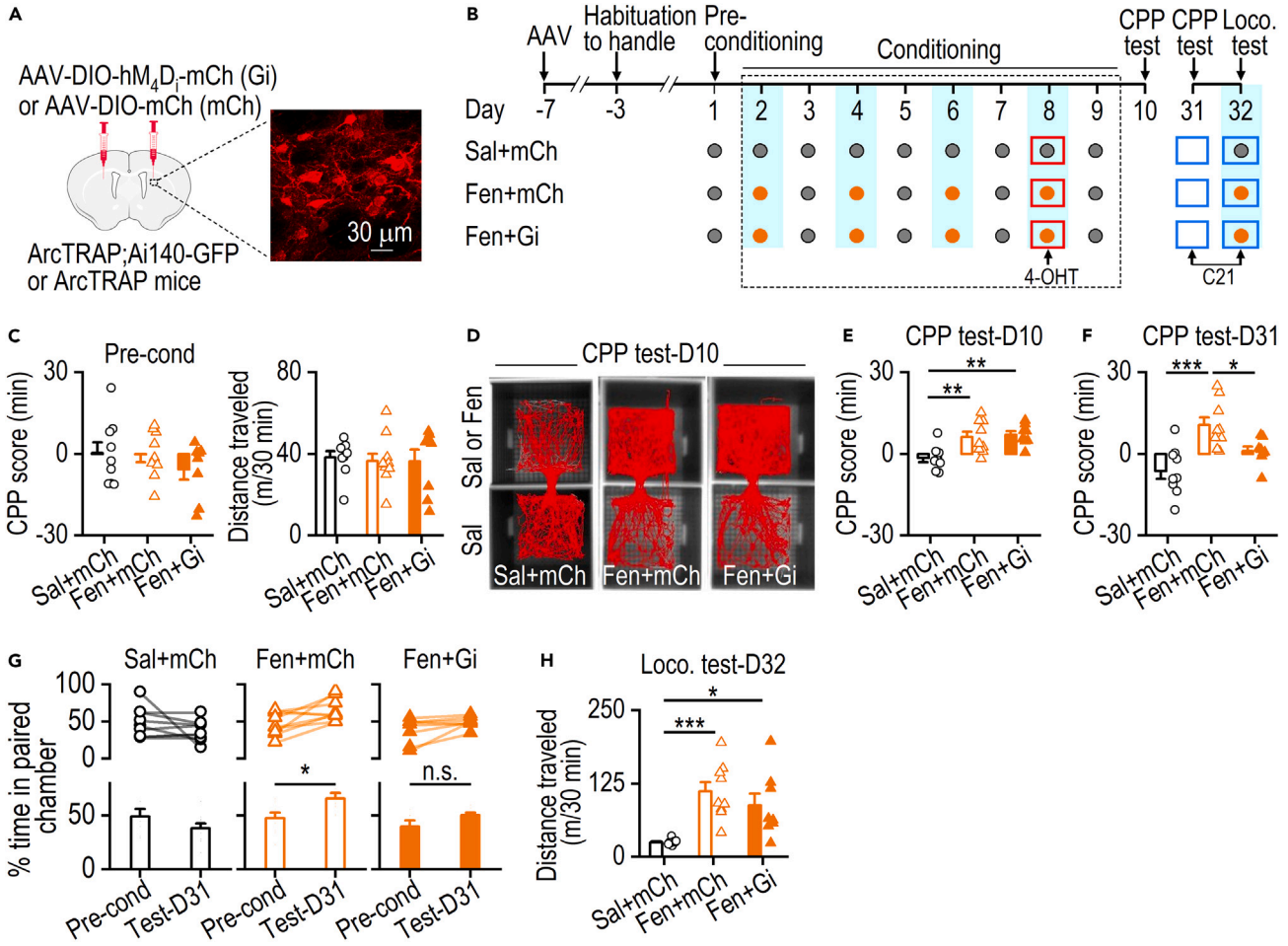

Figure 6. Representative data from fentanyl-induced conditioned place preference and chemogenetic inhibition of fentanyl-activated neurons

(A) Virus infusion in the DMS. ArcTRAP and ArcTRAP;Ai140-GFP mice are infused with AAV-DIO-hM$_4$D$_i$-mCherry (Gi) or control virus AAV-DIO-mCherry (mCh) into the DMS (left). Sample confocal image (right) showing the Gi expression.

(B) Protocol timeline. During the pre-conditioning (day 1), all mice receive saline injections and are placed in the CPP chamber (with the center gate open) for 30 min to record their baseline preference and locomotor activity. From days 2–9, mice in the fentanyl groups (Fen+mCh and Fen+Gi) receive fentanyl injections on days 2, 4, 6, 8, and are restricted in their designated drug-paired compartment for 30 min. These mice receive saline injections on days 3, 5, 7, 9 and are restricted to the non-drug-paired compartment for 30 min. Mice in the saline group (Sal+mCh) receive saline injections for all the conditioning days and are restricted in either compartment in alternate order. After the last drug conditioning (day 8), mice receive 4-OHT injections to tag neurons activated during the conditioning. On day 10, all mice are placed in the CPP chambers (with the center gate open) for 30 min to test their drug-induced baseline preference. After three weeks to allow virus expression (day 31), mice receive C21 injections and re-enter the CPP chambers (with the center gate open) for 30 min to test the effect of chemogenetic inhibition of tagged striatal neurons on their fentanyl memory retrieval. On day 32, mice receive C21 injections and then fentanyl injections, and are restricted in their drug-paired compartment to test the effect of chemogenetic inhibition of tagged striatal neurons on their fentanyl-induced locomotor activation. Loco. test, locomotion test.

(C) CPP scores and distance traveled during pre-conditioning are similar among groups.

(D) Sample CPP tracks.

(E) Fentanyl induces conditioned place preference in both fentanyl groups. **$p < 0.01$ by one-way ANOVA.

(F) Chemogenetic inhibition of tagged DMS neurons significantly reduces CPP scores. *$p < 0.05$, ***$p < 0.001$ by one-way ANOVA.

(G) Chemogenetic inhibition of tagged neurons abolishes the fentanyl-induced increase in time spent in the paired chamber (day 31 versus pre-conditioning). n.s. not significant; *$p < 0.05$ by paired *t* test.

(H) Chemogenetic inhibition of tagged DMS neurons does not suppress fentanyl-induced hyperlocomotion. *$p < 0.05$, ***$p < 0.001$ by one-way ANOVA. N = 9 mice (Sal+mCh), 9 mice (Fen+mCh), and 8 mice (Fen+Gi). Data points represent individual animals, with bars representing the mean ± SEM.

⚠ CRITICAL: Make sure the room light is kept the same for all recording sessions. The room light may become a context feature that animals can use to establish the learning.

b. Use clean, new cages to bring the mice to the room.
c. Gently transfer the mice cages from the transporting cart to the rack.

⚠ CRITICAL: Leave the mice undisturbed for at least 30 min before starting the behavioral testing.

d. Load the protocol in the EthoVision system (Figures 2F and 2G). Set the trial duration to 30 min.
e. Switch to the acquisition window and prepare to start the experiment (Figure 2G).

⚠ CRITICAL: Ensure there is no overexposure of the background, which may confuse the system to track the bright point (overexposed point) in the background instead of the animal body.

f. Use a 1-mL insulin syringe to prepare 0.9% sterile saline.

Note: Each mouse will have its own syringe. Based on the prior calculation, withdraw the same volume of the saline as the required fentanyl.

g. Administer 0.9% sterile saline intraperitoneally to the mouse.
h. After injection, put the mouse back in the cage. Leave undisturbed for 15 min.

Note: The waiting time is necessary to allow the drug to take effect. Depending on the specific drug, the onset of its effects may vary. Therefore, it is important to adjust the waiting time accordingly for each drug to ensure that it has taken full effect before proceeding with the experiment.

i. Hold the entire cage as close to the CPP chamber as possible, gently transfer the mouse by grabbing the tail and put it on the center of the CPP chamber near the guillotine door.
j. Check if the EthoVision can capture the bodies of all the mice.
k. Click the recording button to start the trial (Figure 2G, dashed red circle).
l. Once the trial is finished, gently grab the mouse from the CPP chamber back in the cage.
m. Leave mice undisturbed for 30 min before returning to the home cage.

Note: If having multiple groups, remove all animal wastes and thoroughly wipe and clean the chamber with 70% ethanol solution. Do not start the next round until the ethanol is evaporated.

Fentanyl conditioning in CPP

Timing: 8 days

This step trains the mice to pair the rewarding effect of fentanyl with the environment where they receive the drug. It is composed of four fentanyl-injection sessions which occur every other day, with four saline-injection days in between (Figure 6B). Tagging of fentanyl-activated neurons will be conducted on the last fentanyl conditioning session. After the last saline conditioning, we will measure the first fentanyl-induced CPP, which will serve as the baseline for the chemogenetic manipulation. Animals will return to the home cage for three weeks to allow DREADD to be sufficiently

expressed in tagged neurons. After three weeks, animals will be tested for CPP again with the injection of the DREADD agonist C21.

8. Determine the assignment of the fentanyl-paired compartment.
   a. Use the analysis function in the EthoVision software, pull out the time spent in each compartment by every mouse during the pre-conditioning day.

   Note: See troubleshooting.

   b. Assign one compartment as the drug-paired compartment to all mice and calculate the CPP score.

   Note: CPP score is calculated as: time in the paired chamber – time in the unpaired chamber.

   c. Compare the mean value for each group.
   d. Change the assignment for each mouse in each group accordingly.

   ⚠ CRITICAL: The goal is that each group has mean CPP scores around "0", which represent equal place preference for two compartments. Also ensure CPP scores are not different between each group (Figure 6C).

   Note: In our protocol, fentanyl is administered on conditioning days 1, 3, 5, 7 and saline is administered on conditioning days 2, 4, 6, 8 for drug groups. This is to ensure fentanyl is completely metabolized during the saline conditioning sessions. However, the order for saline or drug sessions can be switched, such that saline is administered on day 1 and fentanyl on day 2. Saline control group will receive saline injections for all the conditioning days.

9. Fentanyl CPP condition (Figure 6B).
   a. Prepare the testing room and habituate the mice in the room as described in step 7.
   b. Close the center guillotine door.
   c. Load the protocol in EthoVision software, and set the trial duration to 30 min.
   d. Pre-fill 1-mL insulin syringes with fentanyl or saline.

   Note: Each mouse will have its own syringe and the volume of fentanyl or saline is calculated based on the body weight.

   e. Administer the fentanyl or saline intraperitoneally.
   f. Gently place the mouse back in the cage and leave undisturbed for 15 min.
   g. Gently transfer the mouse to its designated drug-paired compartment.

   ⚠ CRITICAL: Make sure not introducing any external disturbance (light, sound, etc.) during the conditioning. Since our CPP chamber is not single-housed in sound-attenuating chambers, animals will be easily distracted. This will introduce unwanted cues to the mice.

   Note: For drug groups, they will be conditioned in the non-paired compartment on saline days. For saline groups, they will be conditioned in both compartments in alternate order.

   h. Check if the Ethovision can capture all the mice's movements.
   i. Click the recording button to start the trial.
   j. Once the trial is finished, gently grab the mouse from the CPP chamber back in the cage.
   k. Leave mice undisturbed for 30 min before returning to the home cage.

Tagging of fentanyl-activated neurons in CPP

Timing: 1.5 h

On conditioning day 7 (the last fentanyl conditioning day), active neurons during this contextual learning will be tagged by 4-OHT injection. This will allow pre-infused DREADDs to be expressed only in those tagged neurons. We will later chemogenetically manipulate these neurons to examine their roles in fentanyl-induced contextual learning.

10. Prepare 4-OHT as in step 3 and tag active neurons as in step 4.

⚠ CRITICAL: After injecting 4-OHT, leave mice undisturbed for the next 3 h before returning to the home cage (Figure 6B).

First CPP test

Timing: 1 day

This step is to measure fentanyl-induced place preference. It will help us confirm that mice have acquired fentanyl-induced contextual learning and that the results will serve as a baseline for the later chemogenetic manipulation.

Note: Mice in the fentanyl group that do not exhibit CPP will be excluded from subsequent experiments. In our batch, 1 out of 18 mice in the fentanyl group did not show CPP. The percentage of animals that exhibit CPP can be influenced by several factors, such as drug type, does, etc.[57] It is a good practice to conduct a pilot experiment to identify the percentage of animals that exhibit CPP, which can help in determining the appropriate number of animals for DREADD experiments.

Note: See troubleshooting.

11. Test CPP on day 10 (Figure 6B).
    a. Prepare the testing room and habituate the mice in the room as described in step 7.
    b. Open the center guillotine door.
    c. Load the protocol in EthoVision software, set the trial duration to 30 min.

Note: No drug or saline will be administered during the test session.

    d. Gently place the mice near the center guillotine door in the same CPP chamber where they undergo conditioning.
    e. Check if the EthoVision can capture all the mice's movements.
    f. Click the recording button to start the trial.
    g. Once the trial is finished, gently grab the mouse from the CPP chamber back in the cage.
    h. Leave mice undisturbed for 30 min before returning to the home cage.

Note: Mice will stay in the home cage for three weeks before the chemogenetic manipulation. This will provide sufficient time for the target gene (e.g., hM4Di) to be expressed.

Chemogenetic manipulation of fentanyl-activated neurons in the second CPP test

Timing: 2 days

Timing: 15 min (for step 12)

Three weeks after tagging, fentanyl-activated dorsostriatal neurons during contextual learning will be chemogenetically inhibited. This loss-of-function manipulation is critical to understand whether these tagged neurons are required for fentanyl-context memory. This part is separated into two days. On the first day, we chemogenetically inhibit tagged neurons during CPP memory retrieval. On the second day, we will chemogenetically inhibit tagged neurons during fentanyl-induced locomotor activation.

12. Prepare C21.

Note: See troubleshooting.

⚠ CRITICAL: Make fresh C21 solution every day for optimal results of chemogenetic manipulations. We suggest making the solution in the morning of the testing day.

a. Make 0.2 mg/mL working solution with 0.9% sterile saline.

Note: We choose this concentration because the dose we use is 1 mg/kg.[58] For 10 g of the mouse body weight, we inject $\frac{1\ mg/kg}{0.2\ mg/mL}$ = 5 mL/kg = 0.05 mL/10 g.

b. Store the working solution in a 4°C fridge until use.
c. Store the original C21 powder in a −20°C freezer.

13. Chemogenetically inhibit tagged neurons during the second CPP test (Figure 6B).
a. Prepare the testing room and habituate the mice in the room as described in step 7.
b. Open the center guillotine door.
c. Load the protocol in EthoVision software, set the trial duration to 30 min.
d. Pre-fill 1-mL insulin syringes with C21.

Note: Each mouse will have its own syringe and the volume of C21 is calculated based on the body weight.

e. After 30-min habituation, administer C21 intraperitoneally.
f. Leave mice undisturbed in the cage for 45 min.

Note: In this protocol, we utilized three groups of mice (Saline+mCherry, Fentanyl+mCherry, Fentanyl+hM4Di) to investigate the functional role of fentanyl-activated neurons in contextual memory retrieval. All groups received C21 injections; however, to control for any factors that may have been induced by the i.p. injection procedure, it is recommended to include control groups (e.g., Fentanyl+mCherry with i.p. saline injection instead of C21) for both CPP test and locomotor activity test.

g. After 45 min, gently transfer the mice from the cage to the same CPP chamber where they undergo conditioning.
h. Place the mice near the center guillotine door.
i. Check if the EthoVision can capture all the movements.
j. Click the recording button to start the trial.
k. Once the trial is finished, gently grab the mouse from the CPP chamber back in the cage.
l. Leave mice undisturbed for 30 min before returning to the home cage.

14. Chemogenetically inhibit tagged neurons during fentanyl-induced locomotor activation (Figure 6B).
a. Prepare the testing room and habituate the mice in the room as described in step 7.
b. Close the center guillotine door.
c. Load the protocol in EthoVision software, set the trial duration to 30 min.
d. For each mouse, prepare two 1-mL insulin syringes.

i. Pre-fill the first 1-mL insulin syringe with C21, and the second syringe with fentanyl or saline.

e. After 30-min habituation, administer C21 intraperitoneally.
f. Leave mice undisturbed in the cage for 30 min.
g. Administer fentanyl or saline intraperitoneally.
h. Leave mice undisturbed in the cage for 15 min.
i. Gently transfer the mice from the cage to their fentanyl-paired compartments.

Note: For the saline group, mice will be placed in either one of the compartments.

j. Check if the EthoVision can capture all the mice's movements.
k. Click the recording button to start the trial.
l. Once the trial is finished, gently grab the mouse from the CPP chamber back in the cage.
m. Leave mice undisturbed for 30 min before returning to the home cage.

EXPECTED OUTCOMES

Histological measurement of fentanyl-activated neurons

Fentanyl, like other opioid derivations, activates the mesolimbic dopamine pathway and enhances striatal dopamine levels.[59–61] As dopamine D1 receptors (D1R) are excitatory whereas dopamine D2 receptors (D2R) are inhibitory, this increased dopamine level likely promote the activation of neurons expressing D1R while suppressing neurons expressing D2R.[34] In the striatum, this biased activation of dMSNs, which mainly express D1R, can induce locomotor activation.[62,63] In accordance with this idea, our representative results showed that fentanyl greatly enhanced locomotor activity compared to saline (Figures 3C and 3D). This locomotor activation is accompanied by increased recruitment of dMSNs, but not putative iMSNs, into fentanyl-activated ensembles in the entire striatum (Figure 5). D1R and D2R are expressed across the whole brain.[64,65] Although we found that the densities of both tagged dMSNs and putative iMSNs were increased after fentanyl in open field (Figures 5K, 5P, and 5U), it is expected that fentanyl exposure may recruit more D1R-expressing cells than D2R-expressing cells into the ensembles that mediate a specific fentanyl-related behavior. However, the composition of ensemble recruitment is also influenced by input strength, connectivity, cell excitability, etc.[2] In addition to dopamine receptors, fentanyl and other opioid derivatives primarily target mu-opioid receptors (MOR).[66–68] For neurons expressing MOR, their activation may be largely suppressed if tagging is conducted during the initial fentanyl exposure, as acute MOR activation are inhibitory in nature.[69,70] However, after repeated opioid administration, MOR-expressing neurons might become hyperexcitable and thus exhibit increased activation.[1,71,72] Our tagging approach can help visualize the component of different cell types activated by fentanyl in specific brain regions if crossing the ArcTRAP mice with reporter lines such as D1-tdT, MOR-mCherry,[73] ChAT-GFP,[74] etc. Tagging during different phases (acute, chronic, withdrawal, etc.) or in different behavioral paradigms (open field, operant, etc.) can further enhance our understanding of the neuronal composition recruited by fentanyl-related behaviors.

Functional manipulation of fentanyl-activated neurons

To evaluate the existence of engram cells, functional manipulation of fentanyl-activated neuronal ensembles provides important evidence about their roles in drug-related memories.[3] Typically, loss-of-function manipulations (e.g., chemogenetic inhibition) can help determine whether tagged populations encode a specific memory by reducing subsequent memory retrieval.[3] The striatum is critically involved in the context-drug association and drug-motivated behaviors.[25,27] We found fentanyl-exposed mice exhibited stronger place preference for the drug-paired compartment as compared to the saline mice even three weeks after conditioning (Figures 6D and 6E). This fentanyl-context memory was suppressed by chemogenetic inhibition of fentanyl-tagged dorsostriatal neurons (Figures 6F and 6G), indicating that the activation of these tagged striatal neurons is required for remote fentanyl-memory retrieval. Similar results can be expected if ensemble-specific inhibition is conducted in other regions that are involved in context learning, such as the prelimbic cortex, CA3 region of the hippocampus, or basolateral amygdala.[75,76]

However, it is worth noting that inhibiting learning-tagged neurons may not always lead to impaired memory retrieval. A single engram may contain multiple functionally distinct ensembles, each of which mediates a specific feature of the memory, such as memory generalization or discrimination.[77] Inhibition of only one of these ensembles may only impair a single feature of the memory, rather than the entire memory itself.[77] In our study, fentanyl was found to recruit both dMSNs and iMSNs in the striatum (Figure 5). While chemogenetic inhibition of the general dorsostriatal ensembles led to a reduction in fentanyl memory retrieval, the dMSN and iMSN ensembles may have distinct roles in encoding the memory. In addition, chemogenetic inhibition of tagged dorsostriatal neurons was not sufficient to suppress fentanyl-induced locomotor activation (Figure 6H). This suggests that ensembles required for a specific memory may not be required for more general locomotor features. Therefore, given the unique roles of different brain regions in specific memories, if functional manipulation of learning-tagged ensembles in a region-of-interest fails to affect memory or behavioral expression, it may be worthwhile to investigate the specific components of the learning paradigm to determine if individual features of the memory are affected.

QUANTIFICATION AND STATISTICAL ANALYSIS

For the open field test, the animal performance is recorded by the Activity Monitor software, in which the total distance traveled can be directly read out. A two-way repeated measures (RM) analysis of variance (ANOVA) with appropriate *post hoc* comparisons can be used to examine the effect of fentanyl versus saline across multiple injection days. We generate a MATLAB code to plot the heat map and has deposited the code in GitHub. Export the data file from the Activity Monitor as "Export (Legacy)" in Notepad format. This file can be directly read by our MATLAB code.

For histological measurements of fentanyl-activated neurons, we use confocal microscopy to scan the striatal sections. Sections are 50 μm thick and mounted with VECTASHIELD mounting media (with DAPI). Images are acquired by Olympus Fluoview 1200 confocal microscopy. For each slice, we scan a total of 5 layers from the center with each layer 3-μm thick. After acquiring the images, we use IMARIS 8.3.1 to count the cells. For each striatal subregions, we first define a region of interest (ROI) with 500-μm radius (Figures 5A and 5E, dash-circled region for DMS analysis). We then use the automatic counting function within the IMARIS to generate a raw number of cells for each fluorescence channel (green channel for Ai140-GFP, red channel for D1-tdTomato). A blinded experimenter manually counts the neurons with overlapping green and red fluorescence, representing tagged dMSNs. The number of layers and layer step size are used to calculate the volume of ROI. The density of each type of cells is calculated by dividing the raw number of cells from the ROI volume. The results are manually checked by another blinded experimenter.

> ***Note:*** Ensure sections from different animals are scanned with the same parameter (e.g., number of layers, layer step size, laser power, high voltage (HV), gain, offset, airy disk size, averaging, etc.). For comparison of each cell type between saline and fentanyl groups, a student's *t*-test can be used.

For the CPP test, the movement-related information (distance, velocity, etc.) and the time spent in each compartment are directly read out from the EthoVision XT. Export the data as Microsoft Excel and analyze accordingly. Compare the results using student's *t*-test for two groups and one-way ANOVA with appropriate *post hoc* comparisons for three or more groups.

LIMITATIONS

The current protocol is established based on the ArcTRAP approach. ArcTRAP is an excellent tool to tag neurons with low baseline firing rates, such as striatal MSNs. However, we observe that in some areas ArcTRAP has high background expression without 4-OHT, including multiple cortical regions. It thus requires specific caution when using the ArcTRAP approach to tag cortical ensembles. Such background expression may blur the drug-induced neuronal activation. Alternatively, the FosTRAP approach may be suitable for use on cortical regions. However, FosTRAP has low expression in the

striatum.[5] We suggest researchers thoroughly test the expression level between ArcTRAP and FosTRAP to choose the best option for specific brain regions.

Daily drug/saline injections are stressful experience. We have tried to minimize the effect of stress and pain during the 4-OHT injection, but it is difficult to reduce these effects during the daily injections. If using this approach to study drug-related emotional processing in regions like limbic structures, specific cautions should be taken to explain the results.

TROUBLESHOOTING

Problem 1

Before you begin, step 2: It may be challenging to generate mice with specific genotypes.

Potential solution

To increase the likelihood and number of offspring with desired genotypes, we recommend using one male with two females instead of the traditional mating pairs of one male and one female. Create multiple mating pairs simultaneously for maximum efficiency. For each genotype, we suggest using both sexes to ensure optimal breeding outcomes, particularly if a breeder of a specific sex has a low reproductive rate. For example, instead of creating two mating pairs with one Ai140-GFP male and two ArcTRAP;D1-tdT females, we recommend creating one pair with one Ai140-GFP male and two ArcTRAP;D1-tdT females, and another pair with one ArcTRAP;D1-tdT male and two Ai140-GFP females. Breeder age, especially for females, should be less than eight months, although some male breeders may still be reproductively active at 12 months of age.

Problem 2

Step 2, Fentanyl-induced hyperlocomotion: It is possible that fentanyl may not cause hyperactivity in some strains of mice. It has been shown that 129Sv and DBA2 mice showed less locomotor activity change than C57BL/6 mice after morphine injections.[78] Indeed, we observed that Long-Evans rats did not exhibit locomotor activation after fentanyl injections (unpublished results).

Potential solution

We recommend carefully evaluating the strains for the behavior you are studying. Select and cross-breed the strains that exhibit drug-related behaviors of your interest. Since the activity of striatal MSNs mediates locomotor activity,[63] we chose open field tests to ensure that the fentanyl did affect the striatum by inducing hyperlocomotion. It can thus help us remove the animals which did not show hyperlocomotion after the fentanyl from the histological analysis, which may be due to wrong injections or individual variance. Alternatively, evaluate the drug dose to choose the optimal one that can induce the behavior of your interest.

Problem 3

Step 7, Fentanyl CPP conditioning: The circadian rhythm may affect the rewarding effect of the drug and thus the learning related to the drug.[79]

Potential solution

For specific mouse/group, we suggest conducting the behavior around the same time during the day across the entire experiment period to minimize the effect of circadian changes on the drug effect. In addition to that, we recommend reversing the light/dark cycle of the mice in drug conditioning and administering the drug during the middle of the active phase (dark cycle).

Problem 4

Step 8, Pull out CPP data from EthoVision: EthoVision may lose object tracking occasionally. This situation is worse when the room light is dim.

Potential solution

Before starting the CPP experiment, ensure to use the same type of animal (same fur color) and room light as during the experiment to calibrate the EthoVision tracking system. This will reduce the possibility of lost tracking. However, in our experience, it was unavoidable to have lost tracking. Therefore, before analyzing the result, make sure to check the raw video file from each animal and manually adjust the tracking point if necessary.

Problem 5

Step 11, First fentanyl CPP test: The chemogenetic manipulation during CPP requires animals been exposed in the CPP chambers twice without the drug. The first CPP test is to measure the "baseline" drug-induced preference and the second CPP test is to examine the effect of chemogenetic manipulation. It is possible that the first CPP may cause extinction learning in the specific context, which will reduce the performance during the second CPP test.

Potential solution

Since fentanyl often induces an incubation effect, in which drug-induced responses will gradually increase after prolonged abstinence, we did not observe any extinction effect for fentanyl during the second CPP test. Even though, specific caution is needed when explaining the result. A drug group without DREADD may be served as a control to compare with the DREADD manipulation. Alternatively, it is also plausible to skip the first CPP test and directly conduct chemogenetic manipulations three weeks after the tagging. However, animals in the drug group may not acquire CPP at all. The first CPP test allows us to remove mice without showing CPP from the manipulation results. Researchers need to take caution when only conducting one CPP test, as two CPP tests (before and after DREADD inhibition) can also provide within-subject comparisons.

Problem 6

Step 12, Chemogenetic manipulation of fentanyl-activated neurons: It has been a long debate that the agonist for DREADDs may cause side effects. Even the newly generated C21 can induce some off-target outcomes.[80]

Potential solution

Appropriate control groups (e.g., animals without DREADD receive the drug and C21) should be included to mitigate this issue. If off-target effects are observed in the control animals, optogenetic manipulations may be used, which might also be a less stressful intervention than C21 i.p. injections.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jun Wang (jwang188@tamu.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The MATLAB code for the open field heatmap generated during this study are available at GitHub: [MATLAB code: https://github.com/Xxie119/MATLAB.git], MATLAB code: https://doi.org/10.5281/zenodo.7916669.

The published article includes all data generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

The protocol was conceptualized, designed, and originally published by X.X. and J.W. The manuscript was written by X.X. and J.W. and edited by all authors. X.W. bred transgenic animals and conducted genotyping. MATLAB code was generated by R.C. Representative data were generated by X.X.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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