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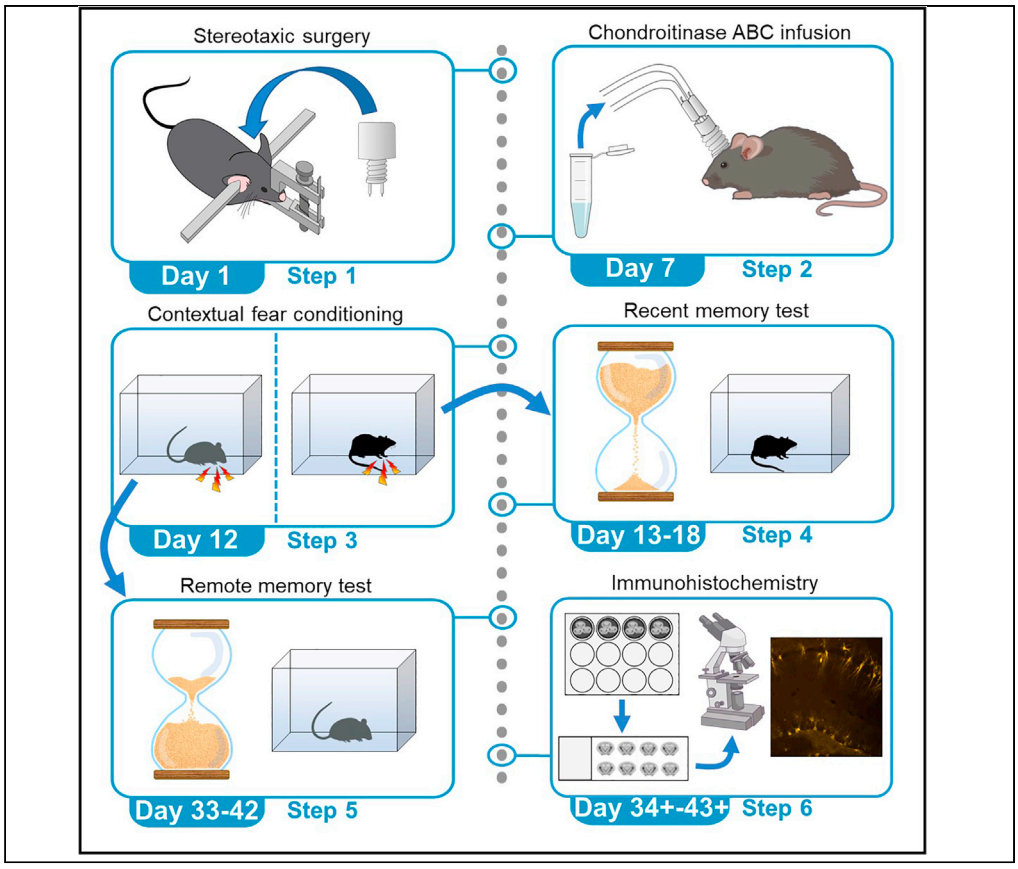
Protocol for assessing the role of hippocampal perineuronal nets in aversive memories



Perineuronal nets (PNNs) are emerging as critical regulators of memory-related neuronal processes. However, their exact contribution depends on type of memory, consolidation stage, or brain region, and remains to be fully investigated. We describe here a protocol to evaluate the importance of PNNs in the dorsal hippocampus in different stages of aversive memories using a mouse model. The protocol provides detailed instructions for surgical implantation of hippocampal cannulas, drug infusion, contextual fear conditioning procedures, and immunohistochemistry for PNN visualization.

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Highlights

Surgical procedure for hippocampal cannula implantation

Chondroitinase ABC infusions into the dorsal hippocampus

Contextual fear conditioning followed by recent and remote memory tests

Immunohistochemistry for perineuronal net visualization

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Protocol for assessing the role of hippocampal perineuronal nets in aversive memories

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SUMMARY

Perineuronal nets (PNNs) are emerging as critical regulators of memory-related neuronal processes. However, their exact contribution depends on type of memory, consolidation stage, or brain region, and remains to be fully investigated. We describe here a protocol to evaluate the importance of PNNs in the dorsal hippocampus in different stages of aversive memories using a mouse model. The protocol provides detailed instructions for surgical implantation of hippocampal cannulas, drug infusion, contextual fear conditioning procedures, and immunohistochemistry for PNN visualization.

For complete details on the use and execution of this protocol, please refer to Jovasevic et al. (2021).

BEFORE YOU BEGIN

PNNs are extracellular matrix (ECM) structures that surround the soma, the proximal and middle dendrites of neurons, often extending to include the axon initial segment (Fawcett et al., 2019). Emerging evidence shows that they are important for proper memory processing. Their involvement has been shown in several subcortical, (hippocampus, amygdala) and cortical (prefrontal, visual, auditory cortices) regions, and in a broad range of memory tasks, testing aversive and appetitive associative memory, or non-associative, recognition memory (Banerjee et al., 2017; Gogolla et al., 2009; Hylin et al., 2013; Romberg et al., 2013; Santiago et al., 2018; Slaker et al., 2015; Thompson et al., 2018; Xue et al., 2014). However, the exact stages of memory processing at which they are involved, and the mechanism through which they exert their activity, remain to be determined. Existing data on the role of PNNs in memory vary greatly, demonstrating both improvement (Morellini et al., 2010; Romberg et al., 2013) and impairment (Hylin et al., 2013; Thompson et al., 2018) of memory with PNN degradation, depending on the brain region in which PNNs are disrupted, timing of disruption, and behavioral task in which memory is tested. Additionally, various methods are used for PNN depletion, such as treatment with chondroitinase ABC (ChABC) (Thompson et al., 2018; Xue et al., 2014), hyaluronidase (Sun et al., 2018), a combination of both (Hylin et al., 2013; Santiago et al., 2018), or genetic manipulations (Romberg et al., 2013). In order to define the role of PNNs in processes related to memory, it is essential to consistently apply standardized protocols in each experimental setting and to minimize variability across different experimental designs. We describe here a protocol for targeted disruption of PNNs in the CA1 region of the dorsal hippocampus (DH) for the purpose of studying their role in the retrieval of aversive episodic-like memories at recent or remote stage, which we have used in our recent publication (Jovasevic et al., 2021). We have used this protocol for targeted delivery of other drugs into DH, and into other brain regions

as well (Corcoran et al., 2011; Guzman et al., 2013; Jovasevic et al., 2015). The disruption of PNNs is achieved through the enzymatic degradation of chondroitin sulfate proteoglycans (CSPGs), a major component of PNNs (Reichelt et al., 2019). Enzymatic degradation of PNNs provides higher temporal specificity than constitutive knockout of a PNN component, and does not run the risk of genetic compensation which can be observed with constitutive knockouts (El-Brolosy and Stainier, 2017). This protocol involves infusion of ChABC into CA1 region of DH guided by a bilateral 26-gauge cannula, followed by behavioral training and testing. Enzymatic treatment is an often used approach for degradation of PNNs, typically with one of the two delivery methods: direct infusion during surgery (Thompson et al., 2018), or a cannula-guided method (Gogolla et al., 2009; Hylin et al., 2013). Infusion during surgery has several disadvantages that in our view makes this approach less suitable for applications with memory tasks. The procedure requires deep anesthesia, and longer recovery, therefore it is not suitable when drug infusions are required soon before memory training or test. The procedure also does not allow multiple infusions of a drug, or a combination of drugs. Although several published reports use cannula-guided ChABC delivery, none provide an in-depth description of the procedure, which is important considering that this procedure is used on mice or rats, and some steps are implemented differently between the two species. Here, we provide a detailed, easy to follow protocol for cannula implantation surgery, drug infusion, and contextual fear conditioning in mice. All procedures involving mice performed in our studies were approved by Northwestern University's Animal Care and Use Committee in compliance with US National Institutes of Health standards. The protocol requires several reagents and instruments to be prepared in advance.

Avertin (2, 2, 2-tribromoethanol) solution

Timing: 12+ h

1. Prepare a stock solution (1.6 g/mL) of avertin.
   a. Weigh 25 g of 2, 2, 2-tribromoethanol, add 15.625 mL tert-amyl alcohol.
   b. Mix in a dark bottle at room temperature (20°C–22°C) to dissolve (~12 h). Use either a magnetic stirrer or a shaker. Stock solution can be stored at room temperature for up to one year.
2. Prepare a working solution solution (14 mg/mL) of avertin. The volume depends on the number of mice that will undergo surgery. We would suggest 1 mL/mouse. Although quite less will be actually used, the extra volume allows for easier dispensing, particularly towards the end of the procedure.
   a. Add stock solution to an appropriate volume of 0.9% saline solution.
   b. Mix thoroughly and vigorously, by vortexing or by using magnetic stirrer, in a dark or an aluminum foil-covered bottle.
   c. Filter the solution though a 0.2-micron filter into a dark or foil covered container and store at 4°C. The working solution can be stored for a month; however, we would advise to prepare fresh working solution one day prior to use.

⚠ CRITICAL: Occasionally 2, 2, 2-tribromoethanol will precipitate when saline is added to the stock solution, and can be very difficult to dissolve again. This can be prevented by incrementally adding small volumes (1 mL) of saline solution and vortexing after each addition. It is very important that all of 2, 2, 2-tribromoethanol is in the solution, otherwise proper level of anesthesia will not be achieved.

Note: We strongly suggest using avertin as anesthetic of choice, which we have reliably and reproducibly used for many years. Unlike isoflurane, avertin anesthesia lasts long enough for cement and cannulas to be well affixed to the skull, and it preserves the resting functional connectivity in the mouse brain better than isoflurane (Xie et al., 2020). If an alternative anesthetic is preferred, ketamine/xylazine also preserves the resting functional connectivity (Xie et al., 2020); however, in our experience anesthesia lasts a very long time, recovery is slower, and

mortality is higher, possibly due to the more severe effects on cardiac function (Hart et al., 2001).

Sterilization of cannulas and surgical instruments

Timing: 2 h

3. Put surgical instruments into an autoclave box. Put cannulas into a glass bottle with an airtight lid.
4. Autoclave at 121°C for 30 min, dry for 30 min.

⚠ CRITICAL: Close the bottle with cannulas only once it has somewhat cooled. If it is closed while still hot, it will be very difficult to open it later.

Preparation of critical equipment

Timing: ~2 h

5. **Stereotaxic instrument and surgical equipment:** Stereotaxic instrument and all other equipment necessary for surgical implantation of cannulas should be set up before the procedure (Figure 1). This includes:
   a. stereotaxic instrument
   b. digital display of coordinates
   c. flexible arm light
   d. centering scope
   e. stereotaxic alignment indicator
   f. drill
   g. micro bead sterilizer
   h. surgical instruments (autoclaved)

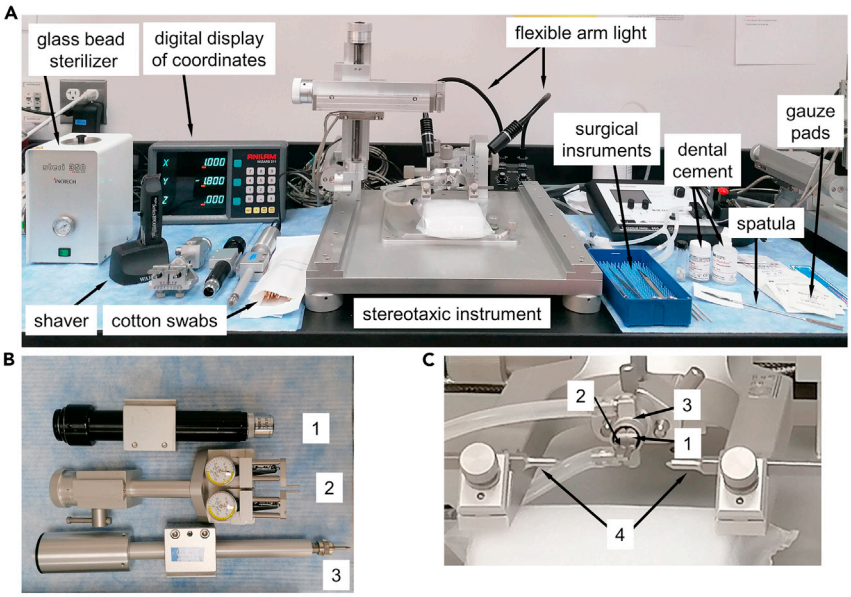

Figure 1. Setup for stereotaxic surgery

(A) Stereotaxic instrument with all surgical and accessory instruments indicated.

(B) Components of the stereotaxic instrument: centering scope [1], stereotaxic alignment Indicator [2], and drill [3].

(C) Section of the stereotaxic instrument showing mouthpiece [1] with the teeth hole [2], nose clamp [3] and ear bars.

i. spatula (autoclaved)
j. cannulas (autoclaved)
k. heated pad
l. cotton swabs
m. small animal shaver (or depilatory cream)
n. ophthalmic ointment
o. 10% povidone/iodine solution
p. carboxylate cement (powder and liquid component)
q. 4% lidocaine gel
r. scale
s. 1 mL syringes
t. 25 gauge needles
u. 70% ethanol
v. 0.02% sodium hypochlorite
w. sterile gauze
x. surgical pads (sterile or autoclaved)
y. sterile gloves.

Note: Stereotaxic frame should be connected to the digital display of coordinates. We use a display with a precision of 0.001 mm for maximum performance. X-, Y-, Z-nobs, antero-posterior tilt, and medio-lateral tilt nobs should be cleaned and lubricated.

Note: The entire surgical area and the stereotaxic instrument should be cleaned with 70% ethanol before starting the procedure.

6. **Infusion equipment:** Intrahippocampal infusions require the use of isoflurane and should follow all necessary precautions. They should be performed in a well-ventilated room, in a fume hood. All required equipment and instruments should be set up just before starting. The detailed instructions will be provided later in the text, as part of the infusion protocol. The required equipment includes:
   a. infusion pump
   b. Hamilton syringes (25 µL)
   c. tubing (two pieces, approximately 5 cm length)
   d. internal cannulas (injectors)
   e. two forceps
   f. isoflurane anesthetizer with an induction chamber.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Coronal brain sections; C57BL/6N mice, 5 days after ACSF infusion into DH + 21 days after contextual fear conditioning	This paper	N/A
Coronal brain sections; C57BL/6N mice, 5 days after chondroitinase ABC infusion into DH + 21 days after contextual fear conditioning	This paper	N/A
Chemicals, peptides, and recombinant proteins		
2, 2, 2-tribromoethanol	Millipore-Sigma	Cat#T48402
tert-amyl alcohol	ThermoFisher	Cat# A730-1
Ethanol, 200 proof (1 x 4 gallon)	Decon Labs, Inc.	Ca#: 2701
Sodium Hypochlorite Solution, 1 L	Fisher Scientific	Cat#: SS290-1
Isoflurane solution, 250 mL	Covetrus	Cat#: 029405

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chondroitinase ABC	Amsbio	Cat#: E1028-10
Artificial cerebrospinal fluid (ACSF)	Harvard Apparatus	Cat #: 59-7316
Biotinylated Wisteria Floribunda Lectin	Vector Biolaboratories	Cat#: B-1355-2
Tissue-Plus™ O.C.T. Compound Tissue-Plus™ O.C.T. Compound	ThermoFisher	Cat#: 23-730-571
Carbo-Free Blocking Solution	Vector Biolaboratories	Cat#: SP-5040-125
Corning Mediatech Cell Culture Phosphate Buffered Saline (10×), 1 L	Fisher Scientific	Cat#: MT-46013CM
Thimerosal	Millipore-Sigma	Ca#: T5125-10G
Hydrogen Peroxide, 30%, 500 mL	Fisher Scientific	Cat#: H325-500
Triton™ X-100, 100 mL	Millipore-Sigma	Cat#: T8787-100ML
Tween-20, 500 mL	Millipore-Sigma	Cat#: P1379-500ML
FluorSave	Millipore-Sigma	Cat#: 345789-20ML
Critical commercial assays		
VECTASTAIN Elite ABC HRP Kit (Peroxidase, Standard) PK6100	ThermoFisher	Cat#: NC9313719
Streptavidin/Biotin Blocking Kit	Vector Biolaboratories	Cat#: SP-2002
Experimental models: Organisms/strains		
C57BL/6N mouse, male, 9 weeks old	Envigo	Order code: 044
Software and algorithms		
Prism 9	GraphPad	https://www.graphpad.com
Other		
Model 1900 Stereotaxic Alignment Instrument	Kopf	http://kopfinstruments.com/product/model-1900/
Model 1940 Micro Manipulator with Digital Display Console	Kopf	https://kopfinstruments.com/product/model-1940-micro-manipulator-with-digital-display-console/
Model 1911 Drill	Kopf	https://kopfinstruments.com/product/model-1911-stereotaxic-drill/
Model 1905 Stereotaxic Alignment Indicator	Kopf	https://kopfinstruments.com/product/model-1905-stereotaxic-alignment-indicator/
Model 1915 Centering Scope 40×	Kopf	https://kopfinstruments.com/product/model-1915-centering-scope-40x/
SomnoSuite Low-Flow Anesthesia System	Kent Scientific	https://www.kentscientific.com/products/somnosuite/
Sliding Top Induction Chamber	Kent Scientific	Cat#: SOMNO-0530XXS
CMA 400 Microdialysis Infusion Pump	Harvard Apparatus	https://www.harvardapparatus.com/cma-4002-microdialysis-syringe-pump.html
Guide cannula	PlasticsOne	C235G-2.0-SPC GUIDE 39624 DBL 26GA 2MM C-C CUT 2MM BELLOW PEDESTAL
Internal cannula	PlasticsOne	C235I-SPC INTERNAL DBL 38981 33GA FIT 2MM C235G-2.0 W 0.5MM PROJ
Dummy cannula	PlasticsOne	C235DC-SPC DUMMY DBL .008in- .2MM FI 2MM C235G-2.0 W 0.5MM PROJ
Dust cap	PlasticsOne	303DC/1
Micromedical tubing, 100 feet intravenous, 0.027 I.D. x 0.045 O.D.	Scientific Commodities Inc.	Cat#: BB31785V/3A
Narrow Pattern Forceps	Fine Science Tools	Cat#: 11002-12
Student Fine Scissors	Fine Science Tools	Cat#: 91460-11
Fisherbrand Double-Ended Micro-Tapered Stainless Steel Spatula	Fisher Scientific	Cat#: 21-401-10
TSE Multi Conditioning System	TSE Systems	Model #: 256060
Leica DM2500 Optical microscope	Leica Micosystems	Product#: DM2500
ColorFrost Plus Microscope Slides	Fisher Scientific	Cat#: 12-550-16A
Mountain Falls 10% Povidone Iodine Solution (4×4oz)	Amazon	https://www.amazon.com/gp/aw/reviews/B07KY778YD
Durelon™ Carboxylate Luting Cement, powder	3M	Cat#: 38236

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Durelon™ Carboxylate Luting Cement, liquid	3M	Cat#: 38216
Steri-350 Glass Bead Sterilizer	ThermoFisher	Cat#: NC9449759
Hamilton 1702N Gastight Syringe, 25 µL, (22s/2in)	VWR	Cat#: 60376-656
Exel International Disposable Hypodermic Needles, 25 gauge (pack of 100)	Fisher Scientific	Cat#: 14-840-84
Topicaine 4% lidocaine gel	Amazon	https://www.amazon.com/TOPICAINE-Lidocaine-Anesthetic-Piercing-SHIPPING/dp/B0028MIGA4
Artificial Tears 15/83% Ophthalmic Ointment Sterile 3.5g/Tube	Henry Schein	1371627
Total Mro LLC 23 X 36 Disposable Underpads	Fisher Scientific	Cat#: NC9248636
K&H Pet Products Electric Small Animal Heated Pad Tan 9 X 12 Inches	Amazon	https://www.amazon.com/Pet-Products-Animal-Outdoor-Heated/dp/B000NVC7DO

MATERIALS AND EQUIPMENT

65 U/mL ChABC solution

Reagent	Final concentration	Amount
ChABC, 10 U (lyophilized)	65 U/mL	10 U
Artificial cerebrospinal fluid	n/a	153.8 µL
Total	**n/a**	**153.8 µL**

Store as 10 µL aliquots at −80°C in 0.2 mL tubes for up to six months. Avoid repeated thawing/freezing.

70% ethanol

Reagent	Final concentration	Amount
Ethanol (absolute)	70%	700 mL
ddH_2O	n/a	300 mL
Total	**n/a**	**1000 mL**

Store at room temperature for up to six months.

0.02% sodium-hypochlorite (bleach) solution

Reagent	Final concentration	Amount
Sodium hypochlorite solution (6%)	0.02%	3 mL
ddH_2O	n/a	897 mL
Total	**n/a**	**900 mL**

Store at room temperature for up to one week.

STEP-BY-STEP METHOD DETAILS

Stereotaxic surgery—cannulation

Timing: 1 day (15–20 min/mouse)

The purpose of this step is to implant and affix cannulas for intrahippocampal delivery. Stereotaxic coordinates for the dorsal hippocampus are 1.8 mm posterior, ±1.0 mm lateral and 2.0 mm ventral to bregma, according to the mouse brain atlas (Franklin and Paxinos, 2013). In comparison to drug delivery via microsyringe infusions, this approach has several advantages: it allows for multiple drug deliveries and requires a much shorter recovery time (20 min, vs several days for microsyringe infusions). All steps below are repeated for each mouse. For our experiments we use 9-week-old male

C57BL/6N mice (Envigo). Please follow your institutional guidelines on aseptic and analgesic managements during and after the surgery.

1. Anesthetize mouse.
   a. Weigh the animal.
   b. Calculate the volume of 14 mg/mL working solution of avertin to be delivered. Mice should be injected with 350 mg/kg. We would suggest making in advance a table ranging from 15 to 35 g, with injection volumes for each 1 g increment.
   c. Inject an appropriate volume of avertin intraperitoneally and return the animal to its home cage.
   d. Determine that the mouse is fully anesthetized before proceeding by using an indicator of deep pain recognition (e.g., the pedal reflex, the tail pinch reflex, the eyelid reflex) (Tsukamoto et al., 2015). It will take approximately 5 min.

   Note: Avertin is lipid soluble, so the exact dose may depend on body fat content. Dosage can vary from 250–500 mg/kg. If necessary, adjust the dose so the mouse is fully anesthetized.

2. Prepare the mouse for surgery.
   a. Apply ophthalmic ointment to prevent eyes from drying.
   b. Put the mouse in the stereotaxic instrument.
      i. Hook the front teeth to the hole of the mouthpiece.
      ii. Fix the nose in place with the nose clamp.
      iii. Fix the head using ear bars. They should be positioned against the skull, just behind the ears.
   c. Shave the head using small animal shaver (or depilatory cream).
   d. Disinfect the surgical area using 10% povidone/iodine solution and alcohol at least three times, ending with povidone/iodine.
3. Proceed with the surgery.
   a. Make an incision, approximately 1 cm, along the midline using surgical scissors or sterile scalpel.
   b. Remove the periosteum using cotton swabs.
   c. Remove any blood and dry the skull with fresh cotton swabs.
   d. Using anatomical features as guides (Figure 2A), align the head with the stereotaxic instrument.
      i. Adjust the head position so it is leveled. The head is properly leveled when: (1) bregma and lambda are at the same height (Figure 2B), and (2) two points, equidistant from the sagittal suture (1 mm, or as close to it as the instrument allows), are at the same height (Figure 2C). We use a stereotaxic alignment indicator to achieve the proper head position (Figure 2D). However, if one is not available, leveling of the head can be accomplished by using the tip of the drill bit, by adjusting the position of the head until the digital display of the coordinates shows the same Z-value when the tip is touching bregma or lambda (repeat for the two lateral points).
      ii. Using X- and Y-nobs, position the head so that bregma is in the center of the crossed lines of the centering scope (Figure 2E). If the head is properly positioned, vertical line of the centering scope should be aligned with the sagittal suture.
      iii. Adjust the digital display of the coordinates to show zero in X- and Y- coordinate. Z-coordinate is not of a concern since the depth of infusion is predetermined by the cannula.
   e. Remove centering scope and fit the stereotaxic instrument with the drill. The tip of the drill bit should be centered at bregma (Figure 3A).
   f. Move the drill along the X-axis to the correct coordinate. For DH cannulas this coordinate is 1.8 mm posterior to bregma (Franklin and Paxinos, 2013).
   g. Move the drill laterally along the Y-axis to 1 mm.
   h. Drill a hole. If bleeding occurs, press a cotton swab to the hole until it stops.

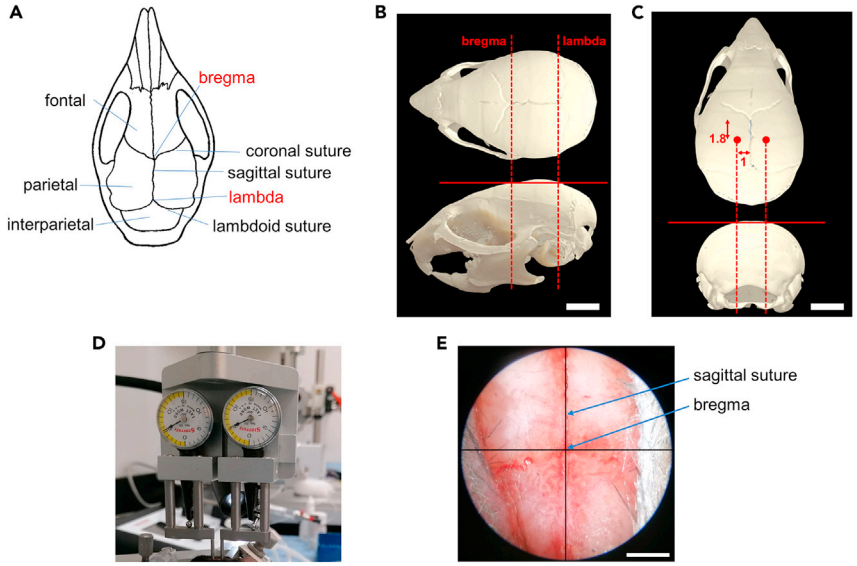

Figure 2. Head alignment during surgery

(A) Anatomical features of the mouse skull used as guides to position and level the head.

(B) Top view of the mouse skull (top), and a proper anterior-posterior alignment (bottom). Scale bar: 3 mm.

(C) Top view of the mouse skull (top), and a proper mediolateral alignment (bottom). Distance unit: mm. Scale bar: 3 mm.

(D) Leveler positioned at bregma and lambda shows equal deviation when head is properly aligned.

(E) Intercept of the vertical and horizontal lines of the stereoscope is aligned with bregma. Vertical line is aligned with the sagittal suture. Black lines are superimposed over the lines of the stereoscope for better visibility. Scale bar: 2 mm.

i. Move the drill laterally along the Y-axis to 1 mm in the opposite direction.
j. Drill a hole. The two holes will be 2 mm apart (Figure 3A). Troubleshooting 1

4. Implant a cannula.
   a. Mix the carboxylate cement (we use a sterile 46-well plate, a different well for each mouse): put one scoop of dry cement and one drop of liquid cement into a well. Mix thoroughly to combine. It should have a glue-like consistency (Figure 3B). Adjust if needed by adding dry or liquid cement.
   b. Insert an assembled cannula into the holes drilled in the skull (Figure 3C).
   c. Affix the cannula using the prepared cement. Work quickly so the cement does not dry.

   Note: It is very important to minimize the movement of the cannula once it is inserted in order to prevent tissue damage. Hold the cannula in place with forceps, while gently applying the cement around it.

   d. Pull the skin and adhere it to the cement around cannula to close the wound (Figure 3D).
   e. Apply 4% lidocaine gel (or another local anesthetic) to the skin to alleviate any pain that the animal may experience immediately after the surgery.
   f. Sterilize the instruments in the glass bead sterilizer

5. Return the mouse to its home cage, placed on a heating pad (37°C). Monitor regularly until it awakens (1–2 h).
6. House the mice individually before starting infusions and behavioral experiments. Ideally, they should be housed in the same room where the behavioral manipulations are going to be performed to minimize disturbances on the training and test days.

   ⏸ **Pause point:** Allow at least one week of post-surgical recovery before proceeding with infusions and behavioral tests.

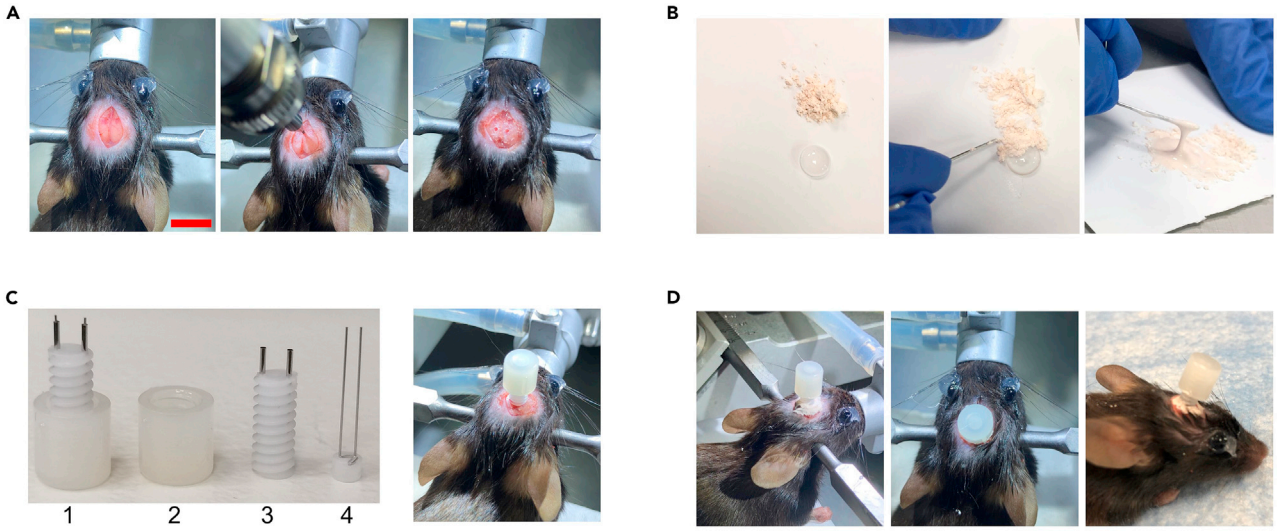

Figure 3. Steps of the stereotaxic surgery

(A) Left: mouse placed in the stereotaxic instrument and secured with ear bars and nose clamp. Skull is exposed, with bregma and lambda visible. Middle: drill tip is positioned at bregma to verify the head alignment. Right: holes in the skull, 2 mm apart, for cannula insertion. Scale bar: 1cm.

(B) Preparation of carboxylate cement to secure cannula, showing the amounts and the consistency of the final mixture. Cement is mixed on a piece of photo film, for a clearer view.

(C) Left: components of a hippocampal cannula - fully assembled cannula [1], dust cap [2], guide cannula [3], dummy cannula [4]. Right: cannula inserted into the skull.

(D) Steps during cannula implantation (from left to right): carboxylate cement applied to the skull, skin pulled around the cannula, mouse at the end of surgery.

⚠ CRITICAL: Make sure the head is completely immobilized during surgery. Any shift in head position during surgery would substantially affect the accuracy of the cannula placement.

⚠ CRITICAL: It is very important that the periosteum is completely removed, and skull dried before the cement is applied. Otherwise, the cement will not adhere properly, and the cannula will fall off before the end of the experiment.

ChABC infusions

Timing: 3 h

In order to determine whether PNNs in CA1 region of DH are involved in memory processing, it is required to disrupt them by enzymatic digestion of CSPGs. In this protocol this is achieved by infusions of ChABC through the implanted cannulas.

7. Assemble the equipment (Figure 4A).
   a. Fill two 25 μL Hamilton syringes with water (a 1.5 mL tube filled with water can be used for this purpose). If air bubbles are present, remove them by forcefully emptying the syringe into the 1.5 mL tube and filling it again. It may be necessary to repeat several times.
   b. Place the syringes into the infusion pump.
   c. Fit the injector with two pieces of tubing and connect to the syringes.
   d. Remove the air from the tubing and the injector by pushing the syringe plunger until a small water drop appears on the tips of the injector.
   e. Set the infusion volume to 250 nL, and infusion rate to 150 nL/min. This volume is sufficient to cause PNN degradation in CA1, but not in the surrounding regions. The infusion apparatus is now assembled and ready for use (Figure 4B). Troubleshooting 2

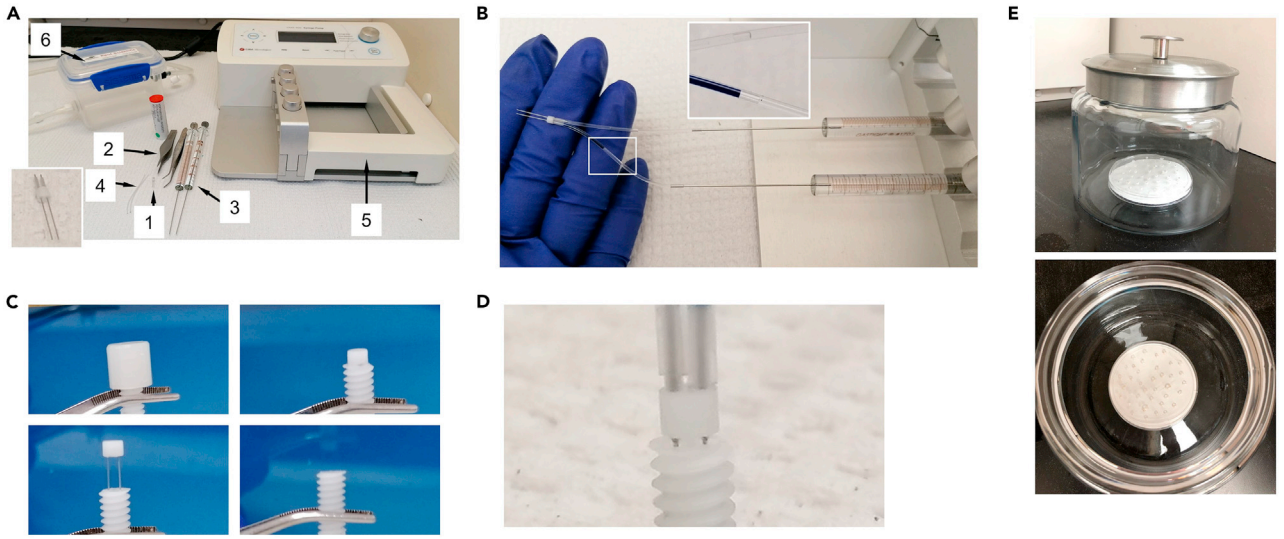

Figure 4. Setup for infusion

(A) Equipment and instruments needed for the infusion; hippocampal injectors [1, insert], forceps [2], Hamilton syringes [3], tubing [4], infusion pump [5], isoflurane induction chamber connected to a low-flow anesthesia system (not in view) [6].

(B) Assembled infusion apparatus. Insert: magnified image of an air bubble used as a barrier between the water in the syringe and the drug. One side is filled with a methylene blue solution for contrast.

(C) Closeups of a cannula on the mouse head showing step by step removal of dust cap and dummy cannula.

(D) Cannula with an injector inserted.

(E) Alternative isoflurane administration for infusions. Jar contains a piece of filter paper covered with perforated petri dish.

f. Fill the infusion apparatus with ChABC solution. Many reagents are either expensive or difficult to obtain, and it is desirable to use them in smallest possible quantities. Instead of filling syringes with the reagent that is to be infused, requiring close to 100 µL (to fill two 25 µL syringes, and two pieces of tubing), we prefer to create a small air bubble in the tubing, which acts as a barrier between the water in syringes and the infusion solution (Figure 4B, insert).
   i. Pull the syringe plungers out until a very small air bubble is formed.
   ii. Insert the injector into the tube with ChABC solution and pull the plungers out gently. Monitor that the air bubbles are moving up while the tubing is filled with ChABC solution.
   iii. Move the pusher of the infusion pump until it is pressed against the syringe plungers (a small drop should appear at the tips of the injector).

8. Infuse ChABC into CA1 Troubleshooting 3
   a. Anesthetize a mouse in the induction chamber, setting the concentration of isoflurane to 5%.

   Note: Closely monitor the breathing. Mouse is anesthetized when it stops moving and breathing slows down. Do not leave a mouse in the chamber too long, as 5% isoflurane may be too high for a longer exposure and may result in death.

   b. Remove the mouse from the induction chamber and transfer it to the assembled infusion equipment, setting the concentration of isoflurane to 1.4%.
   c. Remove the dust cap and the dummy cannula: hold the base of the guide cannula tightly with forceps, unscrew the cap and pull the dummy cannula out (Figure 4C). Forceps should be thin enough to fit around the narrow portion of the cannula base not covered by the cap.
   d. Insert the injector (Figure 4D).
   e. Mark the position of the air bubble in the tubing with a fine marker and start the infusion.
   f. When the infusion is completed leave the injector in the cannula for 1 min. This helps prevent the liquid being pulled upward from the site of injection as the injector is removed.

g. Remove the injector slowly, insert the dummy cannula and screw the cap onto the guide cannula.
h. Return the mouse to its home cage.

Note: Mouse should awaken very quickly, typically just a few moments after it is returned to the cage.

Alternatives: If an induction chamber for anesthesia is not available, a large glass jar can be used for isoflurane delivery instead. Place a piece of filter paper in the bottom of the jar, add 0.5 mL of isoflurane onto the paper and cover with a perforated petri dish (Figure 4E). This should be done in a chemical fume hood.

Pause point: Allow sufficient time for ChABC to degrade PNNs. In our experiments this time was 5 days (Jovasevic et al., 2021).

Behavioral training and tests

Timing: 3 h

This step of the protocol is designed to train the mice in a behavioral paradigm, to acquire an aversive memory, and to determine the strength of the memory in subsequent tests. The protocol uses contextual fear conditioning as a model for aversive episodic memories (Radulovic et al., 1998). This task is particularly suitable for studying long-lasting episodic memories in mouse models due to their hippocampal dependence and their robust and enduring behavioral manifestation (freezing). Contextual fear conditioning is performed in a plexiglass cage, with a stainless-steel rod floor (4 mm diameter, 0.9 cm center to center), within a constantly illuminated box, fitted with a camera to monitor and record the session (Figure 5A). Fear conditioning consists of a single 3-min exposure to a context followed by a 2 s, 0.8 mA footshock (constant current). A test for freezing behavior is performed by re-exposing the mice to the box for 3 min. The freezing response, horizontal activity, exploratory activity, and shock bursts are determined. Freezing should be recorded by trained observers unaware of the experimental conditions while all other data are provided automatically by software analyzing the infrared beam signals from the context. The shock is mildly aversive and causes the mice to either jump or run during its presentation. It is important to use different groups of mice for each experimental condition (vehicle/ChABC, recent/remote memory). Testing the same group of mice for recent and remote memory creates a concern of a possible effect of reconsolidation or fear extinction on the outcome of the remote memory test.

9. Set up the equipment and the room: fear conditioning apparatus should be clean, all instruments connected and turned on. The lights in the room should be dimmed during the experiment (Figure 5B).

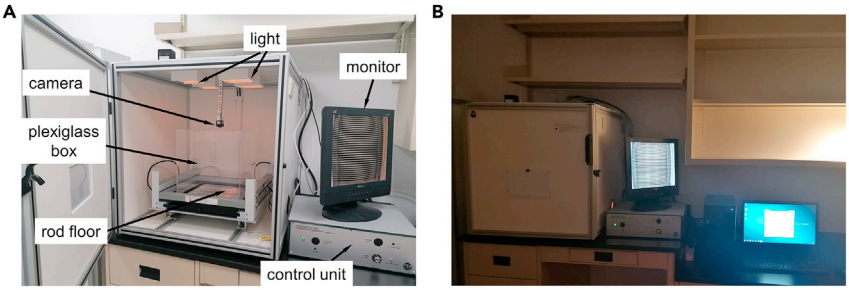

Figure 5. Equipment for contextual fear conditioning
(A) Components of a contextual fear conditioning chamber.
(B) Behavioral testing room during training or testing. The chamber door is closed, mouse behavior is observed on the computer monitor, and the light in the room is dimmed. See also Methods videos S1 and S2.

10. Test the equipment: Set the test current to 0.8 mA. Using a voltameter, verify that the current passing through the floor rods is correct.
11. Train a mouse. Troubleshooting 4
    a. Clean the fear conditioning chamber with 70% ethanol. This step not only cleans the chamber, but also provides scent as the part of the context.
    b. Transfer a mouse from the home cage into the fear conditioning chamber.
    c. Start the program: 3 min context; 2 s, 0.8 mA footshock.
    d. Record whether mouse is freezing every 10 s by briefly (~ 2 s) observing the mouse's behavior. Freezing is manifested as a complete lack of movement, except for respiration (Methods videos S1 and S2, Figures 6A and 6B).
    e. Note whether the mouse responded to the electric shock.
    f. Return the mouse to home cage.
    g. Repeat the procedure (a-f) for each mouse.

Pause point: At this stage the experiment is paused until the recent memory test day.

12. Perform recent memory test. In our protocol it is day 4 after training; however, other time points can be evaluated instead, typically day 1 to day 4. This procedure is for the most part an exact repeat of the training procedure (Step 11 above), comprising of the 3 min context exposure, but without footshock. Troubleshooting 5

Pause point: At this stage the experiment is paused until the remote memory test day.

13. Perform remote memory test. We assessed day 21 for remote memory test. As with recent memory, other time points can be evaluated as well (day 21 – day 35).
14. Quantify the results (see "quantification and statistical analysis" for details).
15. Perfuse animals one day after the remote test with ice-cold 4% paraformaldehyde in phosphate buffer (pH 7.4) following a standard transcardial perfusion protocol (Jovasevic et al., 2021). Remove brains, post-fix for 24 h in the same fixative and then immerse for 24 h each in 20 and 30% sucrose in phosphate buffer. Freeze brains in an embedding medium (we use Tissue-Plus O.C.T. Compound). Cut 50 μm sections on a cryostat and store in 1× PBS/0.4 mg/mL thimerosal at 4°C.

Optional: In addition to manual scoring, freezing can also be determined automatically, using infrared beam monitoring system. We have repeatedly verified the linear correlation between

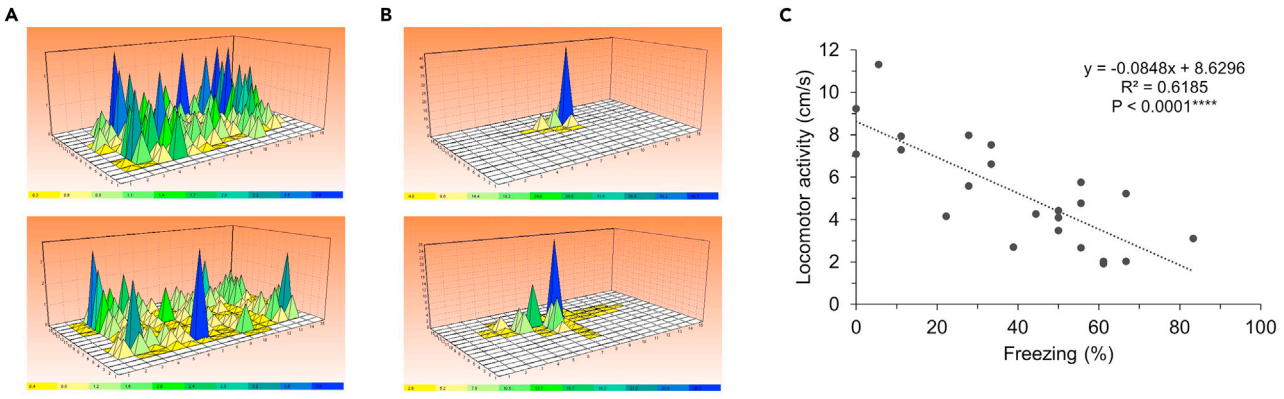

Figure 6. Mouse behavior during contextual fear conditioning training and test sessions

Representative locomotor activity diagrams during (A) contextual fear conditioning training, and (B) test. XY plane indicates position of the mouse during the 3 min exposure to the context, Z axis indicates time spent in each position.

(C) Correlation between manually scored freezing and locomotor activity recorded by the instrument. ****$p < 0.0001$ (Simple linear regression, $F_{1, 21} = 34.04$, $p < 0.0001$).

manually and automatically scored freezing, or between manually scored freezing and locomotor activity (Figure 6C).

Immunohistochemical detection of PNNs

Timing: 1 day

This step of the protocol is performed to evaluate the efficiency of ChABC treatment. PNNs are visualized using Wisteria Floribunda Lectin (WFA) staining. The staining is performed using Vector Biolaboratories reagents, according to manufacturer's instructions (https://vectorlabs.com/media/folio3/productattachments/product_manual/Detect.Glycoproteins.pdf).All steps are performed at room temperature:

16. Wash sections in 1 x phosphate buffered saline, pH 7.4 (PBS)/0.04 mg/mL thimerosal for 5 min.
17. Incubate in 1% hydrogen peroxide in methanol for 15 min.
18. Wash in 1 x PBS containing 0.2% Triton X-100, 3 times for 5 min.
19. Add 6 drops of streptavidin solution from the Streptavidin/Biotin Blocking Kit, incubate for 25 min.
20. Wash in 1 x PBS for 5 min.
21. Add 6 drops of biotin solution the Streptavidin/Biotin Blocking Kit, Incubate for 15 min.
22. Remove the solution. Do not wash.
23. Add 0.5 mL of Carbo-free reagent, incubate for 30 min.
24. Remove the solution. Do not wash.
25. Incubate with 10 μg/mL biotinylated WFA in 1× PBS for 30 min.

Note: Prepare ABC solution at this point (see step 27), as it requires 30 min incubation prior to use.

26. Wash in 1 x PBS/0.05% Tween-20 twice for 5 min.
27. Incubate for 30 min in Vectastain Elite ABC Kit: 1:100 Reagent A, 1:100 Reagent B, in 0.3% Triton X-100/1 x PBS.
28. Wash in 1 x PBS/0.05% Tween-20 three times for 5 min.
29. Incubate for 10 min with appropriate fluorescent dye (FITC for green, Rhodamine for red, or Coumarin for blue) diluted 1:62.5 in amplification diluent.
30. Wash in 1 x PBS, twice for 5 min.
31. Place tissue on Colorfrost Plus slides and let dry overnight.
32. Coverslip with a mounting medium compatible with immunofluorescence microscopy (e.g., Vectashield mounting medium, FluorSave). Tissue can be examined on an epifluorescence or confocal microscope.

EXPECTED OUTCOMES

In this protocol we describe steps for targeted disruption of PNNs in CA1 region of DH for the purpose of evaluating their role in the processing of recent and remote aversive memories in this region. Infusion of ChABC into CA1 should result in complete degradation of PNNs 5 days later. Even if some occasional PNNs remain, they are structurally compromised, and typically appear as a faint ring surrounding only soma (Figure 7A). The earliest time point at which we tested the efficiency of PNN degradation was 5 days after ChABC infusion. If an experimental design requires a memory test at a time point earlier than 5 days, we recommend a test experiment to determine whether PNN degradation is achieved at that time point. During contextual fear conditioning training phase mice should be freely exploring the entire context (Methods video S1, Figure 6A) and their locomotor activity should not differ between the experimental and control groups (Figure 7B). During the presentation of the footshock mice show an outburst of locomotion (typically running and jumping),

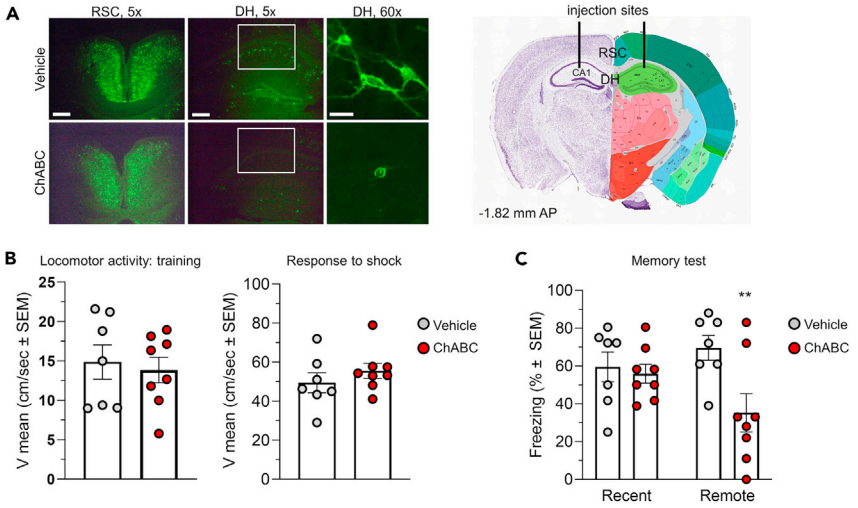

Figure 7. Expected results

(A) Representative images of PNNs in RSC and DH after the infusion of vehicle or ChABC into the CA1 region of DH. White rectangle indicates the region of DH where vehicle/ChABC were infused. Scale bar: 150 μm (5 ×), 25 μm (60 ×). Right: Allen brain atlas image with infusion sites indicated.

(B) Locomotor activity of mice infused with vehicle or ChABC, during contextual fear conditioning training or as the response to 2 s electric shock. Data are represented as mean ± SEM (unpaired t-test, two-tailed; n = 7 (vehicle), 8 (ChABC)/group; training: $t_{13} = 0.3773$; p = 0.7120; response to shock: $t_{13} = 0.9661$; p = 0.3516).

(C) Effect of PNN depletion on recent and remote memory. Mice infused into CA1 with vehicle (ACSF), or ChABC were fear conditioned, and tested at recent or remote time point. Separate sets of mice were used for each experimental group.

Data are represented as mean ± SEM (two-way ANOVA, n = 7 (vehREC, vehREM), 8 (ChABC REC, ChABC REM)/group, effect of treatment: F1, 13 = 5.062, p = 0.0424; test × treatment interaction: F1, 13 = 5.019, p = 0.0432; post-hoc: p = 0.9332 chABC REC vs vehREC, **p = 0.0080 chABC REM vs vehREM). See also Methods videos S1 and S2.

accompanied by vocalization. This heightened locomotor activity should not differ among the groups (Figure 7B). The contextual fear conditioning protocol described here produces long-lasting memory (over 3 months) in control group, manifested as freezing responses (Methods video S2, Figure 6B). We typically observe about 50% time spent freezing. In the control group, this memory can be retrieved at both recent and remote phases (Figure 7C). Freezing levels will vary from experiment to experiment [in our hands typically 40%–60% (Yamawaki et al., 2019)]; therefore, only groups from the same experiment should be compared. In contrast to the control group, the PNN-depleted group shows a distinct pattern of memory responses. In recent test, memory is intact, while in remote test it is strongly impaired (Figure 7C).

QUANTIFICATION AND STATISTICAL ANALYSIS

Contextual fear conditioning testing consisted of 3 min exposure to the conditioning context, during which freezing was measured every 10 s. Freezing was expressed as a percentage of the total number of observations during which the mice were motionless (freezing observations, FO) using formula FO/18*100. Locomotor activity is recorded automatically by an infrared beam system and expressed as mean velocity (cm/s).

We perform statistical analyses using GraphPad Prism software; however, any other comparable software can be used instead (e.g., IBM SPSS Statistics). Only those mice with PNNs that are completely degraded, or structurally compromised, (as described in Expected outcomes) in CA1 should be considered for the analyses. The results are analyzed using two-way ANOVA with group (vehicle or ChABC) and time length as factors. Homogeneity of variance is confirmed with Levene's test for equality of variances. When significant main and interaction effects are observed, two-way

ANOVA is followed by Sidak's multiple comparison test. All comparisons are conducted using two-tailed tests and the p value for all cases is set to <0.05 for significant differences. Group sizes are determined using power analysis assuming a moderate effect size of 0.5.

LIMITATIONS

There are several possible limitations to the experimental protocol that should be taken into consideration, or kept in mind if this protocol is to be modified:

Behavioral tests can be affected by external environmental factors such as loud noises, vibrations in the building, smells, etc. Any exposure to such stressful stimuli can result in various abnormal behaviors, such as freezing during training (before footshock presentation), lack of freezing or extreme freezing (approaching 100%) during the test. In such cases the experiment should be repeated with a new set of mice. If possible, the behavioral equipment should be placed in a well-controlled environment, with minimal disturbances. Any environmental abbreviations should be noted during the experiment.

ChABC degrades CSPGs, a major constituent of PNNs (Deepa et al., 2006), and therefore allows a general assessment of the role of PNNs, but cannot be used to determine the role of individual components of PNNs, which are known to have unique roles (Fawcett et al., 2019). In order to precisely define the role of PNNs in memory it will be required to conduct studies with specific manipulations, targeting individual components of PNNs. However, this is likely to be challenging as well because the constituents of PNNs also play other roles in the organization of the extracellular matrix (Bukalo et al., 2001; Edamatsu et al., 2018; Hirono et al., 2018).

Our approach for PNN detection uses Wisteria floribunda agglutinin (WFA) staining. While the majority of PNNs are WFA-positive, there are also WFA-negative PNNs, such as those surrounding cortical output neurons (Matthews et al., 2002). Although this is not a concern for our experimental protocol, considering it examines PNNs in the hippocampus, it is important to keep in mind for studies of other brain regions.

TROUBLESHOOTING

Problem 1

PNN degradation in a wrong brain region (Step 3)

This problem occurs when cannulas are misplaced. This is rarely a problem for DH and other large brain regions, but could be an important confound when smaller areas are targeted. There are several possible reasons:

(1) Centering scope and/or drill are not properly aligned. This would result in a consistent shift in the coordinates and cannula position.

(2) Inexperienced experimenter. Typically, the source of the problem is that a person new to the procedure is not able to reliably identify bregma. This results in a high proportion of misplaced cannulas, with random shifts in the position.

Potential solution

(1) Verify that the centering scope and the drill are properly aligned with each other. Once the scope is centered on bregma, fit the stereotaxic instrument with the drill, and lower it until the top of the drill bit touches the skull. The drill bit should also be positioned at bregma. Use a magnifying glass if necessary. If this is not the case, realign the instrument. We would advise that any repairs to the stereotaxic instrument are performed by the manufacturer.

(2) Re-training should be implemented until the experimenter is confident in all steps of the procedure, and can reliably perform it, with correctly placed cannula.

Problem 2

PNN degradation is not observed throughout the entire CA1, or extends into surrounding regions (Step 7)

This problem arises when the infusion volume is too small or too large, respectively.

Potential solution

Adjust the volume of ChABC that is infused until PNNs are degraded in the entire CA1, but not in the regions neighboring the CA1.

Problem 3

Enzymatic degradation of PNNs was not successful/incomplete (Step 8)

There are several possible reasons:

(1) ChABC lost its activity. This a likely reason when the degradation of PNNs was not achieved in CA1 of both hemispheres.

(2) ChABC was not infused into DH. If PNNs were degraded in DH on one side, but not the other, a very likely reason is that the injector was clogged during the infusion.

(3) Guide cannulas are plugged, resulting in damage to the injector.

Potential solution

(1) Use a freshly prepared solution of ChABC. Always store the solution at −80°C, in small aliquots to avoid repeated thawing/freezing.

(2) Carefully monitor the movement of the air bubbles in the tubing during the infusion. Mark the start and end position with a fine marker. If one side of the injector is clogged, the bubble will not move from its start position. If the injector is clogged, replace with a new one.

(3) Clean guide cannulas carefully after every use. Remove cement from the outside of the guide cannula. If needed, using a fine wire (or an old dummy cannula), clean the inside of the guide cannula to remove any traces of cement. Put the guide and dummy cannulas into distilled water with a few drops of liquid soap and sonicate for 15 min. Rinse the cannulas several times with distilled water, and once with 70% ethanol. Leave to dry completely before autoclaving.

Problem 4

No response to electric shock during conditioning (Step 11)

There are several possible reasons:

(1) Treatment (pharmacological, surgical) rendered the mouse unresponsive. This issue is typically not observed when using ChABC; however, it does occasionally occur with other pharmacological manipulations. We observed this problem with intrahippocampal applications of extrasynaptic $GABA_AR$ agonist gaboxadol, which at higher doses produces impairing effects on locomotor activity (Jovasevic et al., 2015).

(2) The electric circuit was disrupted.

(3) Shock threshold is increased. We have never encountered this problem, but if this happens it may be due to impaired shock sensitivity in some genetic mouse lines.

Potential solution

(1) Adjust the dose of the drug so it would still have desired pharmacological effect, but without deleterious effects on locomotor activity. As we mentioned, this typically does not occur with ChABC, but in an unlikely scenario that a very high dose is used which results in impaired locomotor activity, the dose should be gradually reduced so it produces desired depletion of PNNs, but without differences in locomotor activity between vehicle and ChABC groups (as illustrated in Figure 7B).

(2) Frequently, the cause of disrupted electrical circuit is mouse feces that bridges two floor bars, resulting in short circuit. In this case, carefully open the door of the fear conditioning chamber and clean the bars, disturbing the mouse as little as possible. Once the bars are cleaned run a "shock only" program in the fear conditioning system (which should be programmed in advance), consisting of only 2 s, 0.8 mA shock.

(3) Increase footshock intensity after careful titration from 0.8 to 1.5 mA until a response to shock can be observed (outburst of locomotion, vocalization). We would not recommend increasing the shock intensity above 1.5 mA.

Problem 5

Lack of freezing in control group at recent memory test (Step 12)

There are several possible reasons for this:

(1) Mouse did not receive a footshock at the end of the training session.

(2) Mouse was under the influence of stressful environmental factors that impaired its normal physiological responses to the footshock or the ability to associate context with the footshock.

Potential solution

(1) Observe animals carefully during the training session. When the footshock is administered animals should display increased locomotion and vocalization. Additionally, the reaction to footshock can also be confirmed based on the recorded locomotor activity data (see Figure 7B). If the mouse is not reacting to the shock, apply one of the solutions to Problem 3. Additionally, if the absence of the footshock was observed after all animals were trained or tested, they can be trained again. However, in that case the results should be cautiously interpreted, since the unplanned exposure to the context, without a footshock, deviates from the standard protocol. The data could be useful and informative; however, the experiment should be repeated, with a new set of animals.

(2) Avoid any potential sources of stress that can be controlled (e.g., cell phone ringing, loud talking, wearing perfume, maintenance or construction work nearby, etc.). If these problems do occur, repeat the experiment with a new set of animals, after the environmental factors causing the problem have been removed.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Jelena Radulovic (jelena.radulovic@einsteinmed.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets or program code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100931.

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AUTHOR CONTRIBUTIONS

V.J. performed the experiments and wrote the manuscript. J.R. revised the manuscript. Z.P. helped with preparing figures, H.Z. with PNN labeling, and A.C. by providing videos of the fear conditioning procedure.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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