Characterization of synaptic plasticity changes via optogenetic manipulation between defined neuronal populations is important for mapping neural circuits involved in normal brain functions and disorders including neuropathic pain. However, determining the strength of synaptic transmission based on optogenetic manipulation is challenging due to variability in opsin expression. This protocol describes the use of slice electrophysiology combined with optogenetics to examine synaptic transmission in genetically defined neuronal populations from neuropathic mice. We detail surgical procedures of spared nerve injury for inducing neuropathy.
Protocol for detecting plastic changes in defined neuronal populations in neuropathic mice

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SUMMARY
Characterization of synaptic plasticity changes via optogenetic manipulation between defined neuronal populations is important for mapping neural circuits involved in normal brain functions and disorders such as neuropathic pain. However, determining the strength of synaptic transmission based on optogenetic manipulation is challenging due to variability in opsin expression. This protocol describes the use of slice electrophysiology combined with optogenetics to examine synaptic transmission in genetically defined neuronal populations from neuropathic mice. We detail surgical procedures of spared nerve injury for inducing neuropathy.
For complete details on the use and execution of this protocol, please refer to Huang et al. (2019) and Huang et al. (2021)

BEFORE YOU BEGIN
Specific neuronal populations are identified by using a transgenic Cre mouse line. To record in parvalbumin (PV) GABAergic neurons, or glutamatergic neurons (e.g., in the prelimbic cortex, PrL), a Cre driver line such as PV-Cre or Vglut2-Cre mice is crossed with a reporter line (such as Ai9 mice). Whole-cell patch clamp electrophysiology is performed in tdTomato fluorescent neurons of brain slices from these transgenic mice using an upright microscope. An adeno-associated virus (AAV-CaMKIIz-ChR2-EYFP) is used to target upstream glutamatergic projection neurons (e.g., in the basolateral amygdala, BLA) and to selectively stimulate the glutamatergic input fibers (e.g., in the PrL) using LED blue light. Transgenic mice and AAVs can be easily purchased from commercial sources.

Breeding animals

© Timing: 4–5 months prior to experiment

1. Transgenic mice: Recombinase Cre driver line PV-Cre or Vglut2-Cre mice and reporter line Ai9 mice are set up to breed litters containing fluorescent tdTomato in PV GABAergic neurons (PV-Cre::tdTomato) or in glutamatergic neurons (Vglut2-Cre::tdTomato). It takes 3 weeks for gestation and 8–9 weeks for the litter to be ready for further virus injection. Certain transgenic lines require more time to breed than others. This has to be taken into consideration when planning. The protocol applies equally to male and female mice (8–12 weeks old). Due to our experimental design, we only used male mice in these specific experiments.
Figure 1. Tools and procedures involving virus injection, SNI/Sham surgery, obtaining brain slices and electrophysiological recording

(A) Surgical tools used for virus injection. From left to right: Ideal drill, operation scissors (small), ear punch for identification of mice, forceps, scalpel, needle holder and spatula (top, helping for placement of ear bar and fixing the mice on frame).

(B) A mouse (body covered with pad) is placed on an Ultra Precise digital stereotaxic frame with Nanoliter injector attached to the arm of the frame.

(C) A needle (tip size 10–30 μm, pulled from glass pipette, yellow arrows) attached to Nanoliter injector is used to measure the relative height of bregma and lambda at the AP axis and two symmetrical points on the left and right of the sagittal suture of the skull at the LR axis.

(D) Surgical tools for SNI/Sham surgery. From left to right: operating scissors (small), needle holder, ear punch, forceps, tip polished glass hook and scalpel.

(E) A small incision is made on the side of the left leg to start the dissection. The visible white line (fascia) (inset, yellow arrowheads) can be used as a landmark for longitudinal blunt dissection of the muscles underneath.

(F) To better illustrate the nerves and adjacent structures, the cut wound is intentionally enlarged. Common peroneal and tibial nerves form a nerve bundle (green arrow) and gradually branch out into two separate nerves distally (upper left). A red vessel runs between the two nerves with the common peroneal nerve on the left and tibial nerve on the right. The sural nerve is the third branch of the sciatic nerve (black arrow). The three branches point towards the hindpaw (upper left, see E). A glass hook (pointing upward) lifts common peroneal and tibial nerves (yellow arrowhead).

(G) Common peroneal and tibial nerves are ligated and transected (green arrow). A 1–2 mm nerve segment is removed from the proximal end of the cut nerve (yellow arrows, the distal end of cut nerve is covered under muscles). The sural nerve is seen intact (black arrow).

(H) Surgical tools for transcardial perfusion and obtaining brain slices. From left to right: operating scissors (large), scalpel, 21 gauge needle for penetrating heart (left ventricle), operating scissors (small), hemostat for clamping and flip rib cage, forceps, large spatula (curved) for transferring brain, Krazy glue and flat spatula for transferring brain blocks.

(I) The thoracic wall was cut open on left, right and bottom side (diaphragm) and flipped up to expose the heart. A 21 gauge needle (yellow arrows) is connected to the perfusion reservoir filled with cutting solution and is about to...
Alternatives: A Cre dependent viral vector carrying a fluorescent reporter protein (e.g., AAV-DIO-eYFP) can be injected in the targeting nuclei of a specific Cre mouse line to identify the recorded neurons.

Virus injections

- **Timing**: 7 weeks prior to experiment

All animal experiments/procedures have to be approved by the Institutional Animal Care Committee. To optogenetically activate glutamatergic BLA inputs into the PrL, we expressed the excitatory opsin ChR2 in the BLA and illuminated terminals of BLA originating fibers in the PrL using blue LED light. To this end, a variety of CaMKIIα promoter viruses of different serotypes can be used. Virally mediated gene expression varies according to the types of the virus and the infected brain areas. Therefore viral serotypes should be validated according to their brain region of interest to obtain the appropriate concentration, dosage and expression level needed.

2. **Load virus**: Attach Nanoliter injector (Drummond Co., PA) on the arm of a stereotaxic frame (Figures 1A and 1B). Prefill the needle (tip size 10–30 μm) with low molecular weight mineral oil. Bleed mineral oil and backfill the needle with AAV. 100–500 nl/mouse, 1–5×10E12 particles/mL or according to dosage tested. Move the arm away from the center for easy handling of the mouse.

3. **Anesthetize the mouse with isoflurane (Vaporizor, Patterson Veterinary, CO): weigh the mouse, place in the induction chamber, once in anesthetized state (slow breathing, slight response to hind paw pinching), remove hair on the head.

4. **Fix the head in the stereotaxic frame (Stoelting Co., IL) (Figure 1B):** first place the bite bar between the jaws of mouse (two front incisors in the hole of the bar), put ear bars into the ear canal on both sides, gradually move the two ear bars closer toward each other until the head is tightly clamped. Gently tighten the nose clamp to hold the head on frame firmly. Apply eye gel (Optixcare Eye lube) to prevent corneal desiccation.

5. **Expose the skull**: Clean the surgical area using surface disinfectant (Betadine and 70% alcohol alternate 3 times). Make a midline incision along anterior-posterior axis. Remove connective tissue on the skull by rubbing the skull surface with a cotton swab.

6. **Place the head at standard position (Figure 1C):** First adjust left and right ear bars to the same height using the adjusting screw. To level the head on the left-right (LR) axis, first find the sagittal midline of the skull and move the needle of the Nanoliter injector to slightly touch the skull at the midline (roughly the middle of the skull along the anterior-posterior (AP) axis). Then move the needle 2 mm (or 3 mm) to the left across the midline, and measure the height of the skull at that point. Then move the needle to the right the same distance and measure the height. If one side is lower than the other side, then increase the height of that side by raising the ear bar on that side using.
the adjusting screw until the two sides are at the same level. In most cases if the ear bars are placed in
the correct position and at the same height, the head should be level on the LR axis. Using similar
strategies, we find the bregma and lambda at the AP axis, measure the height of both points and
adjust them to the same level by changing the height of the nose bar (Figure 1C).

7. Drill a burr hole into the skull: Find the AP and LR coordinate on the surface of skull using the need-
le (for BLA: AP: $-1.3$ mm; LR: $-3.4$ mm). Make a burr hole using an Ideal drill (Stoelting Co., IL)
with 0.6 mm drill bit. Be careful not to damage the brain surface.

8. Inject virus: Remove dura using a 21 gauge needle. Clean/absorb blood or cerebral spinal fluid
with a cotton swab. Lower the needle slowly to the coordinate of the brain region of interest
(BLA AP: $-1.3$ mm; LR: $-3.4$ mm; DV: $-4.9$ mm). Push Inject button to inject 50.6 nl (e.g., AAV5-
CaMKIIz-hChR2(H134R)-EYFP) in bolus at slow speed (23 nl/s). Inject 50.6 $\times$ 2 nl/min until reaching
100–500 nl/mouse (1–5 $\times$ 10E12 particles/mL or dosage tested appropriate). Raise the needle
100 $\mu$m after 5 min. Then wait for another 10 min to slowly lift the needle completely out of the brain.

9. Suture the skin with Vicryl suture (4-0) (Ethicon). Apply polysporin to cut wound. Remove the
mouse from frame and return to home cage following recovery on a heated pad.

\[\triangle \text{CRITICAL: 1. The accuracy of viral injection is crucial for successful experiments. A new}
coordinate must be tested by injection of colored solution (e.g., fluorescent retrobeads,}
100–200 nl) and dissection of the brain area of interest immediately afterwards. The coordi-
nate must be adjusted depending on the actual color distribution according to the mouse
brain Atlas (Paxinos and Franklin, 2001). The actual coordinate for a brain nucleus is affected
by the age and weight of the mice and must be tested and adjusted accordingly. 2. Be cautious
of bleeding when drilling at certain locations of the skull. Use absorbable hemostat (Surgicel
Nu-Knit™, Ethicon) to stop bleeding. 3. Residual dura on the brain surface can easily break the
needle or deviate the needle from the normal path, thus leading to inaccurate injection sites.
Make sure that the brain surface is free of dura, blood clots or bone debris. 4. To avoid back-
flow of injected virus, injections must be slow (e.g., speed 23 nl/s, 50 nl/bolus/30s). The injec-
tion needle must stay at location after the injection for at least 15 min before slowly being
lifted completely. Avoid a second injection if the first injection is not successful (e.g., due to
blocked needle of the first injection).

Neuropathic model: Spared nerve injury

\[\copyright \text{Timing: 2–3 weeks prior to experiment}

The Spared Nerve Injury (SNI) neuropathic pain model involves surgical ligation and cutting of the
tibial and common peroneal nerves without damaging the sural nerve. It produces reliable long-last-
ing tactile hypersensitivity in the skin territory of the spared, intact sural nerve. It usually takes at
least two weeks for tissue healing and the inflammatory response to disappear. Therefore, we use the an-
imals for experiments two weeks after surgery. Below, we describe this procedure in the context of
our experience, and for further reading please refer to Bourquin et al., 2006.

10. Anesthetize the mouse using isoflurane, remove hair on the side of left leg. Disinfect the surface
as described for viral injection (Figures 1D and 1E).

11. Make an incision (~1 cm) on the skin at the mid-thigh level using a scalpel (Figure 1E). Make blunt
dissection between muscles along a visible line of white fascia with scissors (small) and forceps,
then use a tip-polished glass hook to expose (by sliding along the nerves) the three branches of
the sciatic nerve under the biceps femoris muscle. Blunt dissection between muscles instead of
cutting the muscles prevents bleeding. A small artery (red) can easily be seen between the com-
mon peroneal and tibial nerve perpendicularly to the nerves (Figure 1F). Slide a 6/0 silk suture un-
der the common peroneal and tibial nerves proximal to the above-mentioned small artery. Ligate
and sever both nerves on the distal side of the ligation leaving the sural nerve intact. Remove
1–2 mm of nerve from the proximal end of the cut nerve (Figure 1G). For control/sham surgery,
nerves are dissected without ligation and transections but a 3 mm 6/0 silk suture is placed along the nerves at the location corresponding to ligation and transection site.

12. Close muscle and skin with 6/0 silk suture and 4/0 Vicryl suture, respectively.

13. Return the mouse back to home cage after recovery on a heated pad.

△ CRITICAL: 1. An occasional challenge in SNI surgery is to identify the nerves and the correct section of nerves for ligation and transection. First identify the nerve bundle (bright and white) formed by common peroneal and tibial nerves under biceps femoris muscle and follow it distally until it branches out into two separate nerves. At this point, as mentioned above, a red artery runs perpendicularly between these two nerves (Figure 1F). The nerve section before branching is normally a good position for nerve ligation and transection to avoid the sural nerve which branches out slightly more proximally. 2. Pain thresholds should be examined to verify that the surgery has resulted in a hypernociceptive phenotype. Importantly, the sensory test must be applied on a narrow strip of glabrous plantar surface on lateral external side of the ipsilateral (nerve injured) paw that corresponds to the skin territory of the non-injured sural nerve.

Preparation of stock solutions for electrophysiology

⊙ Timing: One or a few days prior to experiment

14. Make 4× stock brain cutting solution. There are multiple recipes for cutting brain slices (e.g., choline-based solution, NMDG based solution). The key to obtaining viable brain slices is to replace the Na⁺ in the extracellular solution with other Na⁺ channel impermeable cations to block action potentials and to prevent cell toxicity. Another important factor is that the cutting solution should be ice-cold (4°C, place cutting solution in −20°C for ~1 h to form an ice and water mix). We choose to use NMDG-based extracellular solutions for brain slice cutting (Adapted from Ting et al., 2014). We make a 4× solution and separate it into 50 mL/tube and freeze it at −20°C for later use. See Recipe Cutting solution (NMDG based extracellular solution, 1×).

15. Make Cs-methanesulfonate based intracellular solution and separate into 700 μL/Eppendorf tubes for later use. See Recipe Intracellular solution (Cs based) (1×).

Note: Almost as effective as the NMDG based cutting solution, sucrose and choline based solutions can also be used for cutting brain slices to prevent cytotoxicity. There are other options for intracellular solutions. For synaptic current recordings, both Na⁺ channel and K⁺ channel blockers (QX-314 and Cs, respectively) are included in the intracellular solution to eliminate contamination from voltage dependent currents and to allow for recording of EPSCs and IPSCs in the same cells (EPSC at −70 mV and IPSC at 0 mV). Intracellular Cs blocks K⁺ current and improves space clamp. High Cl⁻ intracellular solution (symmetrical Cl⁻ intracellular and extracellular) depolarizes the Cl⁻ reversal potential to 0 mV and offers the advantage of large inward IPSC signals at physiological resting membrane potentials (Graziane and Dong, 2016). Potassium based intracellular solution (used with negatively charged organic molecules, e.g., gluconate, methanesulfonate) is mostly used in current clamp to record action potentials. If the membrane potential is held at resting potentials without depolarization, this intracellular solution can also be used in voltage clamp to record EPSCs (Graziane and Dong, 2016).

Preparation of working solutions

⊙ Timing: 2 h prior to patch clamp electrophysiological experiment

On the day of experiment, prepare working solutions (cutting solution and extracellular solution).

16. Make cutting solution: Dilute NMDG-based extracellular stock solution (4×) from 100 mL to 400 mL using purified water (Millipore Milli-Q Water purification system). 150 mL of NMDG
solution is set for incubating brain slices at 33.5°C. Keep the remaining solution at –20°C until a thin layer of ice is formed around the wall of the beaker (see next section). Part of the solution is used for transcardial perfusion (50 mL), part for cooling the mouse head in a 100 × 15 mm petri dish (50 mL), and the rest is for use in the vibratome chamber for slice cutting.

17. Make extracellular solution: Make solution according to Recipe Normal extracellular solution (1 ×).

△ CRITICAL: 1. For making stock cutting solution, add MgSO₄·7H₂O after titrating the pH of the NMDG solution from very basic state to pH 7.4 to avoid precipitation. Leave out CaCl₂·2H₂O and glucose in stock NMDG solution and add them to working solution just before the experiments (a few hours before experiments) to avoid degradation or contamination by for example bacteria and mold growth. 2. Make sure the pH of the solution is accurate.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Bacterial and virus strains | | |
| AAVS-CaMKIIa-HChr2(H314R)-EYFP | Addgene | 26969 |
| Experimental models: organisms/strains | | |
| Mouse: PV-Cre: B6;129P2-Pvalbtm1(Cre)Arbr/J | The Jackson Laboratory | Stock #: 008069 |
| Mouse: Vglut2-Cre: Slc17a6tm1(Cre)Arbr/J | The Jackson Laboratory | Stock #: 016963 |
| Mouse: a9: B6.Cg-Tg(Rosa26Sor-tdTomatoHey1)J | The Jackson Laboratory | Stock #: 007909 |
| Chemicals, peptides, and recombinant proteins | | |
| NMDG | Sigma-Aldrich | Cat #: M2004 |
| KCl | Sigma-Aldrich | Cat #: M3911 |
| NaH₂PO₄ | Sigma-Aldrich | Cat #: S0751 |
| NaHCO₃ | EMD Millipore Co | Cat #: SX0320-1 |
| HEPES | Sigma-Aldrich | Cat #: H4034 |
| D-(+)-Glucose | Sigma-Aldrich | Cat #: G8270 |
| (+)-Na L-ascorbate | Sigma-Aldrich | Cat #: A7631 |
| Na pyruvate | Sigma-Aldrich | Cat #: P2256 |
| CaCl₂·2H₂O | Sigma-Aldrich | Cat #: C3881 |
| MgSO₄·7H₂O | Sigma-Aldrich | Cat #: M2773 |
| NaCl | Sigma-Aldrich | Cat #: S7663 |
| Cs methanesulfonate | Sigma-Aldrich | Cat #: C1426 |
| CsCl | Sigma-Aldrich | Cat #: C4036 |
| EGTA | Sigma-Aldrich | Cat #: E4378 |
| ATP-Mg | Sigma-Aldrich | Cat #: A9187 |
| GTP-Na | Sigma-Aldrich | Cat #: G8877 |
| QX-314 (Cl⁻) | Tocris | Cat #: 2313/50 |
| TTX | Alomone Labs | Cat #: T-550 |
| 4-AP | Sigma-Aldrich | Cat #: A-0152 |
| (-)-Bicuculine methiodide | Tocris | Cat #: 2503/10 |
| DNPQX disodium salt | Tocris | Cat #: 2312/10 |
| DL-AP5 | Tocris | Cat #: 3693/10 |
| Mineral oil (low molecular weight) | Sigma-Aldrich | Cat #: M5904 |
| Eye gel (OptixCare Eye Lube, for animal use only) | CalciMedica | N/A |
| Lumafluor Retrobeads™ (fluorescent latex microspheres) | Lumafluor Inc | Green retrobeads |
| Alcojet powdered detergent | Alconox - Critical Cleaning Expert | Cat #: 1404-1 |
| Software and algorithms | | |
| pClamp 10 Data Acquisition and Analysis Suite | Molecular Devices | pClamp data acquisition and analysis software |
| Multiclamp 700B Commander | Molecular Devices | Software for operating Multiclamp 700B |

(Continued on next page)
## MATERIALS AND EQUIPMENT

### Cutting solution (NMDG based extracellular solution, 1 x)

| Reagent                  | Final concentration (mM) | Amount (g) for 400mL |
|--------------------------|--------------------------|----------------------|
| NMDG                     | 93                       | 7.2614               |
| KCl                      | 2.5                      | 0.0746               |
| NaH₂PO₄                  | 1.2                      | 0.0576               |
| NaHCO₃                   | 30                       | 1.0080               |
| HEPES                    | 20                       | 1.9064               |
| Glucose                  | 25                       | 1.8016               |
| (+)-Na L-ascorbate       | 5                        | 0.3962               |
| Na Pyruvate              | 3                        | 0.1320               |
| CaCl₂·2H₂O               | 0.5                      | 0.0294               |
| MgSO₄·7H₂O               | 10                       | 0.9860               |
| Thiourea (CH₄N₂S)        | 2                        | 0.0609               |

Aliquot into 50 mL/tube and store at −20°C to be used within one month.

### Normal extracellular solution (1 x)

| Reagent                  | Final concentration (mM) | Amount (g) for 1000 mL |
|--------------------------|--------------------------|------------------------|
| NaCl                     | 120                      | 7.0128                 |
| NaHCO₃                   | 26                       | 2.1840                 |
| Glucose                  | 25                       | 4.5040                 |
| KCl                      | 2.5                      | 0.1864                 |
| NaH₂PO₄                  | 1.25                      | 0.1500                 |
| CaCl₂·2H₂O               | 2                        | 0.2940                 |
| MgSO₄·7H₂O               | 1.3                      | 0.3204                 |

Extracellular solution is made on the same day of the experiment.
STEP-BY-STEP METHOD DETAILS

Obtaining brain slices

⏱ Timing: 2 h

This step is crucial for obtaining viable brain slices. Ice-cold NMDG (cutting) solution is used for transcardial perfusion and cutting in the chamber of the vibratome. Brain slices are incubated first in NMDG solution (33.5°C) for 11 min and then in normal extracellular solution (33.5°C) for 50 min (or less) and finally in normal extracellular solution at room temperature (~22°C–24°C) until recording (30°C–32°C). Gentle and skillful handling of the brain tissue is important. The cutting process (from transcardial perfusion to brain slices in incubating chamber) should be short (typically 20–25 min).

1. General preparation
   a. Surgical tools: Scalpel, flat spatula, broad spatula (curved), hemostat, forceps, operating scissors (small), operating scissors (large) suitable for cutting tissues and bones. (Figure 1H)
   b. Razor blade: Wash thoroughly with powdered detergent free of residue. Rinse again with 70% alcohol and deionized water. Fold and break the two-edged blades into separate two blades, one blade for the vibratome, the other for trimming the brain block.
   c. Glue: Krazy glue. It is quick to dry. Be careful to cap the tube properly.
   d. Incubating solutions: Place a small beaker holding 150 mL of NMDG solution in an incubating water bath at 33.5°C. Place another similar sized beaker holding 150 mL of normal extracellular solution in the same bath. We use home-made mesh baskets immersed in solution (1 cm deep) for keeping brain slices in both solutions (see below). Solutions are bubbled with 95% O₂ and 5% CO₂.
   e. Cutting solution: Take out cooled cutting solution (NMDG) from ~20°C and stir to obtain an ice water mix (~1/4 ice particles in the solution). Pour 50 mL into the perfusion reservoir at a height ~0.5 m above the mouse. Pour 50 mL in a 100×15 mm petri dish which sits on a box of crushed ice.
   f. Anesthetics: isoflurane
   g. Vibratome (Leica VT 1200S): Pack crushed ice around cutting chamber. Put the metal plate (brain-block is to be glued to this plate) in the chamber to cool down.

2. Transcardial perfusion and brain block
   a. Anesthetize the mouse in a small closely sealed plexiglass box using 0.3 mL isoflurane. For added protection, this can be done in a fume hood or with an anesthesia gas veterinary scavenging system (Patterson Scientific, WI) to avoid exposure of the experimenter to the anesthetics. Once the mouse exhibits a slowed breathing rate and little response to a hindpaw pinch, remove the animal from the box and insert the nose of the mouse into a small tube (diameter 2 cm) containing cotton balls dabbed with a small amount of isoflurane (0.1–0.2 mL) to keep the mouse being anesthetized.

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| Intracellular solution (Cs based) (1×) | Final concentration (mM) | Amount (g) for 25 mL |
|--------------------------------------|--------------------------|---------------------|
| Cs Methanesulfonate                  | 130                      | 0.7410              |
| CsCl                                 | 4                        | 0.0168              |
| EGTA                                 | 2                        | 0.0190              |
| ATP-Mg                               | 4                        | 0.0507              |
| GTP-Na                               | 0.3                      | 0.0039              |
| HEPES                                | 10                       | 0.0596              |
| QX-314 (Cl)                          | 5                        | 0.0374              |

Aliquot stock solution into 600 µL/tube and store at −20°C. It can be used for months. Take one tube (600 µL) for each experiment to thaw at least 30–60 min before patching.
b. Pin the front pawns. Open thoracic chest with operating scissors (large) to expose the heart (Figure 1I). Puncture the left ventricle with the perfusion needle (21 gauge) for perfusing NMDG cutting solution (bubbled with 95% O2 and 5% CO2) through the heart to the whole body and at same time cut the right atrium to drain blood (Figure 1I). When the fluid exiting the heart becomes white (clear), normally after ~10–15 mL of perfusion, stop perfusion and decapitate the mouse with large operating scissors.

c. Cut the front and left side of the skull in the cold cutting solution of the 100 x 15 mm petri dish, flip the skull (Figure 1J), and gently remove the brain from the skull cavity using a flat spatula. Place the brain on the cold metal plate and block the brain according to the brain region of interest. In our case, we record in the prefrontal cortex, and thus we cut the brain coronally into two parts, an anterior and a posterior block at roughly the middle of the brain. Place brain blocks back into the cutting solution.

d. Clean the metal plate. Paint a thin layer of Krazy glue and put the anterior block of the brain containing the PrL on the glue with the cutting surface down (Figure 1K).

e. Place the metal plate with the brain block in the chamber of the vibratome. Pour the rest of the cutting solution into the chamber (bubbling with 95% O2 and 5% CO2) (Figure 1L).

3. Obtaining brain slices

a. Set Vibratome blade front and back cutting limit. Cut brain block cortex surface first at a speed of 0.14 mm/s and amplitude of 1 mm.

b. Coronal slices containing the part of the brain of interest (e.g., the PrL) are collected. The thickness of the brain slices (200–500 μm) is determined by brain location and experimental design. We recorded from 300 μm slices containing the right side PrL. For PrL recording, we collect the right side of the coronal brain sections by physically separating the left and right sides of the brain (left and right are separable at this location due to the absence of the corpus callosum). For other brain areas such as the periaqueductal gray, to mark the sides of the slices, make a cut on the non-recording side of the slice (Figure 1M).

c. One slice is collected every minute at this cutting speed. Slices are first placed in the NMDG beaker at 33.5°C, then transferred to normal extracellular solution at 33.5°C after 11 min. Count the time for each slice. Finally move the beaker with normal extracellular solution from the water bath (33.5°C) to room temperature (50 min timed from the first slice that was collected into the NMDG beaker). After approximately 1hr at room temperature, slices are ready to be used for electrophysiological recording.

△ CRITICAL: 1. Ice-cold cutting solution for transcardial perfusion and slice cutting in vibratome chamber is critical for obtaining viable brain slices. 2. Gentle and skilful handling of brain tissues is also helpful in maintaining healthy brain slices.

Whole-cell patch clamp

○ Timing: 4–5 h

Whole-cell patch clamp is the gold standard for studying neuronal function and synaptic transmission in acute brain slices. Blue LED light (Thorlabs NJ), Multiclamp recording system (700B amplifier and DigiData 1440A digitizer) and software (pClamp electrophysiology data acquisition & analysis suite) (Molecular Devices, CA) are used to induce and record the optically evoked excitatory and inhibitory postsynaptic current (oEPSCs and oIPSCs) (Figure 1N).

4. General preparation

a. Intracellular solution: Pipette solution should be at room temperature for at least 30 min to completely thaw before use. The needle used to fill patch pipette should be clean. A Millex®-GV syringe filter unit (0.22 μm) is used to filter the Intracellular solution.
b. Pull glass pipettes: A glass pipette puller (Zeitz-Instruments Germany) is used to pull and polish glass pipettes (Borosilicate glass, Sutter Instrument). Tip resistance should be around 4–6 MΩ.

c. Perfusion solution: extracellular solution is added to perfusion reservoir and bubbled with 95% O₂ and 5% CO₂. The solution passes through an inline solution heater (Warner Instruments, MA) to keep solutions in patching chamber at 30°C–32°C. Flow rate of perfusion solution is 1.5–2 mL/min.

d. Air table: is used to keep patch rig free from vibration. Turn on before experiment.

5. Whole-cell Voltage Clamp recording of evoked postsynaptic current:
To identify recorded neurons, a Xenon short arc lamp and filter set for tdTomato (Excitation 554/ Emission 581) were used to illuminate and observe tdTomato fluorescence signal in cell bodies of the labeled neurons. We recorded from layer 5 PV GABAergic interneurons of the PrL in brain slices from PV-Cre::tdTomato mice and glutamatergic neurons of the PrL in brain slices from Vglut2-Cre::tdTomato mice. We also recorded from layer 5 putative glutamatergic neurons in paired recordings of both GABAergic and glutamatergic neurons in the same brain slices. To activate axon terminals of the BLA-PrL projections in the PrL, we injected AAV (AAV5-CaMKIIa-hChR2(H134R)-EYFP) in the BLA and activated ChR2-expressing input fibers using LED light.

a. Transfer one brain slice to the recording chamber perfused with normal extracellular solution (30°C–32°C). Place an anchor on top to stabilize the slice.

b. Lower the tip of the optic fiber (200 μm core, 0.22 NA) into the recording solution and aim at the area to be recorded. The tip of the fiber is normally at around 100–300 μm away from recorded cells. The optic fiber is connected to an LED (Thorlabs, NJ) to deliver blue light. It may also be possible to use whole field illumination to activate the opsin.

c. Fill patch pipette with Cs based intracellular solution for voltage clamp. Apply slight positive pressure to pipette before lowering into solution.

Alternatives: Symmetrical Cl⁻ solution (with equal Cl⁻ in intracellular and external) could be used for recording IPSC and potassium based internal solution (gluconate or methanesulfonate salt) could be used for EPSC. Membrane potential is held at −70 mV in both conditions.

d. Turn on pClamp acquisition/analysis software, and Multiclamp 700B commander software.

e. Identify a fluorescent cell with clear contour using a 40× water immersion objective. Bring the electrode tip close to the cell to be patched using DIC mode. Press electrode onto the cell at 1/3 of the cell body using slow speed until an obvious dimple is formed on the cell. Release positive pressure of the electrode and apply a subtle negative pressure or voltage (−70 mV) to help the seal. In Clampex software change from Bath to Patch, a −70 mV voltage is applied to cell automatically. Once a Giga-ohm seal is formed, apply a slight suction to break into whole cell patch configuration.

f. Perform Capacitance and Access resistance compensation. Sampling rate is set at 20 k/s and holding potential at −70 mV. Wait for 7–10 min for the cell to settle down and start to record.

g. Apply one blue light pulse (5–10 ms) every sweep (5 s/per sweep) every 20 s. Increase light power from low to high intensity to find a threshold intensity which can induce an inward (downward) response (oEPSC) when the holding potential is set at −70 mV (GABA_A receptor reversal potential) or an outward (upward) response (oIPSC) if holding potential is set at 0 mV (AMPA receptor reversal potential).

Note: 1. Temperature in the recording chamber (30°C–32°C) and the distance of the stimulating optic fiber to the recorded cells as well as light intensity all affect the amplitude of the postsynaptic response. They should be kept constant throughout the experiments. 2. When recording one of the postsynaptic currents, the holding potential is chosen at the reversal potential of the other postsynaptic current species to minimize interference. This is especially true when no blockers of that current are added to the perfusate. The reversal potential can be estimated using the Nernst equation if only one ion species is permeable for that
channel, or by using the Goldman-Hodgkin-Katz equation if there are more than two permeant ions. Due to the existence of a liquid junction potential between the patch pipette and bath solution (at the interface between intracellular solution and external solution) when the pipette is in bath and offsetting (zeroing) of pipette current during patching, a small voltage error can be introduced into the recording after establishing the whole-cell configuration (where there no longer is a liquid junction potential). This must be considered when interpreting data. In our case, we did not correct junction potentials. Both the IPSC reversal potential (for Cl\(^-\), \(-70\) mV) and EPSC reversal potential (for Na\(^+\), K\(^+\) and Cs, 0 mV) are calculated (\(-69.5\) mV and 2.8 mV, respectively) and we verified this in real experiments. A High intracellular Cl\(^-\) may affect other cellular processes and IPSC decay time course, which should be taken into consideration when using symmetrical Cl\(^-\) intracellular solution to record IPSCs at resting membrane potentials.

6. Determine monosynaptic connections:

To confirm ChR2 induced depolarization of axon terminals and release of neurotransmitters is action potential dependent, we apply the sodium channel blocker TTX in the perfusion solution. To confirm that neurotransmitter release occurs in a monosynaptic (direct) connection, both TTX and 4-AP, a K\(^+\) channel blocker, are perfused to block action potential dependent polysynaptic transmission, to depolarize opsin-expressing axon terminals and to restore the synaptic connection and neurotransmitter release (Cho et al., 2013; Little and Carter, 2012; Petreanu et al., 2009). Here we recorded in both PV GABAergic interneurons (PV-Cre::tdTomato mice) and glutamatergic neurons (Vglut2-Cre::tdTomato mice) to confirm that the BLA inputs form synaptic connections with both neuronal types.

a. Hold the membrane potential at \(-70\) mV. Increase the LED blue light power (by increasing current amplitude and/or pulse duration) to more than threshold intensity (e.g., double threshold intensity) to get a 50–500 pA oEPSC without inducing massive compound electrical activity.

b. When the amplitude of oEPSC stabilizes after a baseline recording (10 min), apply TTX (1 \(\mu\)M) in perfusate. If the oEPSC is action potential dependent, it should gradually decrease and eventually disappear (\(~7–10\) min).

c. Apply the K\(^+\) channel blocker 4-AP (100 \(\mu\)M) in the presence of TTX (1 \(\mu\)M) and record for 10 min. If an oEPSC reappears, presynaptic glutamate release is restored. This indicates that the ChR2 expressing terminals have synaptic contact with the recorded postsynaptic neurons.

d. In separate experiments before application of TTX and/or 4-AP, we recorded optically evoked oEPSCs (at \(-70\) mV) and oIPSCs (at 0 mV) in glutamatergic neurons to analyze the latency of the oEPSC (monosynaptic) and the oIPSC (disynaptic) (see section 7 and section 8), and recorded neuronal response to high frequency (20 Hz) light stimulation to further confirm the monosynaptic nature of the BLA to the PrL glutamatergic neuron input.

**Note:** A monosynaptic connection is characterized by constant short latency, low jitter (jitter: variability of latency, represented by Standard Deviation or SD of the latency) and the ability to follow 20 Hz (light) stimulation without missing time-locked responses (Doyle and Andresen, 2001; Ji et al., 2010). These characteristics distinguish mono- from polysynaptic connections and can be easily tested by using the appropriate pClamp protocol and further verified by above experiments (step 6).

7. Determine relative synaptic strength of BLA input to GABAergic and glutamatergic neurons:

Selective opsin expression in BLA glutamatergic input is achieved using a glutamatergic neuron specific promoter (CaMKII\(\alpha\)) virus. However, variability in virus expression may compound the accurate quantification of synaptic strength (i.e., amplitude of oEPSCs). To overcome this we recorded oEPSCs in GABAergic and glutamatergic pairs (adjacent cells within 100–200 \(\mu\)m that may share similar input and output features and presumably express opsin at similar levels) in the same
brain slices from SNI and Sham mice. LED light is used to achieve more stable mono-wavelength light source than most other available ones such as diode pumped solid state (DPSS) lasers.

a. Record oEPSCs in fluorescent PV GABAergic and non-fluorescent large pyramidal putative glutamatergic neuronal pairs in layer 5 of the PrL of the same brain slices either from SNI or Sham PV-Cre::tdTomato mice (PV-Cre x ai9 crosses). We recorded in PV GABAergic and in glutamatergic neurons in alternate order.

b. Use same LED light power for all cells and brain slices. When the amplitude of the oEPSC stabilizes after a baseline recording (10 min), apply TTX and K+ channel blocker 4-AP to restore presynaptic glutamate release and wait for drugs to take full effects (10 min). Measure the amplitude of the restored oEPSC in both PV GABAergic and glutamatergic neurons from the same brain slices. The ratio of oEPSCs from both neurons is used for analysis to eliminate opsin expression variation among different slices and mice as a confounding factor.

8. Determine E/I balance in layer 5 output neurons of the PrL:

   Like in other cortical regions, layer 5 output neurons of the PrL integrate inputs from excitatory and inhibitory sources before sending output information to other brain centers. E/I balance is important to determine the strength of layer 5 neuronal output. To assess the relative contribution of the BLA direct monosynaptic excitatory and BLA derived disynaptic inhibitory (feedforward inhibition) inputs, we recorded oEPSCs (holding −70 mV) and oIPSCs (holding 0 mV) in the same layer 5 output neurons across brain slices from either SNI and Sham mice.

   a. Record light-evoked oEPSC at a holding potential of −70 mV (IPSC or Cl− reversal potential) for 5–10 min to obtain stabilized recording. Switch to a holding potential of 0 mV (EPSC reversal potential) to record the oIPSC in the same layer 5 output neurons of the PrL for another 5–10 min. We used Vglut2-Cre::tdTomato mice (Vglut2-Cre x Ai9 cross).

   b. Measure the amplitude both oEPSC and oIPSC from the same neurons. The ratio between oEPSC and oIPSC from each neuron recorded in SNI and Sham mice is used for analysis to cancel out opsin expression variation among different slices and mice.

   c. At the end of each recording, apply DNQX (10 μM) and AP-5 (50 μM) to block the oEPSC to confirm glutamate release. The disappearance of the oIPSC after application of oEPSC blockers suggests that the oIPSC is secondary to the oEPSC and that the oIPSC is due to feedforward inhibition.

Note: Bicuculine, TTX or 4-AP were not included in the extracellular solution in experiments described in section “Determine E/I balance in layer 5 output neurons of the PrL”.

△ CRITICAL: 1. An LED light source is recommended due to its stability compared to the most available powerful DPSS laser light. Strong (light) stimulation could cause polysynaptic and complex compound electrical responses, and should be avoided. 2. In experiments determining the relative synaptic strength (Step 7), voltage clamp is performed in a solution containing bicuculine (10 μM), TTX (1 μM), and 4-AP (100 μM) to ensure a monosynaptic connection. To assess input-output relations of synaptic transmission, the amplitude of the oEPSC in re-established synapses can be used for analysis, but for paired pulse ratio assessment, intact action potential dependent release is necessary to examine the function of presynaptic release, and thus the above strategy cannot be used (not shown in this protocol). 3. In experiments for determination of E/I balance (Step 8), neither bicuculine, TTX nor 4-AP are added to solution to keep polysynaptic conduction.

EXPECTED OUTCOMES

The following are the expected results from recording in layer 5 PV GABAergic neurons and glutamatergic neurons of the PrL following selective optogenetic stimulation of BLA glutamatergic input.
Monosynaptic connections

We obtained oEPSC responses in both PV GABAergic neurons and glutamatergic neurons in layer 5 of the PrL following blue light mediated optogenetic stimulation of BLA inputs (Figures 2A and 2B, top). When we applied TTX, the oEPSC disappeared in both neurons (Figures 2A and 2B, middle). When we applied 4-AP in the presence of TTX, the oEPSC returned in both neurons, suggesting that BLA inputs have monosynaptic (glutamatergic) contact with both neurons (Figures 2A and 2B bottom) (Cho et al., 2013; Little and Carter, 2012; Petreanu et al., 2009). From separate experiments without TTX/4-AP in the perfusate, the latency of oEPSCs (delay from the onset of light stimulus to the onset of oEPSC, holding potential at $-70 \text{ mV}$) from glutamatergic neurons was analyzed and compared with the latency of olPSCs (holding potential at $0 \text{ mV}$) in glutamatergic neurons. $p < 0.0001$ (Unpaired t test with Welch’s correction for unequal variances, F test, $p = 0.0491$). (E) Latencies of oEPSCs and olPSCs were binned into 1 ms bins. (F) oEPSC follows 20 Hz light stimulation. Note: Blue bars indicate blue light stimulation in panel (A, B, C, and F).

Relative synaptic strength of the BLA input to GABAergic and glutamatergic neurons in SNI and sham mice

Restored oEPSCs were recorded in both PV GABAergic and glutamatergic neurons in the same brain slices from SNI and Sham mice (Figure 3A). When the ratio of the amplitude of restored oEPSCs in PV GABAergic neurons and glutamatergic neurons from the same slices are compared between SNI and Sham mice (Figure 3B), we found that nerve injury increased this ratio. This result suggests that the efficacy of GABAergic synapses is enhanced in SNI mice. Figure 3B is reprinted with permission from (Huang et al., 2019).
E/I balance in layer 5 output neurons of the PrL

We performed recordings of oEPSCs (holding potential at $-70$ mV, Figure 4A lower) and oIPSCs (holding potential at 0 mV, Figure 4A upper) in glutamatergic neurons of Vglut2 cre x ai9 mice. At the end of the experiments, application of DNQX and AP-5 blocked both oEPSC and oIPSC in SNI and Sham mice were used for data analysis in panel B. Blue bars indicate blue light stimulation.

When the ratio between the oIPSC and the oEPSC from each of the output neurons in layer 5 of the PrL was compared between SNI and Sham mice (Figure 4C), we found that neuropathic injury shifted the E/I balance in output neurons toward a more inhibitory state. Figure 4C is reprinted from Figure 1, (Huang et al., 2019) with permission.

QUANTIFICATION AND STATISTICAL ANALYSIS

1. We alternate SNI and Sham mice for each day’s electrophysiological recording. We sample 1–2 cells from each brain slice. If pharmacology is involved we only sample one cell in each slice. We use 2–3 slices from each animal depending on experiments and location of the brain area of interest, but in total we only sample a maximum of 3 cells from each animal/day. For each group of data we sample from at least 4–5 mice.

2. In all recordings, any cells with the following properties were excluded: a leak current more than $-150$ pA, or a series resistance more than 20 MΩ after series resistance compensation, or with a 20% increase at the end of the experiments.

3. Data are represented by individual data points overlapped with group means (bar) and standard error of means. We compared the group means using two tailed, unpaired Student’s $t$-test. *P<0.05.

LIMITATIONS

Whole-cell patch clamp electrophysiology is the gold standard for studying neuronal functions and synaptic transmission. It becomes a powerful tool to study neuronal and circuit mechanism of neurological disease when combined with optogenetics. However, the synaptic strength or synaptic input output relations cannot be easily assessed and compared among animals by quantifying the amplitude of light evoked postsynaptic current in the traditional way due to variable opsin expression. Experiments have to be carefully designed and controlled. For example, we compared recorded parameters from one type of neuron or between two or more neurons in the same brain slices in this...
protocol (Huang et al., 2019, 2021). Furthermore, any data on synaptic transmission derived from TTX and 4-AP rescue experiments must be interpreted carefully. For example, a lack of detection of monosynaptic connections could simply mean there is not enough opsin expression in axon terminals even though there exist monosynaptic connections. Furthermore, the 4-AP mediated, opsin induced neurotransmitter release is not physiological. It is therefore important to not draw detailed conclusions on natural synaptic release dynamics because of Ca++ permeability of opsin channels (Lerner et al., 2016). Lastly any cellular and circuit level mechanism derived from experiments using this approach has to be tested and verified at the behavioral level in a whole animal system.

TROUBLESHOOTING

Problem 1
(Step: whole-cell patch clamp)

Whole-cell patch clamp electrophysiology is technically demanding and sometimes low throughput (steps 1 - 5).

Potential solution
Viable brain slices are the key to successful whole-cell patch experiments. It is crucial that all surgical tools are free of harmful chemicals such as fixatives. The cutting blade has to be washed thoroughly with interfering-residue free detergent (e.g., Alcojet powdered detergent, Alconox) and rinsed with alcohol and finally with deionized H2O. All solutions should have correct components with correct pH and osmolarity. The handling of brain tissue should be gentle and skillful. The whole process of brain
slice cutting should be short and in ice cold conditions to increase tissue tolerance to handling. Another factor that affects the yield of patch experiments is the patching process (see problem 2).

Problem 2
(Step: whole-cell patch clamp)

In certain conditions, it can be difficult to form a giga-ohm seal (step 5).

Potential solution
In most cases the possible reason is a clogged patch pipette electrode. One should always see a continuous flow of intracellular solution out of the pipette tip under a microscope after application of a slight positive pressure to the patch pipette. The outflowing solution keeps the tip from debris and blows away tissue particles from the cell surface to help form a clean giga-ohm seal. To prevent patch pipette clogging, the intracellular solution has to be filtered using 0.22 μm filter. The intracellular solution has to be fully thawed at room temperature for at least 30 min to eliminate any precipitations. If a successful giga-ohm seal and whole cell configuration is achieved, but the cell is quickly lost due to large current, this may be caused by a faulty intracellular solution (e.g., incorrect pH). Intracellular osmolality (mOsm/kg) also affects effective giga-ohm seal. Normally equal or slightly lower intracellular osmolality (compared to extracellular solution) is helpful in forming a giga-ohm seal. High intracellular osmolality (e.g., more than 295 mOsm/kg) increases the difficulty to make a seal and it is easy to cause cell swelling and loss of the giga-seal. Therefore, low osmolality (hypotonic) intracellular solution is recommended. The composition of extracellular solution also affects the giga-ohm seal. Accidentally omitted divalent cations (e.g., Ca++) in extracellular solution reduces the adhesion force between glass pipette and cell membrane and decrease seal resistance and makes a giga-seal almost impossible (Priel et al., 2007). Occasionally extracellular solution can be filtered to remove debris or particles that may affect giga-seal to improve patch clamp performance. Finally the size and shape of the tip of the patch pipette also affects patching. A larger than ideal sized tip (e.g., 1–4 MΩ) is difficult to use in patch clamp experiments. A small sized tip (e.g., 6–8 MΩ) is easier for patching, but this makes it harder to break into whole cell mode. A polished, ideal sized (e.g., 4–6 MΩ) pipette is helpful in forming a giga-ohm seal.

Problem 3
(Step: virus injections)

There is little or no virus expression in injected brain location (step 8).

Potential solution
The possible reasons that cause unexpected loss of expression after choosing the right virus dosage (titers and volume) may be multiple fold. A blocked needle can be avoided by cleaning the needle tips using sterile saline and slightly pressing the Empty button on Nanojector to expel air or any debris on needle tip before lowering the virus-loaded needle into the brain location of interest. Back flow of injected solution from injecting track back to surface of the brain could be another cause of loss of expression. This could be caused by a shallow injection site, broken needle or re-injection after failures of the first injection or damaged brain tissue that cannot hold the injected solution. Using low injection speed or volume, careful surgical handling (e.g., drilling, removing dura), and avoiding re-injection could potentially solve these problems. It is also possible that the virus may diffuse to other brain locations through open space, cavities or loose tissue in the brain. Correct targeting during injection could potentially solve this. Mistargeting or injection in the wrong locations is almost always caused by variation of the landmarks on the skull (bregma and lambda) that leads to mis-judgment of the location of interest. Multiple practices of injection before actual virus injection using colored solution (e.g., fluorescent microspheres from Lumafluor Inc., the fluorescent color is readily identifiable under the dissection microscope) is a good way to determine a personalized
coordinate for each brain location of interest and to establish strict criteria to follow. Multiple prac-
tice runs and careful surgical procedure can help achieve near perfect targeting.

**Problem 4**
(Step: neuropathic model - spared nerve injury)

Why did we fail to obtain an SNI phenotype (step 11)?

**Potential solution**
SNI surgery is a well-defined procedure involving ligation and transection of common peroneal and
tibial nerves. One probable reason for failing to obtain a behavioral phenotype is ligation and tran-
section of the wrong nerves or damaged/stretched sural nerve. The location of the initial incision on
the skin of the left leg and blunt dissection along muscle bundles is key to correct location/identifi-
cation of the nerves underneath the biceps femoris. A small artery that runs perpendicularly to the
nerves can be used as a landmark at the distal end of the nerve segment that is to be ligated and
transected. Always use a dissection microscope to ensure that the sural nerve is not picked up for
ligation and transection. If there is still doubt on the identity of the nerves, it is always a good
idea to use this mouse a Sham. Another possible reason of the failure to obtain an SNI phenotype
is likely a wrong testing location on the ipsilateral hind paw. Note that the area innervated by the
sural nerve is a narrow strip of skin on the lateral plantar side of the glabrous skin (without hairs). Oc-
casionally some mice do not develop an SNI phenotype without obvious reasons. Therefore, pheno-
type testing is necessary two weeks post-operation on all operated mice to exclude those mice from
further experiments.

**Problem 5**
(Step: whole-cell patch clamp)

Why is it difficult to obtain consistent optically evoked neuronal responses and how can one use
selective optogenetic targeting to compare neuronal response in different conditions (steps 6, 7
and 8)?

**Potential solution**
Location and cell type specific, input or output defined neuronal targeting and manipulation is the
core feature of optogenetics used in circuit mapping and behavioral studies. Due to variable
expression of viral vectors it is difficult to quantify neuronal responses mediated by the targeted
opsin proteins in different conditions. To obtain reliable virus expression, different viral serotypes,
concentrations and dosage must be carefully tested and verified. Overexpression of viral vectors
may lead to cell toxicity or cell death and compound quantification of neuronal response and is to
be avoided. A stable light source such as an LED light is better suited to activate opsins than the
most available DPSS lasers for quantification studies. Any comparison of neuronal responses medi-
ated by opsin expression must be carefully controlled, if possible, this comparison should be made
among different parameters from the same cells, or among different cells in the same brain slices
that are in close vicinity to one another and display similar levels of viral expression.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be ful-
filled by the lead contact. Gerald W. Zamponi (zamponi@ucalgary.ca)

**Materials availability**
This study did not generate new unique reagents.
Data and code availability
This study did not generate/analyze [datasets/code].

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AUTHOR CONTRIBUTIONS
Conceptualization, Z.Z. and G.W.Z.; investigation, Z.Z. and G.W.Z.; writing – original draft, Z.Z.; writing – review & editing, Z.Z. and G.W.Z.; funding acquisition, G.W.Z.; supervision, G.W.Z.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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