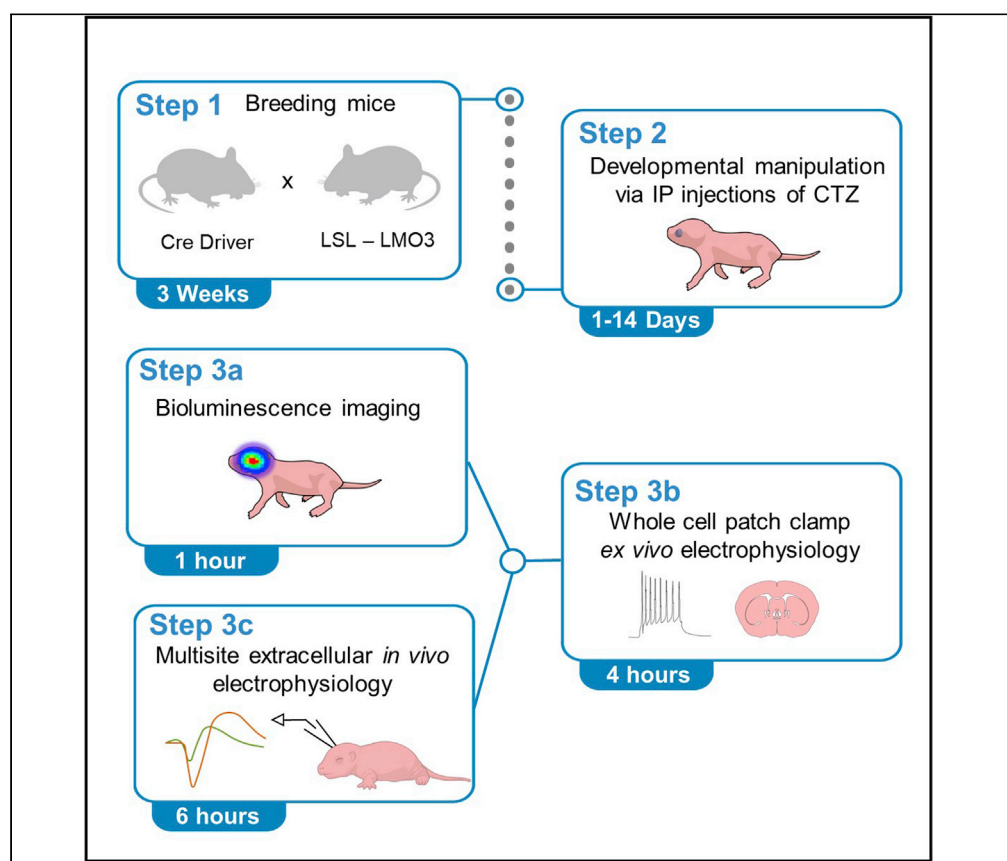


Protocol

Bioluminescent optogenetic (BL-OG) activation of neurons during mouse postnatal brain development



Bioluminescent optogenetics (BL-OG) allows activation of photosensory proteins, such as opsins, by either fiber optics or by administering a luciferin. BL-OG thus confers both optogenetic and chemogenetic access within the same genetically targeted neuron. This bimodality offers a powerful approach for non-invasive chemogenetic manipulation of neural activity during brain development and adult behaviors with standard optogenetic spatiotemporal precision. We detail protocols for bioluminescent stimulation of neurons in postnatally developing brain and its validation through bioluminescence imaging and electrophysiological recording in mice.

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Highlights

BL-OG for cell-specific stimulation of developing neural circuits in postnatal mice

Protocols for validating BL-OG stimulation with imaging and electrophysiology

Tips on administering luciferin in pups and validation with various imaging modalities

Tips for customizing strategies to record from a single neuron and circuit using BL-OG

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Protocol

Bioluminescent optogenetic (BL-OG) activation of neurons during mouse postnatal brain development

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SUMMARY

Bioluminescent optogenetics (BL-OG) allows activation of photosensory proteins, such as opsins, by either fiber optics or by administering a luciferin. BL-OG thus confers both optogenetic and chemogenetic access within the same genetically targeted neuron. This bimodality offers a powerful approach for non-invasive chemogenetic manipulation of neural activity during brain development and adult behaviors with standard optogenetic spatiotemporal precision. We detail protocols for bioluminescent stimulation of neurons in postnatally developing brain and its validation through bioluminescence imaging and electrophysiological recording in mice.

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

BEFORE YOU BEGIN

There are several neuromodulation methods available, including sono- and magneto- in addition to opto- and chemogenetic methods. However, not all these methods are suitable for modulating neurons in developing postnatal mouse pups, and none affords chemogenetic stimulation and standard optogenetic interrogation of the same neurons in the same animal throughout its lifespan.

Bioluminescent optogenetics (BL-OG) utilizes light emitted from a luciferase when oxidizing its luciferin, a small molecule, to activate optogenetic elements. Generally, the luciferase is tethered to the opsin in a luminopsin, LMO (Berglund et al., 2013). LMO3 is a fusion protein of a mutant variant of *Gaussia* luciferase, sbGLuc, tethered via a 15 amino acid linker to *Volvox* Channelrhodopsin 1 (VChR1), followed by a fluorescent reporter protein (EYFP) (Berglund et al., 2016). VChR1 in LMO3 can be activated by a physical light source (arc lamp, LED) as well as by administration of the luciferin coelenterazine (CTZ). Taking advantage of this bimodality, chemogenetic stimulation via intraperitoneal injection of the luciferin is used to activate genetically defined neurons during specific time windows in the postnatal period of brain development. Standard optogenetic interrogation in adults enables to study the consequences of developmental hyperactivity on neural connectivity. This provides a powerful approach for studying the role of neural activity during brain development in shaping the layout of circuitry that forms the basis for adult behavior and its underlying circuit wiring.



The protocol below describes the specific steps for BL-OG stimulation of developing neural populations and its validation in mice with Cre-dependent expression of LMO3 in Emx1 pyramidal cells. Initial validation should be performed for each new target population before carrying out the neural stimulation studies during brain development.

Generating LMO expressing pups targeting cell-specific developing neonatal circuits

⌚ Timing: weeks

1. Obtain and cross stock mouse lines.
 - a. A conditional, Cre-dependent mouse line for expression of LMO3 (LSL-LMO3) is available from The Jackson Laboratory.
 - b. Cre driver lines are available from The Jackson Laboratory and other sources.
 - i. In the specific example described in the protocols below we used the Emx1-Cre line.
 - ii. We also used Pvalb-, Dlx5/6-, and DAT-Cre driver lines for crossing with LSL-LMO3 mice to developmentally stimulate interneurons and dopaminergic neurons, respectively.
 - iii. Generally, the choice of Cre driver line is determined by the specific research question and the specific population to be developmentally stimulated.
 - c. Cross Cre driver line with LSL-LMO3 line to get LMO3 expressing and control pups.
 - i. For initial exploration of the experimental system it is recommended to cross heterozygous breeders for both partners. This generates litters with all appropriate controls: wildtype, Cre only, LSL-LMO3 only, and Cre-LMO3.
 - ii. For more targeted applications cross homozygotes of one strain with heterozygotes of the other, generating 1:1 expressing and non-expressing pups in each litter.
 - iii. For specific applications, cross homozygotes of both strains to generate entire litters of Cre-LMO3 pups.
2. Genotyping of litters.
 - a. Litters obtained from heterozygous parents are genotyped using PCR techniques on toe clippings. Depending on the time window chosen for developmental stimulation, genotyping occurs during or after chemogenetic stimulation.
 - b. Pups bred from both homozygous parents do not need to be genotyped.

Alternatives: Alternative methods of genetically targeting neural populations for postnatal stimulation are *in utero* electroporation or injection of AAV, either Cre dependent versions or vectors with specific promoters.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Isoflurane	Piramal Critical Care	NDC 66794-013-25
NaCl	Sigma	S7653; CAS: 7647-14-5
KCl	Sigma	P5405; CAS: 7447-40-7
NaH ₂ PO ₄	Sigma	71507; CAS: 10049-21-5
NaHCO ₃	Sigma	S5761; CAS: 144-55-8
D-(+)-Glucose	Sigma	G8270; CAS: 50-99-7
CaCl ₂	Sigma	C8106; CAS: 10035-04-8
MgCl ₂ hexahydrate	Sigma	M2670; CAS: 7791-18-6
NMDG	Sigma	M2004; CAS: 6284-40-8
HEPES	Sigma	H3375; CAS: 7365-45-9
Thiourea	Sigma	T8656; CAS: 62-56-6
Na-ascorbate	Sigma	A7631; CAS: 134-03-2

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Na-pyruvate	Sigma	P2256; CAS: 113-24-6
MgSO ₄ heptahydrate	Sigma	230391; CAS: 10034-99-8
K ⁺ Gluconate	Sigma	P1847; CAS: 299-27-4
Phosphocreatine disodium salt hydrate	Sigma	P7936; CAS: 19333-65-4
Mg-ATP	Sigma	A9187; CAS: 74804-12-9
Na ₂ -GTP	Sigma	G8877; CAS: 36051-31-7
KOH	Sigma	P5958; CAS: 1310-58-3
D-AP5	Tocris	0106; CAS: 79055-68-8
CNQX	Tocris	0190; CAS: 115066-14-3
Picrotoxin	Tocris	1128; CAS: 124-87-8
Tetrodotoxin citrate	Tocris	1069; CAS: 18660-81-6
Cs-methanesulphonate	Sigma	C1426; CAS: 2550-61-0
EGTA	Sigma	E3889; CAS: 67-42-5
QX-314	Sigma	552233; CAS: 21306-56-9
Biocytin	Sigma	B4261; CAS: 576-19-2
Urethane	Sigma	U2500; CAS: 51-79-6
Tergazyme	Alconox	1304
CTZ (water-soluble native coelenterazine)	Prolume Inc. (NanoLight)	3031
Vehicle (water-soluble carrier without CTZ)	Prolume Inc. (NanoLight)	3031C
CTZ Native	Prolume Inc. (NanoLight)	303
NanoFuel Solvent	Prolume Inc. (NanoLight)	399
Experimental models: organisms/strains		
Mouse: LSL-LMO3: B6N.129-Gt(ROSA)26Sor _{tm} 4.1 (CAG-sbGLuc/COP3/EYFP)Ute/J	The Jackson Laboratory	JAX: 034853
Mouse: Emx1-Cre: B6.129S2-Emx1 _{tm} 1(cre)Krl/J	The Jackson Laboratory	JAX: 005628
Mouse: Other Cre driver lines	The Jackson Laboratory	https://www.jax.org/research-and-faculty/resources/cre-repository
Recombinant DNA		
pAAV-hSyn-LMO3 (sbGLuc-VChr1-EYFP)	Addgene	Plasmid #114099
Other		
C&B Metabond (Quick Adhesive Cement System) Kit	Parkell Inc.	SKU: S380
"C" Universal TBB Catalyst	Parkell Inc.	SKU: S371
Black Nylon, Polyurethane-Coated Fabric (1.5 m X 2.7 m) X 0.12 mm (thick)	Thorlabs	BK-5
Yakamoz Mini DIY Hand Drill Battery Powered Rechargeable Cordless Micro Electric Rotary Drill	Yakamoz	N/A
Model 942 Dual Small Animal Stereotaxic Instrument with Model 960 Electrode Manipulator with A.P. Slide Assembly and Model 940-B Linear Scale Assembly with Digital Display Readout Console, 10 micron resolution	Kopf Instruments	Model 942 Model 960 Model 940-B
A1x32 Poly2-5mm-50s-177-OA32LP	NeuroNexus Technologies	N/A
CerePlex-μ digital amplifier	Blackrock Microsystems	N/A
CerePlex Direct data acquisition box	Blackrock Microsystems	N/A
Gorilla All Purpose Epoxy Putty Stick	Hardware stores	N/A
Upright Microscope	Olympus	BX51WI
Multiclamp 700b amplifier	Molecular Devices	N/A
Digidata 1440 digitizer	Molecular Devices	N/A
Carbogen gas (95% O ₂ , 5% CO ₂)	Local supplier	N/A
Digital Imaging System	LI-COR	Odyssey Fc
Stereomicroscope	Leica	MZ10F
High-speed, ultra-sensitive research grade microscopy camera	Teledyne Lumenera	INFINITY3S-1UR

MATERIALS AND EQUIPMENT

- 1000 mL Recording ACSF (artificial cerebrospinal fluid) 1× (storage: 4°C, use within a week)

Reagent	Final concentration (mM)	Amount (mg)
NaCl	121	7013
NaH ₂ PO ₄	1	138
NaHCO ₃	26	2185
KCl	2.8	209
D-Glucose	15	2700
~ 285–290 mOsm (without Ca/Mg), pH 7.3–7.4 with Carbogen gas		

- 100 mL CaCl₂ / MgCl₂ stock solution (200 mM) (storage: 21°C, use within a month)

Reagent	Stock concentration (mM)	Amount (g)	Final concentration in recording ACSF (mM)
CaCl ₂	200	2.94	2
MgCl ₂ Hexahydrate	200	4.07	2

- 2000 mL Protective Recovery Cutting (PRC) Buffer 1×, pH: 7.3–7.4 (with Carbogen), ~310 mOsm (storage: 4°C, use within a week)

Reagent	Final concentration (mM)	Amount
NMDG (N-Methyl-D-glucamine diatrizoate)	92	35.92 g
KCl	2.5	372.8 mg
NaH ₂ PO ₄	1.25	345 mg
NaHCO ₃	30	5.04 g
HEPES	20	9.54 g
Thiourea	2	306 mg
Na-ascorbate	5	1.98 g
Na-pyruvate	3	660 mg
CaCl ₂	0.5	147 mg
MgSO ₄	10	4.92 g
D-Glucose	25	9 g
Adjust pH with 6N HCl		

- 1000 mL Protective Recovery Holding (PRH) Buffer 1×, pH: 7.3–7.4 (with Carbogen), ~310 mOsm (storage: 4°C, use within a week)

Reagent	Final concentration (mM)	Amount
NaCl	92	5.38 g
KCl	2.5	186.4 mg
NaH ₂ PO ₄	1.25	172.5 mg
NaHCO ₃	30	2.52 g
HEPES	20	4.77 g
Thiourea	2	153 mg
Na-ascorbate	5	991 mg
Na-pyruvate	3	330 mg
CaCl ₂	2	294 mg
MgSO ₄	2	493 mg
D-Glucose	25	4.5 g
Adjust pH with 1N NaOH		

- 10 mL Potassium gluconate intracellular solution, pH: 7.25, ~300 mOsm (Filter and store at -20°C as one time use aliquots, use the prep within a month)

Reagent	Final concentration (mM)	Amount (mg)
K ⁺ Gluconate	130	304.5
KCl	10	7.5
HEPES	15	35.75
Phosphocreatine-disodium salt hydrate	5	12.75
Mg-ATP	4	20.3
Na ₂ -GTP	0.3	1.6
Adjust pH with 1N KOH		

- Synaptic blockers (store at -20°C as one time use aliquots, use the prep within a month)

Reagent	Stock concentration (mM)	Amount (mg)	Final concentration in recording ACSF (μM)
1 mL 200 mM Picrotoxin in DMSO (50 μL aliquots)	200	120.5	100
2 mL 15 mM CNQX in DMSO (100 μL aliquots)	15	7	15
1 mL 100 mM D-AP5 in dH ₂ O (50 μL aliquots)	100	20	50

△ CRITICAL: All of the above synaptic blockers (Picrotoxin, CNQX and D-AP5) are toxic and should be handled as per the manufacturer provided safety guidelines.

- Urethane stock solution: Weight 134 mg of Urethane and dissolve in 1 mL of PBS/Saline.

△ CRITICAL: Urethane is toxic and should be handled as per the manufacturer provided safety guidelines.

Note: Urethane stock solution should be prepared fresh for a single day use.

STEP-BY-STEP METHOD DETAILS

Neural stimulation during early postnatal mouse brain development

⌚ Timing: 5–15 days

This step describes the preparation of luciferin solution and injection into mouse pups. Depending on the research question and experimental design, injections will be once per day or more often, over as many days as postnatal neural stimulation is desired. It is recommended to inject all pups of a litter with either CTZ or vehicle.

1. Prepare CTZ or vehicle solutions.
 - a. Take out a vial of water-soluble CTZ or vehicle from the -80°C freezer and let warm to 21°C . Keep protected from light.
 - b. Per 500 μg vial add 500 μL sterile water, using either a syringe or by opening the vial and adding water with a pipette, then putting the rubber stopper back on the glass vial.
 - c. Incubate reconstituted glass vial in 55°C waterbath for a few minutes to completely dissolve powder. Dissolving occurs without formation of bubbles (compared to dissolving by vortexing).

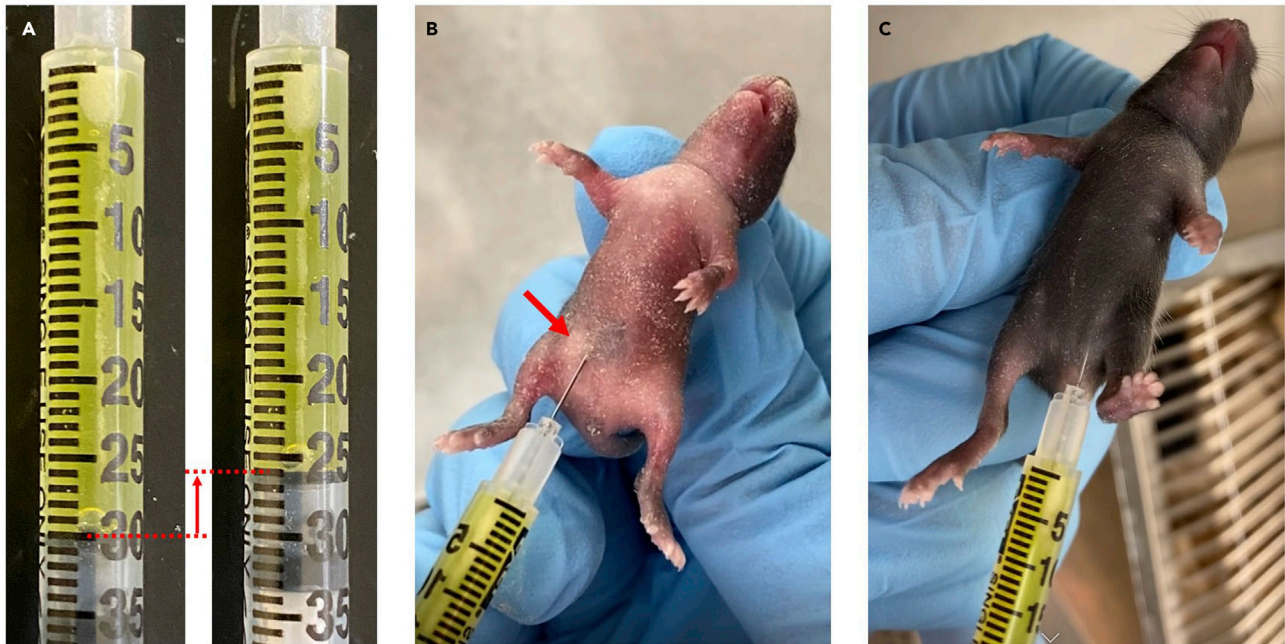


Figure 1. Intraperitoneal injection of CTZ

(A) CTZ solution is pulled up into a 0.5 mL Insulin syringe and desired volume (here: 4 units = 40 μ L, red arrow) is expelled ip.
(B) Postnatal day 5 pup injected ip with CTZ. Spread of expelled CTZ solution can be observed through the skin (red arrow).
(C) Postnatal day 11 pup injected ip with CTZ.

d. Transfer solution into black microcentrifuge tube. Rinse walls of the glass vial to retrieve all CTZ. Remove amount of solution needed for the day with 0.5 mL Insulin syringe. Store leftover at 4°C for use the next day. Loss of activity is <10% after 24 h at 21°C. Discard after 24 h. Do not freeze!

2. Inject postnatal pups.

- a. Pull up CTZ or vehicle solution into 0.5 mL Insulin syringe with a 31 gauge needle as needed for injecting a litter; remove any bubbles. Take black tube with CTZ to animal facility or keep syringe containing CTZ covered in aluminum foil when transporting to animal facility. The dose (CTZ) or volume equivalent (vehicle) is 10 μ g/g or 10 μ L/g body weight (Figure 1A).
 - i. Pups at postnatal days 4 and 5 receive 30 μ L (3 units). Day of birth is postnatal day 1.
 - ii. Pups at postnatal days 6 to 14 receive 40 μ L (4 units).
- b. Inject pups intraperitoneally (ip) with volumes specified in step 2a. Use separate syringes if more than one litter is being injected.
 - i. Pups aged postnatal days 1 to 5 are injected in a lower quadrant (Figure 1B). The needle can usually be visualized through the skin. Care needs to be taken to avoid piercing inner organs, including the stomach.
 - ii. Pups aged postnatal days 6 and above are injected in a lower quadrant (Figure 1C). As due to skin coloration and fur growth the needle can no longer be visualized when injecting, draw plunger back a little before expelling the desired volume into the body cavity.

△ **CRITICAL:** It is best to take out the entire litter, inject all pups, and then return the injected neonates as a group (entire litter) to minimize disturbing the dam.

Validate neural stimulation by bioluminescence imaging in developing mice

⌚ Timing: 1 h

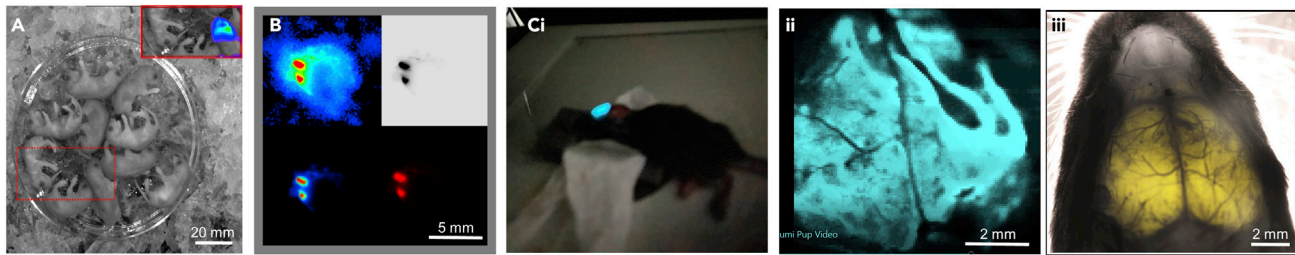


Figure 2. Bioluminescence imaging of pups

(A) IVIS imaging of a litter of Emx1-Cre $\times$ LSL-LMO3 heterozygotes on postnatal day 4. Mice were injected ip with CTZ, anesthetized on ice, and imaged. One pup was Emx1-LMO3 and showed bioluminescence (see red marked inset).

(B) Different displays of image taken after CTZ administration to an adult mouse injected bilaterally into the motor cortex with AAV-hSyn-LMO3. Images taken on a Licor Odyssey FC (designated gel and Western blot imager).

(C) Bioluminescence images of a P14 Emx1-LMO3 pup. (i) Raw picture taken with an iPhone. Urethane-anesthetized pup is placed under stereomicroscope. (ii) Pseudocolored image of bioluminescence emission of same pup as in (i) taken through a stereomicroscope (Leica MZ10F) with an Infinity 3S-1UR camera. (iii) Overlay of brightfield and EYFP fluorescence image taken through stereomicroscope from littermate of pup shown in (i) and (ii). EYFP is strongly visible over the entire cortex.

This step describes one of three ways for validating that CTZ injections lead to bioluminescent light emission in targeted circuits in the brain. The protocol describes bioluminescent imaging using the IVIS (Perkin Elmer In Vivo Imaging System). If this system is not available, alternative approaches for live imaging are described.

3. Prepare CTZ or vehicle solution and inject pups immediately before imaging as described in steps 1 and 2 above.
4. For imaging using IVIS follow manufacturer's protocols, using the Living Image Software. Images are taken in 5-min bins over a 20- to 30-min period, either by setting up an imaging sequence or by taking serial individual images. Bioluminescence emission is expected within 10–15 min after CTZ injection and will decline over the following hour. Bioluminescence emission is expected in LMO expressing, but not in non-expressing, pups injected with CTZ, and in neither group when injected with vehicle.
 - a. Neonates up to 5 days of age are anesthetized by hypothermia during imaging.
 - i. Inject CTZ ip, then place pups on crushed ice in a dish. Pups should be kept on ice no longer than 30 min after induction of anesthesia.
 - ii. As soon as pups stop moving, transfer dish with pups into IVIS chamber and start imaging. Depending on how long the imaging lasts, pups may have to be transferred to a fresh dish with ice.
 - iii. Once imaging is completed, pups are kept on a heating pad until pink and moving.
 - b. Neonates older than 5 days of age are imaged under isoflurane anesthesia (oxygen 2 L/min; isoflurane 1–2%), using the nose cones inside the IVIS chamber.
 - i. Inject CTZ ip, then place pups in induction chamber.
 - ii. Collect images in 5-min bins.

Troubleshooting 1

Alternatives: If an IVIS system is not available, other options are imaging systems equipped with a cooled CCD camera or a sCMOS camera. Image documenting systems for Western blots, for example, are designed for chemiluminescence, such as Fuji Film LAS-3000 or Li-Cor Odyssey Fc (Figure 2B). Depending on the anatomical location, distribution, and level of expression of the LMO bioluminescence can be captured with a cell phone camera or through a stereomicroscope (Figure 2C).

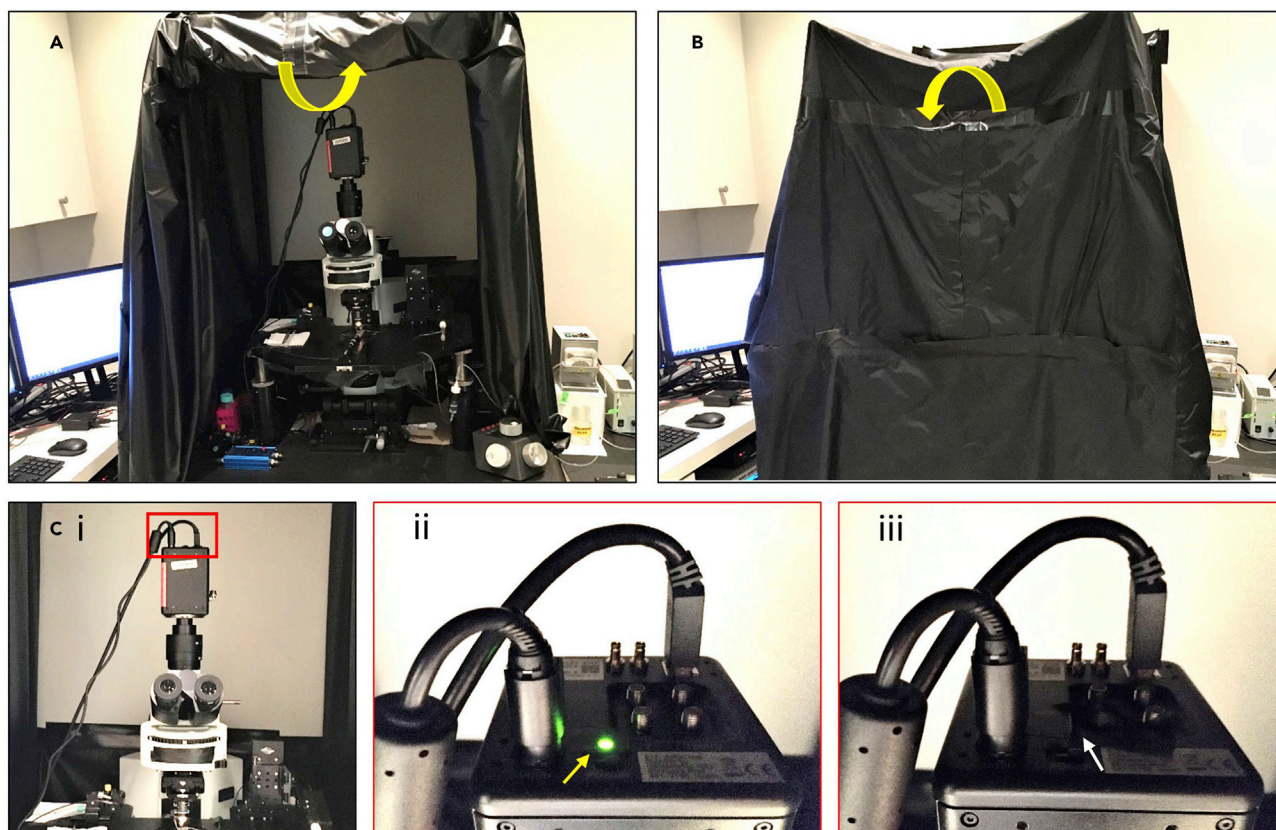


Figure 3. Making the patch rig light-tight

(A) Set-up showing the patch rig covered by the flexible black drape with the front side open and rolled up.

(B) Same set-up as in A, now with the front black drape rolled down to be used for bioluminescence imaging.

(C) Example of a source of light present inside the covered patch rig, such as the LED indicator on the camera (red square, i), zoomed in showing the green LED indicator (yellow arrow, ii), which is covered with a black tape (white arrow, iii).

Validate neural stimulation by patch clamp recordings in slices

⌚ Timing: 4 h

This step describes the use of patch clamp recordings from brain slices for simultaneous assessment of light emission and neural stimulation in the presence of CTZ. The protocol describes critical modifications to standard electrophysiology rigs, details the procedures for preparation and application of the luciferin, and specifies procedures for validation of bioluminescence-elicited neural activation.

5. Make the patch rig light tight.
 - a. Create a light tight compartment around the recording rig (Figure 3) using black drapes (e.g., THOR LABS). Access is provided by keeping the flexible black drape in the front rolled-up (Figure 3A). The front drape is dropped at the time of CTZ treatment and bioluminescence imaging while recording from the patched cell (Figure 3B).
 - b. Cover all light sources (e.g., LED indicators on the camera) present inside the light tight compartment (Figure 3C).
6. Prepare CTZ stocks.
 - a. For ex-vivo applications there is no need to use the more expensive water-soluble *in vivo* preparation of CTZ (#3031). Prepare 50 mM stocks by dissolving 500 μ g lyophilized CTZ (#303) in

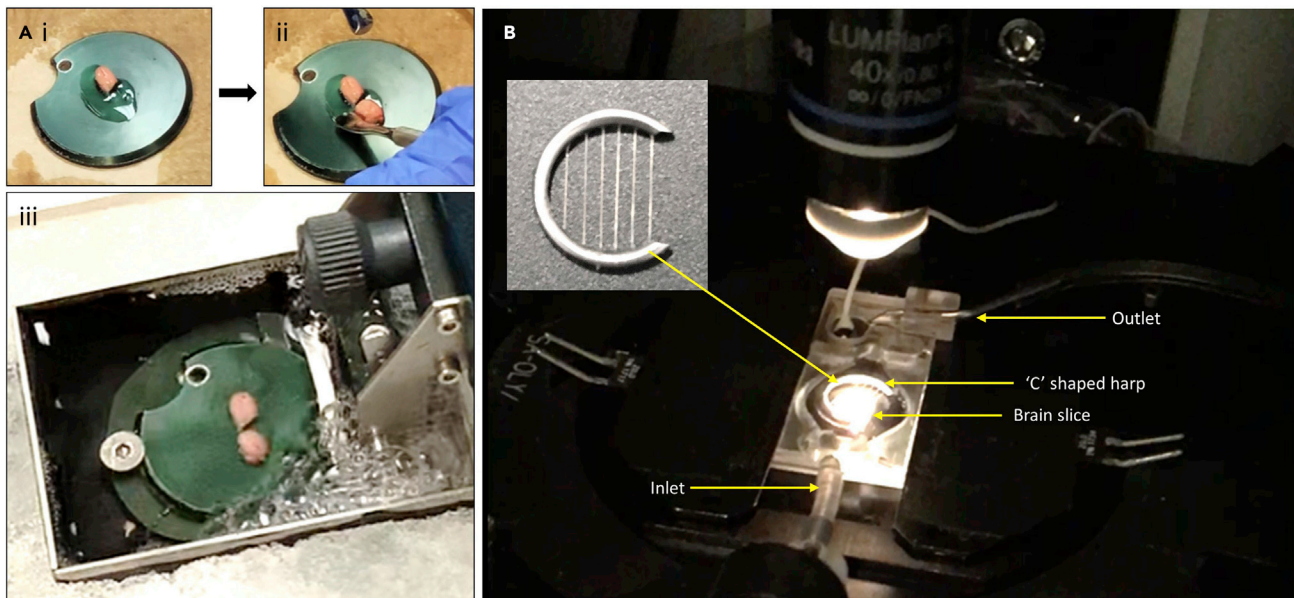


Figure 4. Preparation of brain slices from neonatal pups and mounting the slice into the recording chamber

(A) Vibratome platform with the glue to accommodate up to 2 pup brains (i & ii) for simultaneous slicing using the Vibratome (iii). (B) Patch recording chamber connected with the inlet and outlet tubing for ACSF circulation containing a slice, weighed down by a 'C' shaped meshed harp (see close-up in inset on top left).

- 25 μ L of NanoFuel Solvent. Make sure to dissolve all CTZ along the walls of the vial by pipetting or vortexing. Protect vial from direct light.
- b. Prepare 5 μ L aliquots in 0.5 mL black microcentrifuge tubes and store at -80°C for future use. CTZ dissolved in NanoFuel Solvent does not freeze at -80°C . Aliquots can be removed from and returned to the freezer several times for making working solutions as long as exposure to light and 21°C is kept to a minimum.
7. Prepare brain slices from neonatal pups.
 - a. Anaesthetize the mouse pup via isoflurane inhalation and remove the head with sharp scissors. Isolate the brain immediately and place it in ice-cold PRC buffer bubbled with carbogen gas. Total time taken for the brain isolation and transfer to the PRC buffer should not exceed 60 s.
 - b. Prepare 300 μm thick slices (coronal/horizontal/sagittal, based on the experimental design) from the desired brain region using a Vibratome (e.g., VT1000 s, Leica) and ice-cold PRC buffer continuously bubbled with Carbogen (gas mixture of 95% O_2 and 5% CO_2). Up to 2 pup brains can be glued on the Vibratome platform for simultaneous sectioning (Figure 4A).
 - c. Store the slices in PRH buffer at 25°C for 1 h recovery before transferring to recording chamber.
8. Perform patch clamp recordings from LMO expressing cells.
 - a. Transfer a brain slice to the recording chamber perfused with carbogenated ACSF (perfusion rate: 3 mL/min). The heater temperature for the buffer inlet is set at 39°C to keep the buffer temperature in the recording chamber around 35°C . Place a C-shaped mesh harp over the slice to fix its position in the desired orientation (Figure 4B).
 - b. Under 480 nm excitation light identify a fluorescent (EYFP from LMO3) cell and patch using an established set-up (e.g., Multiclamp 700b amplifier and Digidata 1440 digitizer together with the pClamp recording software (Molecular Devices)) (Figure 5).
 - c. Stimulate the patched LMO expressing cell with light of the appropriate wavelength from a physical source by delivering light through the objective or from a separate LED source. The opsin in LMO3, VChR1, can be activated at either 480 or 540 nm light illumination (30 mW/cm^2 , 1 s). Depending on the age of the pups and the specific cells expressing the LMO

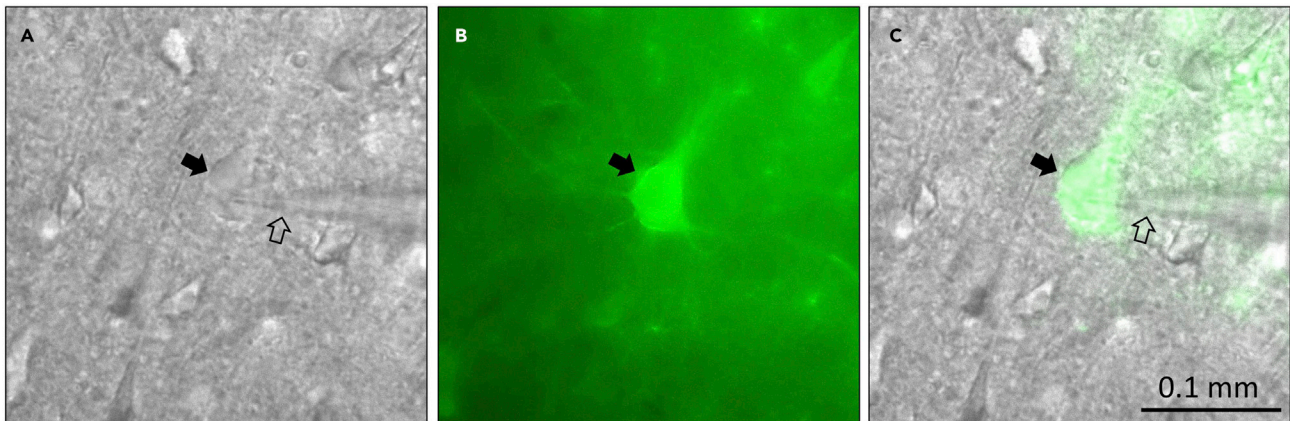


Figure 5. Patching LMO-expressing neurons

(A) Brightfield image of patched pyramidal neuron in medial prefrontal cortex layer 5. Filled arrow points at patched neuron, open arrow indicates patch pipette.

(B) Fluorescent image of the same field as in (A) under 480 nm excitation wavelength.

(C) Merged image of (A) and (B).

neurons may be functionally immature, in which case light stimulation may only generate sub-threshold depolarizations.

- d. Record from the patched LMO expressing cell in the presence of CTZ (Segev et al., 2016). Maintain membrane potential at -70 mV by continuously injecting negative DC current, <100 pA, in current clamp mode while delivering periodic square current injections to evoke firing (e.g., 0.1 Hz, 300 pA, 50 ms). Bath application of CTZ is expected to depolarize the membrane potential and increases the firing response to current stimulation.
 - i. A peristaltic pump continuously circulates carbogenated ACSF from a beaker into the recording chamber and from there back into the beaker. Transfer 250 μ L of carbogenated ACSF from this beaker into a 1.5 mL microcentrifuge tube (Figures 6A and 6B).
 - ii. Add 5 μ L of 50 mM CTZ to the ACSF and mix well (Figure 6C). This 1 mM concentrated CTZ-ACSF will be diluted to ~ 300 μ M final concentration in the recording chamber. The volume and concentration of CTZ can be scaled up or down based on the experimental set-up.
 - iii. While keeping the pump running at the chosen flow rate, take out the inlet tube of the pump from the ACSF beaker and quickly immerse it in the CTZ solution (Figure 6Di), keeping the transition time as short as possible to avoid any air void in the tubing. The time it takes for the CTZ to reach the patched neuron depends on the flow rate and length of the tubing and has to be determined for the specific recording set-up. As an example, for the depicted set-up (Figure 5) with a flow rate of 1.5 mL/min it takes 2.5 min (± 5 s) from the CTZ-ACSF being taken up by the inlet tube to producing bioluminescence from the slice in the recording chamber (see also Note below). The perfusion flow rate is kept constant throughout the entire experiment.
 - iv. Remove the outlet tubing of the pump from the ACSF beaker and place it in a separate 50 mL tube to collect the ACSF with the used reagent. Dip the outlet tubing back into the ACSF beaker after the reagent has exited (Figure 6A).
 - v. Turn off all lights in the room and observe the bioluminescence in the recording chamber upon entry of CTZ (Figure 7A).
 - vi. Roll down the front black drape to make the rig completely light tight if measurement of photon counts is desired (bioluminescence imaging) (Figure 7B).
[Troubleshooting 2](#)
 - vii. The vehicle treatment control is conducted in the same way as described above for CTZ. However, there will not be any generation of bioluminescence, and no increase in firing

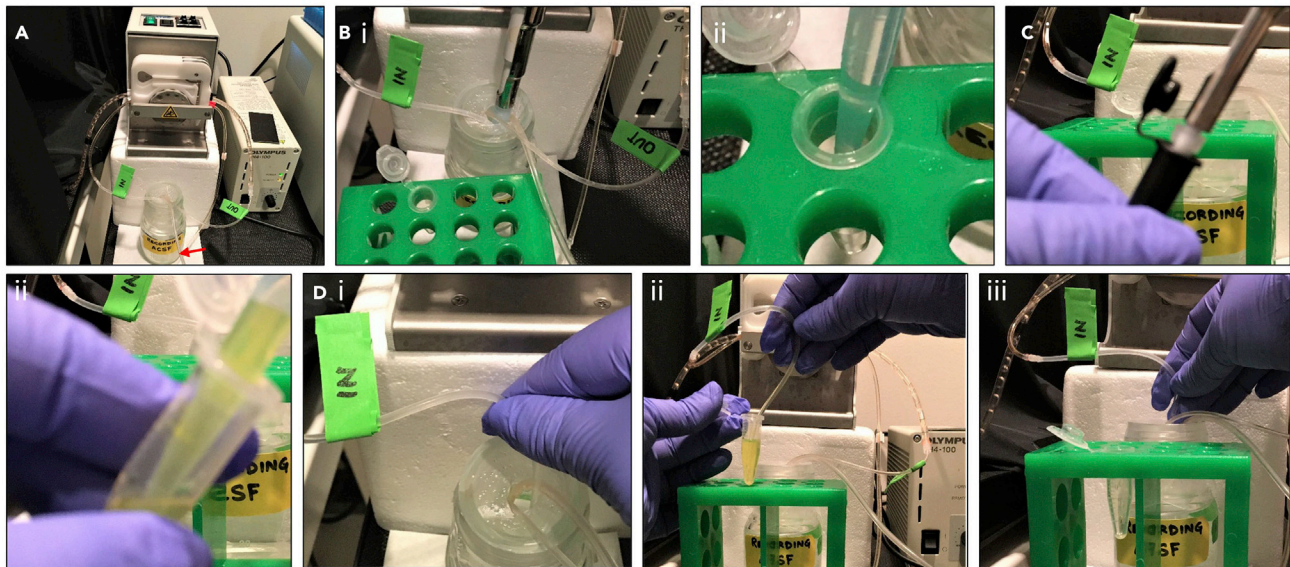


Figure 6. CTZ treatment during patch recording of LMO expressing neuron

(A) Peristaltic pump circulating the carbogenated ACSF present in the glass beaker. Inlet and outlet tubing labeled with green tapes; red arrow points at gas tubing from the Carbogen tank.

(B) Using a P1000 pipetman carbogenated ACSF is pipetted out from the beaker (i) and transferred to an Eppendorf tube (ii).

(C) CTZ (50 mM stock) is pipetted out from the black stock vial (originally stored at -80°C) (i) and mixed well with the fresh ACSF in the Eppendorf tube (CTZ-ACSF) (ii).

(D) The inlet tubing is removed from the ACSF beaker (i) and is quickly dipped into the CTZ-ACSF solution in the Eppendorf tube, as in C (ii). The inlet tube is quickly transferred back to the ACSF beaker once the Eppendorf tube is empty.

response to current stimulation is expected.

Troubleshooting 3

⚠ CRITICAL: Keep the ACSF bubbled with Carbogen at all times to preserve cell viability.

Alternatives: Other materials can be used to make the patch rig light-tight, such as thick black plastic sheets.

Note: Timing for entrance and exit of CTZ in and out of the chamber will depend on the tubing size, chamber volume and pump flow rate. These temporal parameters can be determined by a mock experiment adding food coloring dye instead of CTZ to the 250 μL of carbogenated ACSF. The time needed for eliciting an increase in firing can be tested by adding Bicuculline or Picrotoxin to, or increasing the KCl concentration of, the 250 μL ACSF inlet solution while recording from a patched neuron.

Validate neural stimulation by acute extracellular *in vivo* recordings in postnatal mice

⌚ Timing: 6 h

This step describes the use of multisite acute extracellular recordings *in vivo* from postnatal pups for the assessment of neural stimulation with intraperitoneal (IP) CTZ application. The protocol details how to anesthetize pups, head-mount them into a stereotaxic frame, and record the effect of IP CTZ application.

9. Ahead of time, construct a re-usable head mount for the neonatal skull to be fixed to the stereotaxic frame. The molded "crown" creates a customized structural interface between the skull and

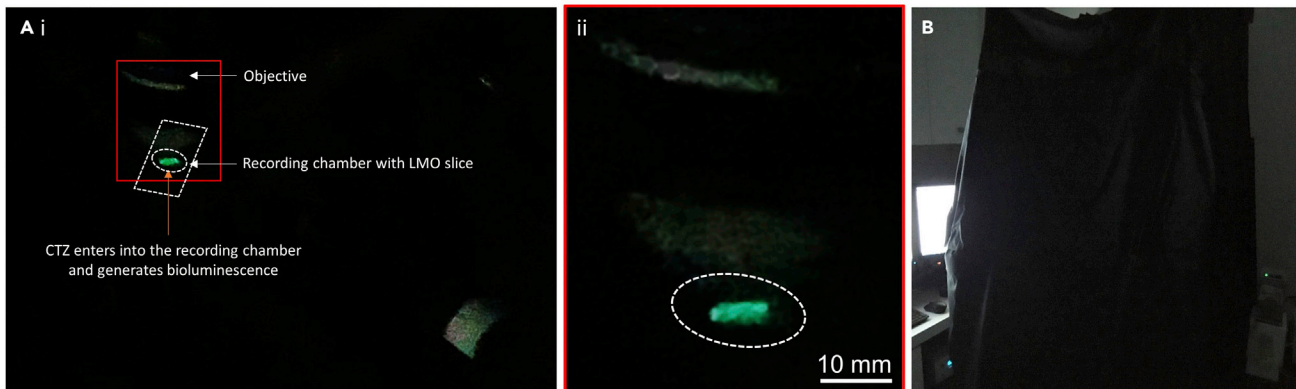


Figure 7. Bioluminescence emission from the LMO expressing neurons in the slice upon CTZ treatment

(A) CTZ-induced bioluminescence from the LMO slice (inside red square) upon its entry into the recording chamber (shown as dashed white boundaries) (i), with further zoomed-in view (ii). Pictures were taken with a cell phone.

(B) Set-up for bioluminescence imaging: all the lights in the room are turned off and the front black drape is rolled all the way down and tucked well to make the patch rig completely light-tight (e.g., the light from computer screen on the left cannot enter the light-tight rig).

the stereotaxic apparatus that serves to stabilize the soft skull of the pup and allows to fix it in a stereotaxic frame through attached ear bars.

- a. Create a custom molding with 'Gorilla Epoxy Putty Stick' (Figure 8A).
 - i. Follow the manufacturer's protocol to get pliable putty.
 - ii. Use mouse heads from different developmental stages to construct molds that reflect the specific age, allow for clear visualization of the skull surface, and ensure a tight fit between the mold and the skull of the pup.
- b. Trim the hardened rough mold with a Dremel, using sanding, carving, and cutting accessories.
- c. Super glue the head mount to flat metal washers, one on each side.
10. On the day of recording, prepare the ephys rig.
 - a. Assemble and check all components needed (drill with small drill bit, electrode, LED optical cable and source, saline).
 - b. Turn on the warming pad (maintained at 37°C until the end of the recording session).
11. Anesthetize the pup.
 - a. Transfer the pup from the parent cage to a holding box (for example, a small Styrofoam box padded with soft tissue). Cover the pup with some gauze to keep it warm.
 - b. Prepare fresh Urethane. Weigh the pup and calculate the amount of Urethane stock to be injected (1.0 g/kg weight of the animal). If the calculated volume of Urethane stock for I.P. injection is less than 100 μ L, dilute it 1:2 with saline and then inject I.P. (Example: a pup weighing 7 g will need 52.24 μ L of the Urethane stock, or 104.5 μ L when 1:2 diluted).
 - c. Inject the Urethane into the abdomen (intraperitoneally) avoiding any organs.
 - d. Note the time of this primary Urethane injection and return the pup to the warm and darkened holding box until it is anesthetized (i.e., does not respond to a toe pinch). Time intervals for complete anesthesia can vary between 30 min to 1.5 h.
 - e. If pup does not move for a while but still responds to a toe pinch, boost anesthesia by acute application of Isoflurane.
 - i. Place a small gauze pad into a 10 mL plastic tube with a screw cap.
 - ii. Add 1–2 drops of Isoflurane to gauze and close lid.
 - iii. Just before making an incision, open the lid of the tube and hold the opening of the tube close to the snout of the pup for 3 s.
12. Fix the head mount to the pup's head.
 - a. Quickly remove the skin from the head using scissors to expose the skull (Figure 8Bi).

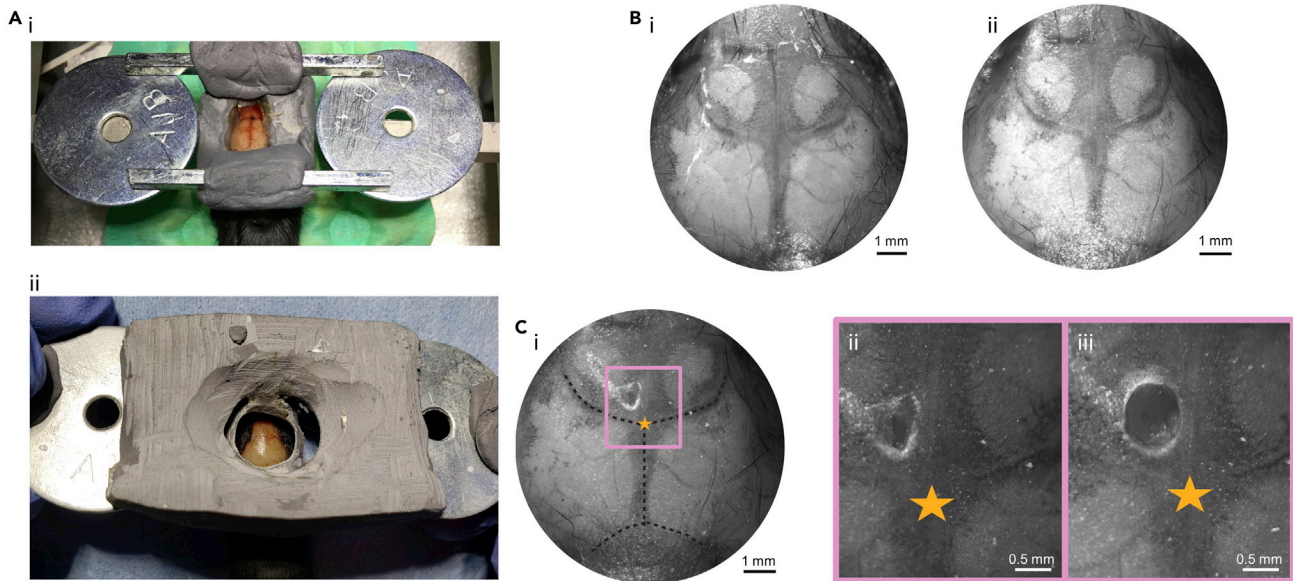


Figure 8. Customized EPOXY and METABOND postnatal mouse skull stereotaxic holder

(A) Two example variations of customized stereotaxic holders (i and ii).

(B) Skull of a P12 mouse after removal of skin allowing visualization of skull landmark structures and the underlying brain before (i) and after (ii) applying METABOND to the bone surface. The METABOND layer continues onto the stereotaxic holder shown in (A).

(C) Overview picture (i) with stippled lines indicating midline (straight line), lambda (lower half circle) and bregma (upper half circle); star indicates convergence of bregma and midline. Zoomed-in pictures show a craniotomy over the left medial prefrontal cortex beginning with a drill hole only through the METABOND layer (ii), then through the skull providing access to the brain surface (iii – center opening inside the drill hole).

- b. Dry the surface with Q- tips and apply Vet bond to glue the skin to the skull. This provides a stable base for the head mount fixation.
- c. Fix the pre-constructed head mount to the prepared skull using C&B METABOND.
 - i. Take out the pre-chilled porcelain tray from -20°C freezer.
 - ii. Mix the METABOND components quickly, following manufacturer's instructions.
 - iii. Align the head mount over the skull, so that the marked Bregma is visible in the center and the window of the molded mount allows for clear visualization of skull landmarks.
 - iv. Apply the METABOND mixture along the circumference of the head mount and across the skull, thereby fixing the skull to the head mount (Figure 8Bii). The goal is to create a stable interface between the skull and the mount.
 - v. Allow the METABOND to solidify while gently holding the pup, making sure that the pup is not weighed down by the head mount. This takes about 2–3 min.
13. Attach the head-mounted pup to ear bars.
 - a. Turn the mouse with attached head mount belly side up.
 - b. Super glue ear bars to the two metal washers of the head mount.
 - c. Allow the glue to bond for at least the recommended time for the specific brand of glue used (1–2 min).
14. Place the pup into the stereotaxic frame via the ear bars. Cover the pup (from neck to tail) with a soft cloth or gauze to keep it warm.
15. Craniotomy of the pup (use surgical stereomicroscope).
 - a. Deliver a booster dose of Urethane (20% of the original dose) to the pup.
 - b. Connect the optrode to the Omnetics holder and bring it right on top of the Bregma mark (Figure 8Ci). Reset the digital stereotaxic apparatus for the anterior/posterior (AP) and medial/lateral (ML) values. Move the electrode to the desired AP and ML coordinates and mark the point (with a thin sharpie) for drilling the hole for the optrode.
 - c. Lift up the optrode holder and move it all the way to the back to protect the electrode while performing the craniotomy.

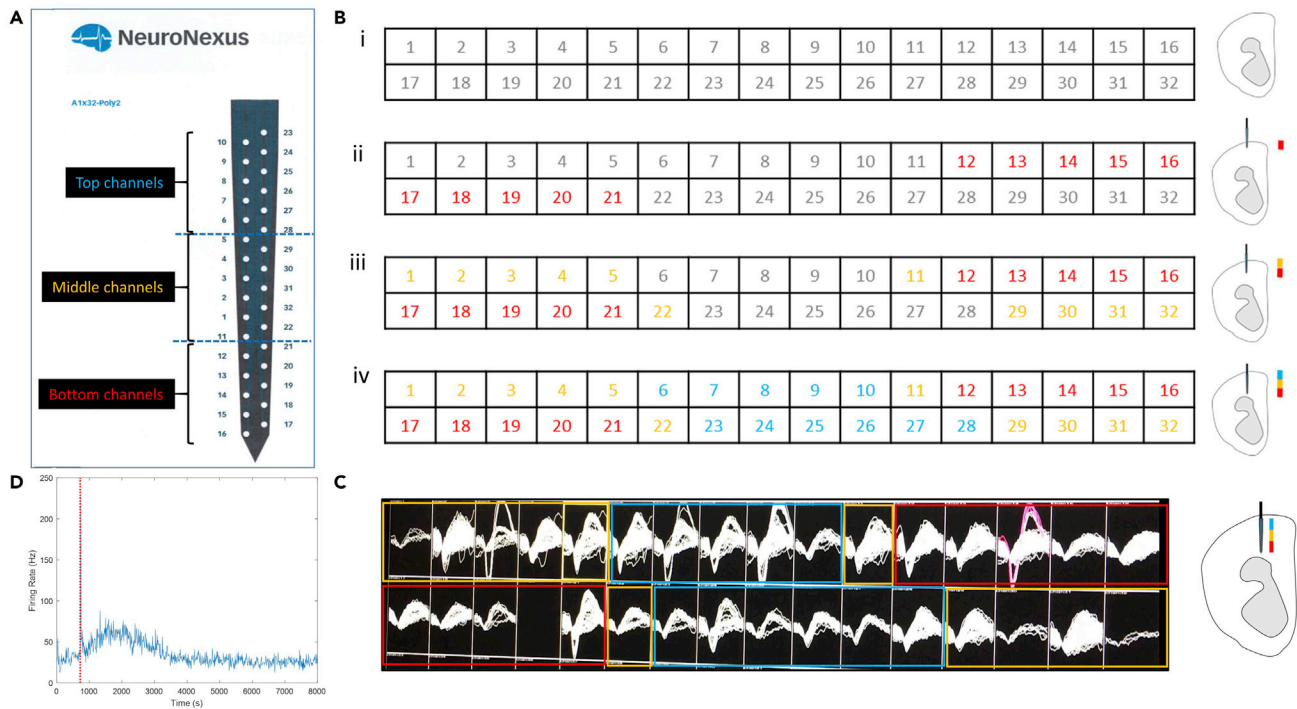


Figure 9. Assessment of dorso-ventral placement of the optrode in the pup brain for ephys recording

(A) Example channel map for a single shank, 32-channel silicon laminar probe (A1x32 Poly2-5mm-50s-177-OA32LP, NeuroNexus Technologies). Color code for DV arrangement of electrodes is maintained throughout figure: bottom (red), middle (yellow), and top (blue). Fiber optic is located 50 μ m above the highest recording site.

(B) Order of appearance of all 32 channels shown as a table (2 $\times$ 16) (as visible on the software screen, Blackrock Microsystems) starting from before inserting until fully inserted (i-iv, left side) and illustrations depicting the respective depths of the optrode in the pup brain (i-iv, right side).

(C) Example of a recording screenshot showing activity in most channels when the probe is fully inserted in the pup cortex.

(D) Example recording from medial prefrontal cortex of P14 Emx1-LMO3 pup. Representative trace of the average firing rate from putative regular spiking units (pyramidal neurons) after pup received an IP injection of CTZ (red dotted line). The average firing rate returns to baseline around 3000 s or 48mins following CTZ administration.

- d. Using a hand-held, battery operated drill, gently drill into the delicate skull, avoiding any bleeding (Figure 8C). Drip some saline on the hole in the skull to avoid any drying and for clear sight of the brain surface.
 - i. Be aware that the dried METABOND on the skull surface needs to be completely drilled through first (Figure 8Cii) in addition to the mouse skull (Figure 8Ciii).
 - ii. Confirm successful craniotomy visually and by gently touching the surface with fine blunt forceps.
- e. Drill a second hole over the cerebellum (no specific coordinates necessary).
- f. Using the X/Y/Z axis micromanipulators of the stereotaxic instrument bring back the optrode to the front and carefully align it over the craniotomy of the target area. Insert, by hand, the silver wire connected to the optrode's ground and reference ports through the second craniotomy into the cerebellum.
- g. Move the optrode down until the tip of the optrode touches the brain surface. At this point reset the dorsal/ventral (DV) values on the digital stereotaxic apparatus.

16. Record from the desired brain region of the pup.

- a. Turn on the ephys set-up and software.
- b. Slowly, turning the micromanipulator in 10 micron steps, move the optrode tip dorso-ventrally.
 - i. Follow progression of the probe into the brain according to the channel map for the optrode used (Figure 9A).

- ii. Watch the panel of optrode channels on the computer screen for neural activity while going down slowly ([Figure 9B](#)). As the optrode moves more ventral, more channels will start showing activity. Once this includes the top channels, the entire probe has been inserted into the brain.
- c. When consistent neural activity is observed in most of the channels ([Figure 9C](#)) at the desired DV position, let the neurons recover for about 30 min.
- d. During the recovery phase, prepare the water-soluble CTZ/vehicle solution (as described in steps 1 and 2 above). Once the recovery phase is over, start the recording with a baseline of about 20 min (before CTZ/vehicle injection).
- e. Deliver a pulse of blue light via the optrode to check that LMO-expressing neurons are being recorded.
- f. For CTZ/vehicle application, lift the pup gently by its tail (without disturbing the upper body) and deliver the CTZ/vehicle intraperitoneally with an insulin syringe. Bring the tail down to place the pup back to its original position.
[Troubleshooting 4](#)
- g. Record the activity for the desired period of time ([Figure 9D](#)).
[Troubleshooting 5](#)
- h. After the recording, carefully take out the optrode from the brain and dip-clean it with distilled water immediately to avoid any drying of debris. Clean further with Tergazyme or any other suitable electrode cleaning reagent.
- i. Remove the pup from the rig and the head mount, and surgically remove its brain. Put the brain in fixative (4% PFA) to do histology later if desired.

△ **CRITICAL:** Step 12: Care needs to be taken to NOT get any Vetbond, Metabond, glue, or any other solutions into the pup's nostrils while mounting the headstage.

△ **CRITICAL:** Throughout the surgery the breathing pattern of the pup needs to be monitored at all times when preparing pups for recordings. If any shallow breathing is being noticed, perform "CPR" by gently rubbing fingers on the chest of the pup for 2–3 minutes, until the breathing rate returns to normal.

Alternatives: Different bonding reagents and glues can be used. The ones listed here are one example of what works.

Different head stages can be used (see for example [Bitzenhofer et al., 2017, 2020](#)). The protocol presented above outlines how to perform acute extracellular recordings in developing anesthetized pups using a standard stereotaxic set-up and customized headstage assembled from materials available in any hardware store. A major consideration when creating a molding for the custom headstage is that the headstage interfaces well with the fragile skull of the mouse through the Metabond. Accordingly, a different molded headstage will be necessary when recording from postnatal pups of different ages.

For the initial anesthesia alternatives to urethane can be used (Avertin, Ketamine/Xylazine). The recordings can be done under continuous isoflurane anesthesia.

Note: Always store the Urethane powder in a desiccator and prepare fresh Urethane for the day.

Note: We dilute the Urethane solution 1:2 to make the injectable volume $\leq 100 \mu\text{L}$, ensuring Urethane to be distributed and absorbed more efficiently after IP injection.

Note: In our experience, every pup responds differently to Urethane. Even pups from the same litter with similar weights showed variations in their responses towards Urethane.

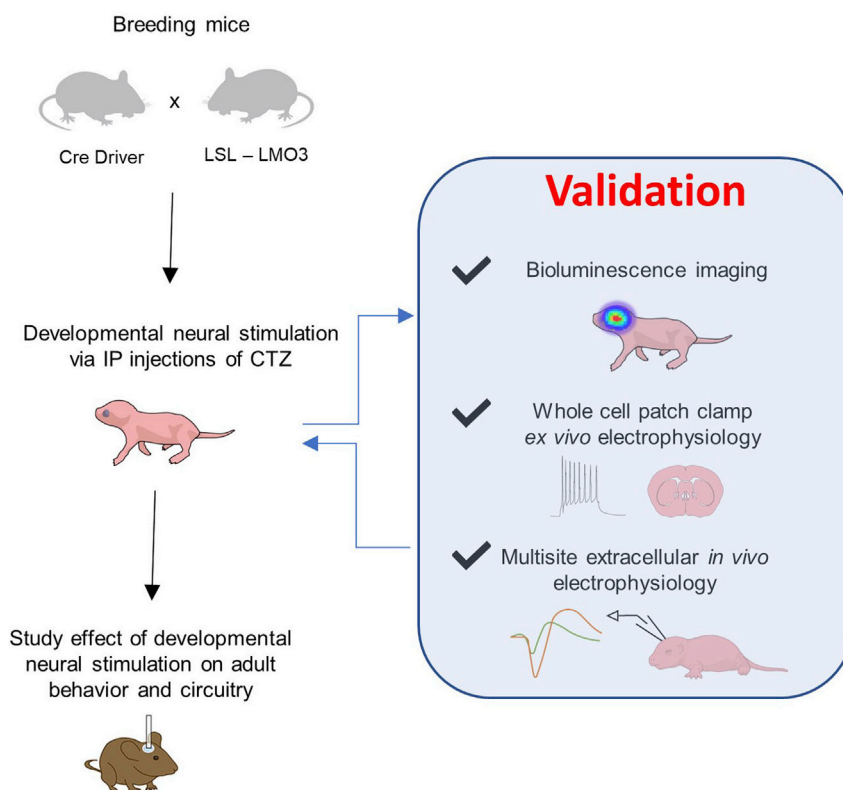


Figure 10. BL-OG approach to studying the role of neural activity during development in shaping adult behavior and its underlying circuitry

The expected outcome of the protocol detailed here is the validation of BL-OG activation in targeted neural populations in developing pups.

Generally, one booster dose (20% of the original) is sufficient to keep the pup anesthetized throughout the entire recording, but occasionally, two booster doses (20% of the original), delivered 20–30 minutes apart, are needed.

EXPECTED OUTCOMES

BL-OG technology facilitates stimulation of targeted neural populations during any time of postnatal development. The effects of this experimenter controlled developmental perturbation can then be studied in adult animals in multiple ways. While the developmental stimulation via bioluminescence is straightforward, it needs to be validated for each newly selected cell population (Figure 10). This protocol is expected to guide users through these initial one-time validation experiments. BL-OG activation of developing circuits can be verified by live bioluminescence imaging, patch clamp recordings from brain slices, and *in vivo* recordings. Once validated, developing pups can simply be injected intraperitoneally with CTZ or vehicle during the time window and at the frequency chosen to generate cohorts of experimental (CTZ) and control (vehicle) animals for further studies. The BL-OG approach will allow for causal cell type mechanistic understanding of early neural activity and its subsequent role in circuit maturation, underlying normal and aberrant behavior.

LIMITATIONS

In principle, there are no limitations to using BL-OG stimulation during postnatal development in mice. No side effects have been observed from CTZ injections in pups. However, initial validation of the specific experimental system will be limited by the extent of expression of the luminescent opsin. Light emission and neural stimulation of genetically targeted populations such as Emx1-positive pyramidal

neurons allow validation by live imaging due to their robust and multi-layer expression across the cortex, as well as patch recordings in slices and *in vivo*. As the number of LMO-expressing cells in a targeted population becomes smaller and/or more sparsely distributed, simple bioluminescence imaging might not be an option. However, single cell recordings from patched neurons in slices and/or multiunit recordings *in vivo* will provide validation of stimulation. If neural stimulation cannot be achieved at the developmental time point selected, animals a few days further along in development might have to be used.

TROUBLESHOOTING

Problem 1

No bioluminescence detected during live imaging (step 4 in [validate neural stimulation by bioluminescence imaging in developing mice](#)).

Potential solution

We used 5-min collection times for bioluminescent imaging of neonatal mice expressing LMO3 in Emx1-positive cells, i.e., pyramidal neurons across the entire cortex. Temporal bins for collection of light emission may have to be shortened or extended depending on the level and anatomical distribution of the respective conditionally expressed LMO3. For imaging with the IVIS system photon collection times can be extended to 30 min per bin. Concentration of CTZ can also be increased to up to 500 μg per animal to initially visualize light emission. Depending on anatomical location (superficial vs deep inside the brain, such as cortical layers vs midbrain structures), distribution (entire cortex vs sparse numbers in single layers or nuclei), and level of expression (ubiquitous strong promoter vs cell-specific weak promoter) bioluminescence emission might be under the detection limit for live imaging of the targeted cell population. In this case, validation should proceed to *ex vivo* and *in vivo* recordings.

Problem 2

No bioluminescence detected in brain slices (step 8 in [validate neural stimulation by patch clamp recordings in slices](#)).

Potential solution

If the level of expression and number of neurons expressing the LMO are expected to lead to robust bioluminescence emission, lack of observed luminescence might have trivial reasons: Is the rig light-tight? Is the concentration of CTZ high enough? Is the CTZ solution freshly prepared without prolonged exposure to light and oxygen, which will reduce its overall activity over time due to auto-oxidation? Is the brain slice preparation of good quality, with visible EYFP expression in healthy neurons? If the level of LMO expression and/or number of expressing cells is low, bioluminescence emission might be under the detection limit for real-time imaging. If the rig is equipped with an EMCCD camera, this will significantly extend the detection limit. Alternatively, the slice can be placed in an IVIS chamber for photon collection over longer time periods.

Problem 3

No increase of neural activity with CTZ application detected in brain slices (step 8 in [validate neural stimulation by patch clamp recordings in slices](#)).

Potential solution

Depending on several parameters, such as age of the pups, expression level, neuronal viability, quality of the recording and illumination strength, light stimulation may evoke depolarizations of varying magnitude. It is helpful to first use an external light source (arc lamp, LED) for stimulation (blue light, one sec pulses with increasing light intensity up to saturation, i.e., $> 0.2 \text{ W/cm}^2$) of the patched neuron to verify the presence of a clear light-induced current/voltage response in the cell at -70 mV (ideally $\geq 500 \text{ pA}$ or $\geq 10 \text{ mV}$). The impact of LMO activation can then be assessed by delivery of CTZ during continuous membrane potential recording in current clamp, where a

depolarization of the membrane potential is expected. Alternatively, the effect of LMO activation can be assessed from the firing response of the cell to depolarizing square current injections prior, during and after CTZ delivery to the recording chamber, where an increase in firing frequency is expected in the presence of CTZ.

Problem 4

No increase of neural activity with CTZ application detected *in vivo* (step 16 in [validate neural stimulation by acute extracellular in vivo recordings in postnatal mice](#)).

Potential solution

Again, it will be helpful to first use an external light source (LED) for stimulation via the optrode to gauge maturity and activation threshold of the targeted neural population. If no response with CTZ is achieved after confirmed stimulation with LED light, increasing the CTZ concentration might be necessary. If still no response can be elicited, test stimulation in older pups.

Problem 5

Increase of neural activity with CTZ as well as with vehicle application detected *in vivo* (step 16 in [validate neural stimulation by acute extracellular in vivo recordings in postnatal mice](#)).

Potential solution

CTZ or vehicle is injected ip into a headfixed pup with the electrode inserted. While the head should not be movable, even the slight repositioning of the body when injecting ip often results in artifactual changes in firing rates. However, these are usually brief (under 5 min duration) and specific differences between CTZ and vehicle application should become obvious during further recordings over 10–20 min. Alternatively, insert a thin catheter into the intraperitoneal cavity before starting the recordings. This allows application of CTZ or vehicle without touching the headfixed pup.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact (Ute Hochgeschwender, hochg1u@cmich.edu).

Materials availability

Plasmids used in this study and their sequences are available at Addgene (LMO3, #114099).

Mouse lines generated in this study are available from The Jackson Laboratory (B6N.129-Gt(ROSA)

26Sortm4.1(CAG-sbGLuc/COP3/EYFP)Ute, Stock No: 034853).

Data and code availability

No new code was generated in this study.

The raw datasets supporting the current study are available from the lead contact on request.

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AUTHOR CONTRIBUTIONS

E.L.C., M.P., A.B., and W.E.M. devised the protocols and performed experiments. N.C.S., D.L., and C.I.M. provided essential input to the work. M.P., E.L.C., A.B., and U.H. prepared the manuscript. U.H. supervised the work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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