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1 **TITLE:**
2 In Vivo Intracerebral Stereotaxic Injections for Optogenetic Stimulation of Long-Range Inputs in
3 Mouse Brain Slices
4

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16 **KEYWORDS:**

17 stereotaxic injection, channelrhodopsin, optogenetics, electrophysiology, acute brain slice,
18 whole-cell patch-clamp recording, neuronal morphology, hippocampus, synaptic transmission
19

20 **SUMMARY:**

21 This protocol describes a set of methods to identify the cell-type specific functional connectivity
22 of long-range inputs from distant brain regions using optogenetic stimulations in ex vivo brain
23 slices.
24

25 **ABSTRACT:**

26 Knowledge of cell-type specific synaptic connectivity is a crucial prerequisite for understanding
27 brain-wide neuronal circuits. The functional investigation of long-range connections requires
28 targeted recordings of single neurons combined with the specific stimulation of identified distant
29 inputs. This is often difficult to achieve with conventional and electrical stimulation techniques,
30 because axons from converging upstream brain areas may intermingle in the target region. The
31 stereotaxic targeting of a specific brain region for virus-mediated expression of light-sensitive ion
32 channels allows selective stimulation of axons originating from that region with light.
33 Intracerebral stereotaxic injections can be used in well-delimited structures, such as the anterior
34 thalamic nuclei, in addition to other subcortical or cortical areas throughout the brain.
35

36 Described here is a set of techniques for precise stereotaxic injection of viral vectors expressing
37 channelrhodopsin in the mouse brain, followed by photostimulation of axon terminals in the
38 brain slice preparation. These protocols are simple and widely applicable. In combination with
39 whole-cell patch clamp recording from a postsynaptically connected neuron, photostimulation
40 of axons allows the detection of functional synaptic connections, pharmacological
41 characterization, and evaluation of their strength. In addition, biocytin filling of the recorded
42 neuron can be used for post-hoc morphological identification of the postsynaptic neuron.
43

44 **INTRODUCTION:**

45 Defining connectivity between brain regions is necessary to understand neural circuits. Classical
46 anatomical tracing methods allow establishing interregional connectivity, and lesion studies help
47 to understand the hierarchical organization of information flow. For example, brain circuits for
48 spatial orientation and head direction signaling involve the directional flow of information from
49 the thalamus to the presubiculum. This has been demonstrated by lesion studies of antero-dorsal
50 thalamic nuclei (ADN) that degrade the head direction signal in the downstream dorsal
51 presubiculum, as well as the parahippocampal grid cell signal^{1,2}.

52

53 The functional connectivity between brain areas is more difficult to establish at a cellular and
54 subcellular level. In the hippocampus, a highly organized anatomy allows to investigate pathway-
55 specific synaptic connections using electrical stimulation in the slice preparation. Stimulation
56 electrodes placed in stratum radiatum of CA1 can be used to specifically stimulate Schaffer
57 collateral input from CA3³. Stimulating electrodes placed in stratum lacunosum moleculare of
58 CA1 will activate the perforant path input to CA1^{4,5}. Electrical stimulation activates
59 neurotransmitter release from axon terminals; however, it activates neurons with somata near
60 the stimulation site as well as axons of passage. It is therefore of limited use for studying afferents
61 from defined brain regions when fibers of different regions of origin intermingle in the target
62 structure, as is typically the case in the neocortex.

63

64 Neurons may also be stimulated with light. Optical methods include the photoactivation of caged
65 glutamate, which can be combined with one- or two-photon laser scanning. Multiple closely
66 spaced sites may be stimulated sequentially, with no mechanical damage to the tissue⁶. This has
67 been successfully used to map synaptic receptors as well as activate individual neurons⁷. While
68 glutamate uncaging can be used for local circuit analysis, it does not allow for specific activation
69 of long-range inputs.

70

71 A method of choice for the investigation of long-range connectivity in neuronal circuits is the use
72 of virus-mediated channelrhodopsin expression. Using in vivo stereotaxic injections as described
73 here, the expression of light-gated ion channels can be targeted and spatially restricted to a
74 desired brain region. In this way, channelrhodopsins are effective for mapping excitatory or
75 inhibitory connectivity from one region to its target. Transfected axons terminals may be
76 stimulated with light in a brain slice preparation, and patch-clamp recordings as a read-out allow
77 examination of the functions and strengths of specific circuit components in the brain⁸. The
78 optogenetic approach combined with stereotaxic injection of a virus offers unprecedented
79 specificity and genetic control⁹. Stimulating with light additionally allows for both high temporal
80 and spatial precision^{10,11}.

81

82 The presubiculum is a six-layered cortical structure at the transition of the hippocampus and the
83 para-hippocampal formation^{12,13}. It receives important synaptic input from the ADN¹¹ but also
84 from several other cortical and subcortical regions¹⁴. Thus, the selective stimulation of thalamic
85 axons terminals within a presubicular slice is not possible with electrical stimulation nor
86 glutamate uncaging. Described in this protocol are methods to determine functional connectivity
87 between brain regions (ADN and presubiculum) using precise stereotaxic injections of viral
88 vectors expressing light-gated channels. Also described is the photostimulation of axons

89 terminals of projecting neurons in their target region, coupled with whole-cell patch-clamp
90 recordings of post-synaptic neurons in the brain slice preparation.

91

92 **PROTOCOL:**

93 All procedures were performed in accordance with the European Community Council Directive
94 (2010/63/EU) and approved by the ethics committee of Paris Descartes University. The
95 experimenter must obtain authorization for the procedure to comply with local regulations.

96

97 **1. Planning of the experiment**

98

99 1.1. Define the brain area to be targeted. Determine the stereotaxic coordinates of the injection
100 site with the help of a mouse brain atlas¹⁵. For the right antero-dorsal thalamic nucleus (ADN),
101 the coordinates are: -0.82 posterior, 0.75 lateral, -3.2 depth (mm) relative to bregma.
102 Coordinates may need to be adjusted for animals of different age, sex, or strain.

103

104 1.2. Confirm and document the exactitude of the coordinates by injecting a fluorescent tracer
105 (150 to 300 nL) observable with an epifluorescence microscope in a pilot experiment (**Figure**
106 **1A,B**).

107

108 1.3. Define the type of virus to be injected. Store the virus in 6 μ L aliquots at -80 °C as
109 recommended by the producer. Bring 1 aliquot placed on ice to the surgery room, for injection
110 of one to six animals on a given day. Biosafety regulations for the use of AAV may depend on the
111 country or institution, and the use of a PSM 2 hood may be required.

112

113 NOTE: Here, we use a AAV2/5 serotype expressing Chronos, a fast channelrhodospin-2 variant,
114 fused to green fluorescent protein under the control of the Synapsin promoter:
115 AAV5.Syn.Chronos-GFP.WPRE.bGH.

116

117 **2. Stereotaxic surgery**

118

119 2.1. Install a stereotaxic frame equipped with a pump holder on a stable standard laboratory
120 bench. Adjust stereoscope so as to clearly see the zone where the animal's head will be placed.
121 Use a LED light source for illumination. Rotate the stereoscope away to access the pump holder,
122 which is not needed for the first steps of the surgery.

123

124 2.2. Install a 10 μ L Hamilton syringe equipped with a 33 G beveled metal needle in the pump
125 holder. Test the ejection system with water.

126

127 2.3. Anesthetize a 4- to 5-week old C57BL6 mouse with an intraperitoneal injection of a mix of
128 ketamine hydrochloride and xylazine (100 mg/kg and 10 mg/kg, respectively). Prepare a mix of 1
129 mL of ketamine and 0.5 mL of xylazine in 8.5 mL of 0.9% NaCl. This will result in 10 mg/mL
130 ketamine and 1 mg/mL xylazine in the mix. Of this mix, inject intraperitoneally 10 μ L per gram of
131 the animal's body weight. Duration of anesthesia is about 1 h.

132

133 2.4. Verify that the animal is well-anesthetized with a toe pinch. Then, pull out the tongue to
134 facilitate breathing. Shave the cranial hair.

135

136 2.5. Inject 20 μ L of lidocaine hydrochloride (4 mg/mL; 2 mg/kg) under the skin of the head for
137 local anesthesia and wait 5 min for the effect to begin. To avoid eye damage due to dryness,
138 cover the eyes with topical ophthalmic ointment.

139

140 2.6. To expose the skull, create a straight cut in the scalp with small surgery scissors. Place the
141 animal in a stereotaxic frame, inserting the ear bars slightly rostral to the actual ear to rest on
142 the bone and pull down the skin, which should create good access to the skull. Tighten into place.
143 Install the nose piece.

144

145 2.7. Maintain the body of the animal horizontally at the level of the head using a height-adjusted
146 support. Place a heating pad under the mouse to keep it at physiological temperature.

147

148 2.8. Clean the skull by applying 0.9% NaCl with a cotton swab to remove soft tissue from the
149 bone. Use the stereoscope for the rest of the surgery.

150

151 2.9. Adjust the skull so that the bregma-lambda axis is level, moving up or down the nose and
152 teeth piece. This necessitates iterative measures of bregma and lambda, as both will change
153 following adjustment of the nose level.

154

155 2.10. Find the location of the injection site on the skull. Adjust the injection needle above the
156 injection site according to posterior and medial coordinates and mark the skull with a disposable
157 needle. Move the injection needle upward by 4 cm.

158

159 2.11. Use a 0.5 mm burr with a drill to realize a 1 mm diameter craniotomy on the mark, at one-
160 half of maximum speed. Swab eventual bleeding with a paper tissue.

161

162 2.12. Empty the water contained in the Hamilton syringe for storage by completely ejecting it
163 with the pump. Only the needle will still be filled with water.

164

165 2.13. Take the centrifuge tube with the aliquot of virus that is to be used for this day. Make sure
166 that the viral solution is not frozen anymore but has remained cooled (close to 0 °C, on ice). Only
167 briefly remove from the ice to obtain 700 nL with a micropipette for small volumes. Deposit the
168 drop on a 5 cm x 5 cm piece of paraffin film. Avoid creating bubbles. Put the remaining viral
169 solution back on ice.

170

171 NOTE: The drop volume should be greater than the desired injection volume (700 nL for 200 nL
172 injected). This will give a safety margin in case some of the liquid is lost during the transfer and
173 allows performing a small test ejection (step 2.16) before proceeding.

174

175 2.14. Place the paraffin film on top of the craniotomy. Plunge the needle in the drop of viral
176 solution without changing the antero-posterior and lateral position.

177
178 2.15. Use the “withdraw” function of the pump to fill the syringe with about 500 nL of viral
179 solution disposed on the paraffin film. Do this under visual control (stereoscope), watch the drop
180 disappear, and make sure not to aspirate air.

181
182 2.16. Make sure the syringe has been filled correctly. Verify the functioning of the ejection
183 system by driving down the plunger to test eject a small drop of liquid of 50 nL under visual
184 control. Wipe the drop.

185
186 2.17. Insert the needle into the brain to the chosen depth, by turning the knob controlling the
187 dorso-ventral axis of the stereotaxic apparatus clockwise. Push the “run” button (speed 15
188 nL/min per volume of 150 nL injected). A small volume (50–300 nL, depending on the virus used)
189 is slowly ejected over 10 min with an automatic pump.

190
191 2.18. Wait 10 min after the injection to avoid leaking from the injection site. Then, slowly remove
192 the needle over 3–5 min by turning the knob controlling the dorso-ventral axis counterclockwise.

193
194 2.19. Rotate the vertical part of the stereotaxic frame with the syringe away from the animal.
195 Immediately wash the needle in clean distilled water by filling-emptying it several times, in order
196 to avoid clogging. Store the syringe filled with water.

197
198 2.20. Remove the mouse from the stereotaxic frame. Suture the skin with 4-0 polyamide suture
199 filament. Make three or four stiches, tied with 2-1-1 standard surgical knots.

200
201 2.21. Place the mouse in a heated cage until it completely wakes up from anesthesia, and provide
202 water and soaked food in a Petri dish placed on the ground. If the heat source is below the cage,
203 use a spacer grid to avoid overheating.

204
205 NOTE: According to local guidelines, a single dose of ketoprofen (2–5 mg/kg, subcutaneously) or
206 buprenorphine (0.05–0.1 mg/kg, subcutaneously) may be applied to prevent pain.

207
208 2.22. When the animal is fully awake, return it to its home cage and monitor its well-being,
209 particularly on the day following injection. Check for signs of pain. If any behavioral modification
210 is observed, the animal is weighed to monitor its body weight.

211
212 2.23. Depending on the virus used, the time for full expression may vary. Here, we allow 3 weeks
213 for expression of AAV5.Syn.Chronos-GFP.

214 215 **3. Solutions for acute slice recordings and fixation**

216
217 3.1. Prepare stock solutions of 10x concentrated cutting solution (125 mM NaCl, 25 mM sucrose,
218 2.5 mM KCl, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄, and 2.5 mM D-glucose) and artificial
219 cerebrospinal fluid (ACSF) solution (124 mM NaCl, 2.5 mM KCl, 26 mM NaHCO₃, 1 mM NaH₂PO₄,
220 and 11 mM D-glucose) in pure deionized water prior to electrophysiology experiments. Store

221 these solutions at 4 °C in 1 L bottles without CaCl₂ and MgCl₂.

222

223 3.2. On the day of the experiment, dilute the stock solutions of cutting solution and ACSF 10x to
224 a final volume of 0.5 L each. Agitate with a magnetic stirrer and oxygenize by bubbling with
225 95%/5% O₂/CO₂. Add divalent ions to obtain final concentrations of 0.1 mM CaCl₂ and 7 mM
226 MgCl₂ for the cutting solution, and 2 mM CaCl₂ and 2 mM MgCl₂ for ACSF.

227

228 3.3. Prepare the potassium-gluconate based pipette solution to contain: 135 mM K-gluconate,
229 1.2 mM KCl, 10 mM HEPES, 0.2 mM EGTA, 2 mM MgCl₂, 4 mM MgATP, 0.4 mM Tris-GTP, 10 mM
230 Na₂-phosphocreatine, and 2.7–7.1 mM biocytin for post-hoc cell morphology revelation. Adjust
231 the solution's pH to 7.3 and osmolarity to 290 mOsm. Store 1 mL aliquots at -20 °C.

232

233 3.4. Prepare 0.1 M PBS by diluting BupH PBS dry-blend powder pouches in 500 mL of distilled
234 water, resulting in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2.

235

236 3.5. To prepare 1 L of 4% PFA solution, dilute 111 mL of 36% liquid PFA and 90 mL of 10x PBS
237 solution in distilled water.

238

239 3.6. Prepare 30% sucrose solution containing 150 g of sucrose in 500 mL of 0.1 M PBS.

240

241 **4. Preparation of brain slices**

242

243 4.1. Prepare the bench space with absorbent bench paper before perfusion.

244

245 4.2. Install a drip about 1 m above the bench for gravity-fed perfusion. Attach a 24 G butterfly
246 needle.

247

248 4.3. Surround the cutting chamber of the vibratome with ice and store it in a freezer.

249

250 4.4. Anesthetize the mouse with intraperitoneal injection of the same ketamine-xylazine mixture
251 used for surgery. Assess the stage of the anesthesia by pinching the toe with forceps. When fully
252 asleep, inject 100 µL of heparin (5000 U.I./mL) intraperitoneally.

253

254 4.5. Fix the animal with adhesive tape on the absorbent paper, lying on its back. Open the
255 thoracic cage by cutting the ribs on the left and right sides with small scissors, from the diaphragm
256 upwards. Maintain the thoracic cage open with the help of adhesive tape.

257

258 4.6. Clamp the descending aorta with a hemostat and perfuse via the left ventricle of the heart
259 with 4 °C cooled and oxygenated (95%/5% O₂/CO₂), cutting the solution through the 24 G
260 butterfly needle. After 5 s, open the right atrium with small scissors.

261

262 4.7. After 5 min of perfusion, when the organs are bloodless, stop the perfusion. Decapitate the
263 animal with big scissors and immerse the head into 4 °C cooled and oxygenated cutting solution
264 in a Petri dish.

265
266 4.8. To extract the brain, cut the skin from neck to nose, then section the last vertebrae from the
267 skull with scissors. Manually retract the skin and use small scissors to open the skull, cutting it
268 along the midline, from caudal to rostral, upward to between the eyes.

269
270 4.9. Carefully remove the parietal bone and caudal part of frontal bone with curved or bone
271 forceps. Extract the brain with a small rounded spatula by inserting the instrument between the
272 brain and the cranial floor, sectioning the olfactory bulb, optic nerve and other cranial nerves,
273 and cerebellum.

274
275 4.10. Gently submerge the brain in ice-cold cutting solution (4 °C) in a beaker. Position the brain
276 on filter paper to gently dry the cortical surface. Glue the brain cortex-down to the specimen
277 holder of a vibratome, with the caudal side facing the blade, in order to cut horizontal brain slices.

278
279 4.11. Fill the cutting chamber with ice-cold oxygenated cutting solution so the brain is fully
280 immersed. Make a cut on the left hemisphere (contralateral to the injected side) to avoid
281 potential left-right ambiguity on slices.

282
283 CAUTION: Always oxygenate the solution and protect slices from light exposure.

284
285 4.12. Cut 300 µm thick slices with the vibratome, at a speed of 0.07 mm/s at 1 mm amplitude. At
286 this stage, it is recommended to briefly check the Chronos-GFP expression in the thalamus using
287 a fluorescent flashlight (440–460 nm) and corresponding filter glasses (500 nm long pass).

288
289 4.13. Isolate the hippocampal region with a scalpel and transfer it to a chamber positioned in a
290 beaker filled with bath-warmed (34 °C), oxygenated (95%/5% O₂/CO₂) ACSF.

291
292 4.14. After 15 min, take the chamber out of the heated water bath and let the slices rest at room
293 temperature, still oxygenated for at least 45 min until use.

294 295 **5. Whole-cell patch-clamp recording**

296
297 5.1. Gently transfer a brain slice containing the hippocampal complex with a custom-made glass
298 transfer pipette to the recording chamber mounted on an upright microscope. A transfer pipette
299 is made of a shortened Pasteur pipette (inner diameter 6.5 mm) attached to a rubber pipette
300 bulb. Continuously perfuse the recording chamber (3 mL) with 34 °C (warmed) ACSF bubbled with
301 95%/5% O₂/CO₂. Set the speed of the peristaltic pump to 2–3 mL/min.

302
303 5.2. Briefly examine Chronos-GFP expression in axon terminals in the region of interest with blue
304 LED illumination (470 nm) and observe with a 4x objective. GFP fluorescence is visualized through
305 an appropriate emission filter, with a CCD camera image displayed on a computer screen.

306
307 5.3. Place a slice anchor made from a U-shaped platinum wire with tightly spaced nylon strings
308 (“harp”) on the slice to maintain it.

309
310 5.4. Change to a 63x immersion objective and adjust the focus. Check for axons expressing
311 Chronos-GFP and choose a pyramidal neuron for patch recording.

312
313 5.5. Move the objective upward.

314
315 5.6. Pull pipettes using a Brown-Flaming electrode puller from borosilicate glass. The puller is set
316 to produce pipettes with approximately 1 μm in tip diameter. Fill the pipettes with K-gluconate-
317 based internal solution.

318
319 5.7. Mount the pipette in the pipette holder on the head-stage. Lower the pipette in the chamber
320 and find the tip under the objective. Pipette resistance should be between 3–8 $\text{M}\Omega$. Apply a light
321 positive pressure with a syringe so as to see a cone of solution outflow out of the pipette and
322 progressively lower the pipette and objective to the surface of the slice.

323
324 5.8. Patch the cell in voltage-clamp configuration: approach the identified neuron and delicately
325 press the pipette tip onto the soma. The positive pressure should produce a dimple on the
326 membrane surface. Release the pressure to create a giga-ohm seal ($>1 \text{ G}\Omega$ resistance). Once
327 sealed, set the holding voltage to -65 mV . Break the membrane with a sharp pulse of negative
328 pressure: this is achieved by applying strong suction to a tube connected to the pipette holder.

329
330 5.9. Record in whole-cell current clamp mode the responses of the neuron to hyperpolarizing and
331 depolarizing current steps (**Figure 2A**).

332
333 NOTE: This protocol will be used to determine active and passive intrinsic properties of the cell.
334 Custom-written MATLAB routines are used for off-line analysis^{10,16}.

335
336 5.10. Record in current- or voltage-clamp postsynaptic responses to whole-field 475 nm LED
337 stimulation of afferent fibers expressing Chronos. Stimulate with trains of 10 stimulations of 2
338 ms durations at 20 Hz (**Figure 2B,C**). Light intensity may vary from 0.1–2 mW.

339
340 NOTE: Light intensity was measured with a digital handheld optical power console equipped with
341 a photodiode sensor, positioned under the objective. Response latencies of 2–4 ms are
342 characteristic for a monosynaptic connection.

343
344 5.11. To investigate the nature of the synaptic transmission between the long-range afferents
345 and the recorded neuron, different pharmacological agents may be used. To pharmacologically
346 distinguish direct, monosynaptic responses from indirect responses via network activation, add
347 1 μM TTX and 100 μM 4-AP to the ACSF.

348
349 NOTE: Bath application of glutamate receptor blockers allows to determine the nature of the
350 neurotransmitter that is released and the identity of postsynaptic receptors. For example, AMPA
351 type glutamate receptors will be blocked by NBQX (10 μM) and NMDA receptors by APV (100
352 μM). Depending on the aim of the study, protocols may be conceived to investigate voltage

353 dependence of synaptic responses or response dynamics over time.

354

355 5.12. Wash with original ACSF solution to patch another cell, or transfer the slice containing a
356 biocytin-filled neuron in a small vial filled with 4% PFA.

357

358 5.13. After overnight fixation in 4% PFA, wash the slice in 0.1 M PBS (2x for 5 min, 1x for 20 min).

359

360 5.14. Store in 30% sucrose at 4 °C.

361

362 **6. Biocytin revelation**

363

364 6.1. Transfer the fixed slices containing biocytin-filled neurons onto a glass blade in a drop of 30%
365 sucrose and perform three cycles of freezing-thawing: place the blade onto dry ice disposed in a
366 styrofoam box for 1 min until drops of sucrose are completely frozen, then press the blade against
367 the hand palm to thaw.

368

369 6.2. Wash the slice 3x in 0.1 M PBS (2x for 5 min, 1x for 1 h and 50 min), gently agitated. Do not
370 exceed 2 h for the last washing.

371

372 6.3. Pre-incubate the slice at RT for 2 h in agitated buffer solution containing 2% milk powder
373 (0.4 g in 20 mL) to saturate non-specific sites and 0.5% Triton X100 (0.1 mL in 20 mL) to
374 permeabilize the membranes in 0.1 M PBS.

375

376 6.4. Incubate overnight at 4 °C in a solution containing 2% milk powder, 1% Triton X100,
377 streptavidin-Cy5 conjugate (1/500), and DAPI (1/1000) in 0.1 M PBS, gently agitated.

378

379 6.5. Wash the slice three times in 0.1 M PBS (2x for 5 min, 1x for 2 h). The last wash can last
380 longer, up to 4 h, to reduce background staining.

381

382 6.6. Before mounting the slice, use an epifluorescence microscope at 10x magnification
383 configured to observe Cy5 fluorescent markers in order to identify the side of the slice containing
384 the marked cell in a chamber filled with PBS.

385

386 6.7. Transfer the slice onto a blade, cell-side up, dry it with a paper tissue, and mount it using
387 high-resistance mounting medium.

388

389 6.8. Use an epifluorescence microscope at 10x magnification in Cy5 and DAPI configuration to
390 examine the cell body location, and in GFP configuration to observe the marked afferents, or a
391 high-resolution confocal microscope at 20x for detailed somatic, axonal, and dendritic
392 morphology (**Figure 2D,E**). Filter settings are detailed in the **Table of Materials**.

393

394 **REPRESENTATIVE RESULTS:**

395 The procedure presented here was used to express a blue light-sensitive channelrhodopsin
396 (Chronos) fused to GFP in the antero-dorsal nucleus of the thalamus (ADN), by stereotaxic

397 injection of anterograde adeno-associated virus. The stereotaxic coordinates were determined
398 according to a mouse brain atlas and tested by injecting 200 nL of fluorescent tracer fluoro-ruby.
399 The animal was sacrificed 10 min after the injection, and the brain was extracted and fixated
400 overnight. Coronal brain sections were prepared to examine the injection site, which was
401 correctly placed in and limited to ADN (**Figure 1A,B**).

402
403 In order to express Chronos-GFP in neurons of ADN, we injected 300 nL of AAV5.Syn.Chronos-
404 GFP.WPRE.bGH. Three weeks after the injection, acute horizontal brain slices were prepared.
405 **Figure 1C** shows a brain slice containing the thalamic injection site in the right hemisphere, with
406 GFP expression in green. Upon inspection with an epi-fluorescence microscope equipped with a
407 4x objective, GFP labeled thalamic axons were observed in the presubiculum (**Figure 1C,D**). It was
408 noted that thalamic axons densely innervated the superficial layers I and III of the presubiculum
409 (**Figure 1D**).

410
411 The activity of presubicular neurons in layer III was recorded in the whole-cell patch-clamp
412 configuration. Hyperpolarizing and depolarizing current steps were applied while recording the
413 membrane potential variations (**Figure 2A**). Data was stored on a computer for later offline
414 analysis of active and passive membrane properties. Presubicular layer III principal cells typically
415 possessed a negative resting potential close to -63 mV and required depolarizing current
416 injections to drive the membrane potential to firing threshold. A full description of their intrinsic
417 properties has been published¹¹.

418
419 Stimulating ADN axon terminals expressing Chronos-GFP elicited excitatory post-synaptic
420 potentials (EPSPs) in presubicular layer III principal cells in current clamp mode (**Figure 2B**).
421 Depending on light intensity, the EPSPs could reach action potential threshold. Postsynaptic
422 responses were also observed in voltage-clamp mode as excitatory post-synaptic currents
423 (EPSCs) were elicited (**Figure 2C**). Onset latencies of EPSCs evoked by light stimulations were
424 short (median, 1.4 ms¹⁰), indicating a direct synaptic contact between thalamic axons and layer
425 III presubicular neurons. Persisting EPSCs in TTX-4AP condition confirmed this monosynaptic
426 activation. It is noteworthy that these cells responded reliably to the light stimulations of afferent
427 axons with a regular firing pattern.

428
429 **FIGURE AND TABLE LEGENDS:**

430
431 **Figure 1: Stereotaxic injection in the anterodorsal thalamic nucleus (ADN).** (A) Schematic
432 representation of the injection. (B) Injection site confirmation with fluoro-ruby in a coronal
433 section. Inset indicates antero-dorsal level and distance from bregma. (C) Horizontal slice
434 following AAV-Chronos-GFP injection in the thalamus. The axonal projections to the ipsilateral
435 presubiculum should be noted. An incision on the left side of the slice (indicated by a black
436 triangle) marks the contralateral hemisphere. (B, C) Scale bar 1 mm. (D) Magnified view of inset
437 in (C) with ADN projections to the presubicular superficial layers. Scale bar = 100 μ m.

438
439 **Figure 2: Presubicular layer III neuron: intrinsic properties, response to light stimulation of**
440 **thalamic afferents, and post-hoc revelation of cell morphology.** (A) Firing pattern and

441 membrane potential variations of layer III neuron for hyperpolarizing and depolarizing current
442 steps. **(B, C)** Responses of layer III neuron to 2 ms light stimulations (blue bars) of thalamic axons
443 recorded in **(B)** current-clamp and **(C)** voltage-clamp modes. **(D, E)** Layer III pyramidal neuron
444 (white, indicated by filled yellow triangle) surrounded by thalamic axons expressing Chronos-GFP
445 (green) in presubicular superficial layers with DAPI staining (blue) in horizontal slice imaged with
446 an epifluorescence microscope **(D)**, scale bar = 100 μm) and confocal microscope at a high
447 magnification **(E)**, scale bar = 50 μm). The cell in **(A)** is indicated with filled yellow triangles. A
448 second, partially filled neuron is present in this slice indicated with empty yellow triangles.

449

450 **DISCUSSION:**

451 In vivo viral injection to express light-sensitive opsins in a defined brain area is a choice method
452 for the optogenetic analysis of long-range functional connectivity^{10,11,17,18}. Stereotaxic injections
453 offer the possibility to precisely target a specific area of the brain. The coexpression of an opsin
454 with a fluorescent reporter conveniently allows evaluation of the successful expression and
455 confirmation of the precise injection site. The use of AAV serotype 2/5 typically restricts
456 expression to the targeted brain region. In this way, a restricted population of neurons is
457 transfected, expressing light-sensitive ion channels in their cell bodies and axon terminals. In
458 subsequent ex vivo slice experiments, it is possible to stimulate these axon terminals with light
459 pulses directly in their target area, while reading out successful synaptic transmission via patch-
460 clamp recording of a post-synaptically connected neuron. The above protocol is robust and
461 convenient, and some additional notes may help performance of successful experiments.

462

463 Different types of anesthesia may be used. Described here is the intraperitoneal injection of a
464 ketamine-xylazine combination as an easy-to-use, short-term anesthesia with convenient
465 analgesia¹⁹. The depth and duration of anesthesia may vary to some extent. In some cases, it may
466 be necessary to inject another half-dose of ketamine-xylazine during the protocol. Isoflurane
467 anesthesia can be a good alternative to induce more quickly and better control the depth of
468 anesthesia. Coordinates of injection sites may be determined with the help of a mouse brain
469 atlas. In practice, coordinates need to be tested and adjusted, if necessary.

470

471 Clean working conditions are also key. It is recommended to use disposable protective gear,
472 including gloves, a mob cap, and a lab coat. When positioning the animal in the stereotaxic frame,
473 special attention should be paid to the comfort of the animal, which will greatly improve
474 efficiency of the anesthesia. The body of the animal should be aligned with the head and neck.
475 The most critical step in positioning the animal and before craniotomy is adjustment of the
476 bregma-lambda axis. Especially when targeting deep brain structures, even a small deviation will
477 generate errors when lowering the injection needle into the brain. In some cases, one may
478 deliberately choose and calculate an oblique needle trajectory.

479

480 The injection volume is a determinant factor for obtaining precisely localized opsin expression. A
481 small volume is ideal to privilege a tightly restricted transfection zone. Higher volumes may be
482 useful to cover the full extent of a large target area. If a large area needs to be covered, such as
483 the septum¹⁸, it may be helpful to place several small injections with a range of neighboring
484 coordinates. The interval until the ex vivo electrophysiological recording is also critical. A

485 minimum time for full expression is necessary. While 3 weeks seem to be an optimal delay for
486 these experiments¹¹, the necessary delay may vary depending on the virus, its serotype, and the
487 distance to the postsynaptic brain region.

488

489 The approach described here is even more powerful when combined with injections in transgenic
490 animals. Previous work has exploited different mouse lines for subtypes of GABAergic neurons,
491 in order to specifically target either PV- or SST-expressing interneurons for patch-clamp
492 recordings²⁰. Simultaneous double recording of neighboring PV and pyramidal neurons or SST
493 and pyramidal neurons then allows comparison of strengths of long-range inputs between two
494 neuron types¹¹. This yields results that are standardized with respect to one neuron type. This
495 standardization is particularly important in cases where the expression levels of opsins vary
496 between different animals or different slices.

497

498 Slice health is essential for high-quality patch-clamp recordings. Constant oxygenation of the
499 slices is crucial, and a slow cutting speed significantly improves slice surface quality. A slice
500 thickness of 300 μm preserves, to some extent, the microcircuit integrity in horizontal
501 presubicular sections, including pyramidal neurons with their cell bodies, dendritic and local
502 axonal ramifications, and local synaptic connections. The type of light-gated channels chosen to
503 induce activation of afferent fibers will greatly influence the stimulation parameters (duration,
504 light intensity). Chronos is a blue light-sensitive channelrhodopsin, and a broad range of
505 illumination wavelengths can be used for activation (peak sensitivity around 500 nm, even with
506 minimal light intensity of 0.05 mW/mm², also activated at 405 nm, and up to 530 nm²¹).
507 Furthermore, Chronos has fast kinetics properties in comparison to classical ChR2, which enables
508 high frequency stimulations and reliable activation of long-range projections²². In combination
509 with the expression of Chrimson, a red-shifted opsin variant, the independent optical excitation
510 of distinct neural populations becomes feasible.

511

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518

519 **DISCLOSURES:**

520 The authors declare no competing financial interests.

521

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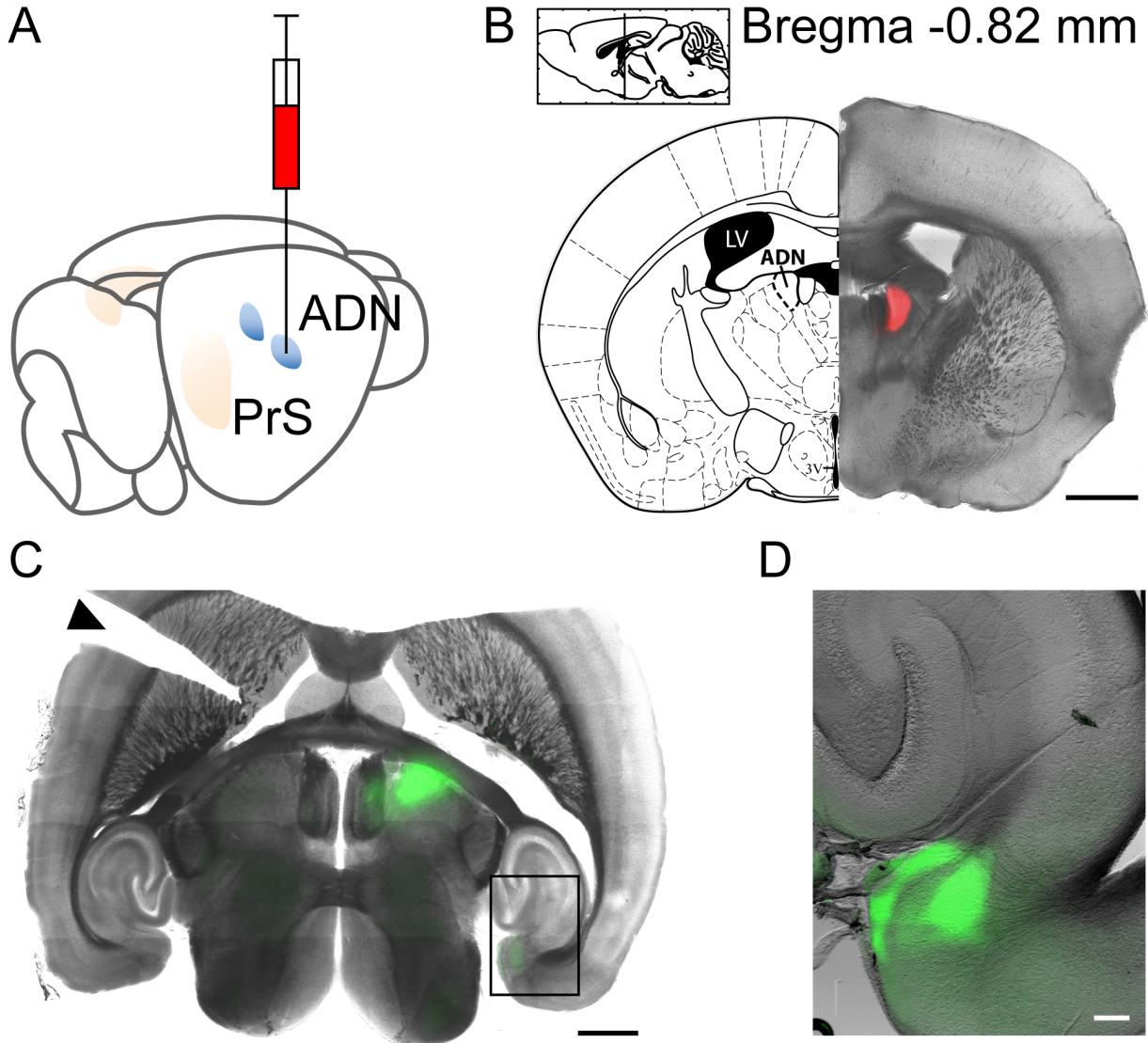
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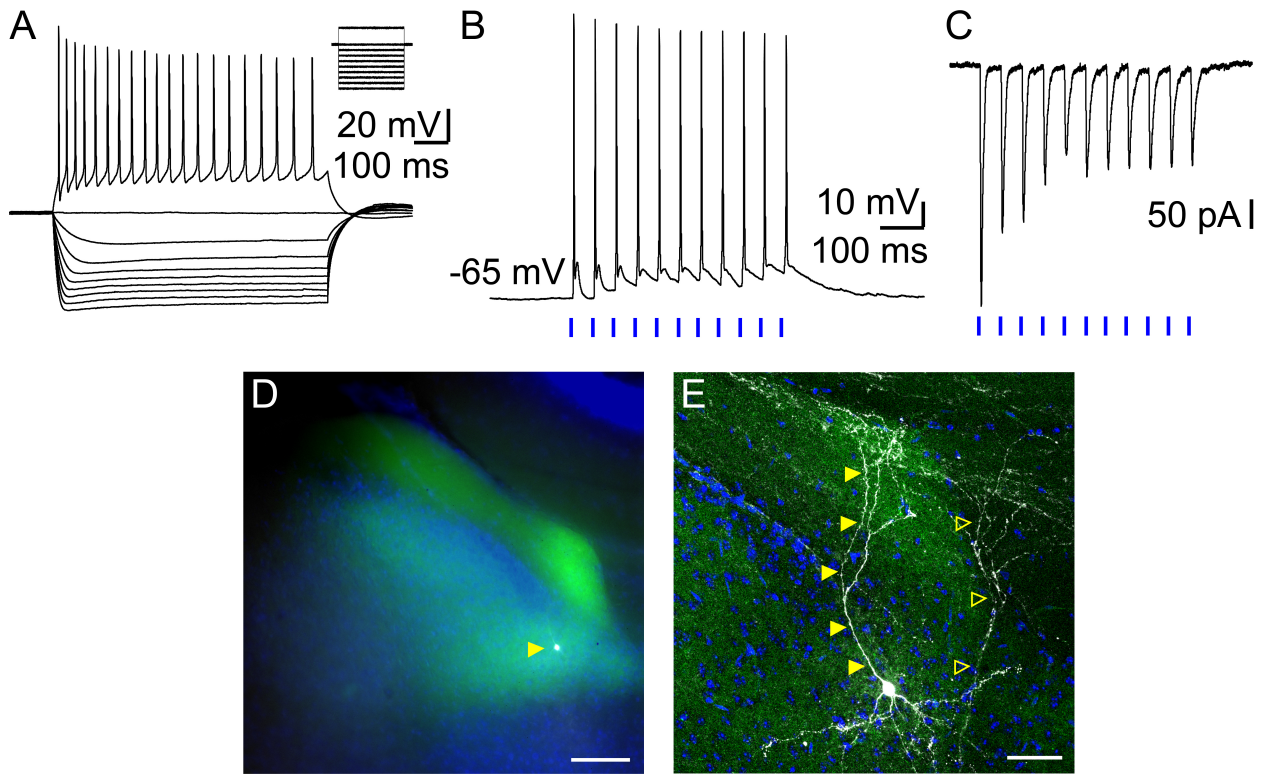
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586

Figure 1.



587

588 Figure 2.
589



590