Journal of Visualized Experiments

Optogenetic manipulation of neuronal activity to modulate behavior in freely moving mice
--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE61023R4
Full Title:	Optogenetic manipulation of neuronal activity to modulate behavior in freely moving mice
Corresponding Author:	Olivia Andrea Masseck, PhD Universität Bremen Bremen, Bremen GERMANY
Corresponding Author's Institution:	Universität Bremen
Corresponding Author E-Mail:	masseck@uni-bremen.de
Order of Authors:	Olivia Andrea Masseck, PhD
	Laura Berg
	Jill Gerdey
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$3000)

1   **TITLE**
2   Optogenetic Manipulation of Neuronal Activity to Modulate Behavior in Freely Moving Mice
3
4   **AUTHORS AND AFFILIATIONS**
5   Laura Berg[1,2], Jill Gerdey[1,2], Olivia A. Masseck[1,2]
6
7   1.   Biology and Chemistry, Synthetic Biology, University of Bremen, Bremen, Germany
8   2.   Advanced Fluorescence Microscopy, Faculty of Biology and Biotechnology, Ruhr-
9   Universität Bochum, Bochum, Germany
10
11   E-Mail addresses:
12   Laura Berg   Laura.Berg@rub.de
13   Jill Gerdey   jill1@uni-bremen.de
14   Olivia A. Masseck   masseck@uni-bremen.de
15

16   **KEYWORDS**
17   Optogenetics, Mice, Optical Fiber, Virus Injection, Implantation, Anxiety, Behavior, Open Field,
18   Novelty-Suppressed-Feeding, Barnes Maze
19

20   **SUMMARY**
21   With optogenetic manipulation of specific neuronal populations or brain regions, behavior can
22   be modified with high temporal and spatial resolution in freely moving animals. By using different
23   optogenetic tools in combination with chronically implanted optical fibers, a variety of neuronal
24   modulations and behavioral testing can be performed.
25

26   **ABSTRACT**
27   Optogenetic modulation of neuronal circuits in freely moving mice affects acute and long-term
28   behavior. This method is able to perform manipulations of single neurons and region-specific
29   transmitter release, up to whole neuronal circuitries in the central nervous system, and allows
30   the direct measurement of behavioral outcomes. Neurons express optogenetic tools via an
31   injection of viral vectors carrying the DNA of choice, such as Channelrhodopsin2 (ChR2). Light is
32   brought into specific brain regions via chronic optical implants that terminate directly above the
33   target region. After two weeks of recovery and proper tool-expression, mice can be repeatedly
34   used for behavioral tests with optogenetic stimulation of the neurons of interest.
35

36   Optogenetic modulation has a high temporal and spatial resolution that can be accomplished
37   with high cell specificity, compared to the commonly used methods such as chemical or electrical
38   stimulation. The light does not harm neuronal tissue and can therefore be used for long-term
39   experiments as well as for multiple behavioral experiments in one mouse. The possibilities of
40   optogenetic tools are nearly unlimited and enable the activation or silencing of whole neurons,
41   or even the manipulation of a specific receptor type by light.
42

43   The results of such behavioral experiments with integrated optogenetic stimulation directly
44   visualizes changes in behavior caused by the manipulation. The behavior of the same animal

45 without light stimulation as a baseline is a good control for induced changes. This allows a
46 detailed overview of neuronal types or neurotransmitter systems involved in specific behaviors,
47 such as anxiety. The plasticity of neuronal networks can also be investigated in great detail
48 through long-term stimulation or behavioral observations after optical stimulation. Optogenetics
49 will help to enlighten neuronal signaling in several kinds of neurological diseases.
50
51 **INTRODUCTION**
52 The modulation of neuronal circuits in the central nervous system and their behavioral outcomes
53 are important for understanding how the brain works, especially in psychiatric diseases and
54 cognitive tasks such as learning and memory. With optogenetics, single cells or cell populations
55 up to whole circuitries can be modulated by light. Common optogenetic tools like
56 Channelrhodopsin2 (ChR2) or Archaerhodopsin (Arch) are able to activate or silence neurons, or
57 increase or inhibit transmitter release at axon terminals projecting to distinct brain regions[1–4].
58 However, Arch needs to be used carefully as it was shown that its activation at presynaptic
59 terminals increases spontaneous transmitter release[5]. Arch is an outward rectifying proton pump
60 that changes the pH value inside the cell. This alkaline milieu induces calcium influx and enhances
61 transmitter release[5]. To specifically modulate intracellular signaling pathways, receptor chimeras
62 composed of a light activatable optogenetic tool, such as rhodopsin or cone opsin, in conjunction
63 with an adequate G-protein coupled receptor, can be created[6–8]. The amount and variation of
64 optogenetic tools available has increased significantly during the last decade[9].
65
66 The purpose of optogenetics is to manipulate neuronal circuitries during behavior. Optogenetics
67 enables, for example, the measurement of acute behavioral changes such as changes in anxiety
68 behavior. Optogenetic tools are delivered into target regions of the brain via viral vectors. With
69 the help of special promoters and enhancers, or the Cre-loxP system, cell type specificity can be
70 ensured for the expression of optogenetic tools (**Figure 1A**). There are several genetically
71 modified mouse lines expressing the enzyme *Cre-Recombinase* in specific cell types only. For
72 example, Nex-Cre mice express the *Cre-Recombinase* in pyramidal neurons in the cortex and the
73 hippocampus under the control of the Nex-promotor[10]. This enzyme is able to invert DNA-
74 sequences, which are flanked by loxP sides[11]. Consequently, the DNA-sequence of a double-
75 floxed optogenetic tool, which is inverted and flanked by loxP sides, can only be transcribed by
76 neurons that possess the *Cre-Recombinase*, but not by other neuronal types[12,13]. In the case of
77 Nex-Cre mice, the optogenetic tool will be solely expressed in pyramidal neurons. Light
78 stimulation of certain brain regions is then achieved via chronic implantation of optical fibers
79 directly above the region of interest. Animals can then be coupled to a suitable light source and
80 freely behave in nearly all kinds of behavioral tests.
81
82 [Figure 1]
83
84 Optogenetic approaches are utilized as it enables both high temporal and spatial resolution[14] and
85 cell type specific modulation. Additionally, it is possible to repetitively use the implanted device
86 without further treatment. After a stereotactic surgery, where the injection of an adeno-
87 associated virus carrying the optogenetic tool and the implantation of the optical fiber is
88 performed, mice can recover for two weeks. We have chosen a recovery time of only 2 weeks,

89 because this is enough time to recover from the surgery and for the virus to express. As the
90 behavioral experiments are followed by immunohistochemistry, we have to ensure that mice do
91 not get too old during the experiment; otherwise the tissue quality is decreased. They show no
92 obvious behavioral impairments from the implant and engage in typical cage behavior. Of course,
93 the implantation is accompanied by a significant surgical lesion; therefore, the mice are
94 monitored intensively. After the surgery, mice need to be single housed, as group housed mice
95 tend to injure each other's fresh wounds and implants. However, housing conditions have a great
96 impact on the anxiety level of male mice, as single housed mice show lower anxiety levels[15] and
97 in general less depressive-like symptoms[16].

99 Chemical or electrical manipulation of brain circuitries lack the high cell type specificity of
100 optogenetics and have a lower temporal and spatial resolution[14,17,18]. Depending on the
101 experimental question, electrical or chemical stimulation can have different advantages. When
102 passing fiber terminals in a specific region also need to be stimulated, electrical stimulation is the
103 best method. Chemical stimulation is a good choice for when transmitter specific receptors in a
104 whole region should be activated by agonists. Another great advantage of optogenetics
105 compared to chemical or electrical stimulation is that endogenously, neurons are not sensitive
106 to light, which avoids the occurrence of side effects[19]. Indeed, high light intensities might induce
107 heating effects[8,20], but due to proper control groups, the behavioral effects due to optogenetic
108 manipulation can be eliminated.

110 Investigating rodent behavior, especially in regards to psychiatric diseases, has greatly improved
111 with optogenetics in freely moving animals, as it enables the direct modulation of single
112 receptors up to specific cell populations[21] and circuitries[22]. The possibility to measure the acute
113 effects of such modulations, as well as the long-term behavioral effects after a defined time[23] or
114 after chronic stimulation[24], enables a wide flexibility of experimental designs and provides very
115 detailed insights into brain circuitries. Light stimulation can be used to modulate neurons located
116 at the injection site of the optogenetic tool. When both the injection and implantation address
117 the same brain region, cell bodies and back projecting axons of principle neurons and
118 interneurons in this region can be targeted[3,6,8]. However, the light fiber can also be implanted in
119 a region different from the injected one. In this case, light stimulation can modulate transmitter
120 release at axon terminals in projection areas of the injected region[25–27].

122 In the study here, optogenetics is used in combination with experiments to analyze anxiety-
123 related behavior. Anxiety-related psychiatric diseases affect more than one third of the world's
124 population[28–30] and cause a high economic burden[31]. Those affected suffer from a feeling of
125 arousal, tension and worry followed by avoidance behavior[32,33]. These chronically occurring
126 negative emotions, which are mainly focused on future events[34], strongly interfere with the daily
127 life of patients. Common treatments like benzodiazepines or selective serotonin reuptake
128 inhibitors (SSRIs) are only successful in some of the patients. A large amount of people do not
129 respond to the treatment at all[35], showing that the mechanism underlying such diseases is not
130 yet fully understood. The medial prefrontal cortex (mPFC) is known to play an important role in
131 the development and manifestation of anxiety[21,25,27,36–38]. Specifically, the overactivation of the
132 infralimbic cortex (IL) region in the mPFC might be part of anxiety-related disorders[39,40]. The

133 example experiment described here could help to understand how modulations in the IL region
134 of the mPFC influence anxiety behavior. Additionally, the development of new therapeutic
135 strategies for anxiety-related psychiatric diseases can also potentially be supported.
136
137 2-6 month old male Nex-Cre mice are used to express ChR2 specifically in pyramidal neurons
138 within the IL region of the mPFC[41]. Nex-Cre mice have a C57Bl/6 background and express the
139 enzyme Cre-recombinase specifically in pyramidal neurons. During a stereotactic surgery, double
140 floxed ChR2-DNA is injected into the IL region via adeno associated viral vectors. The optical
141 implant is placed directly above the region of interest (**Figure 1B**) and the implant is fixed with
142 dental cement. Control animals receive an injection of double floxed tdTomato-DNA in the same
143 region to mimic cell specific expression.
144
145 Animals are group-housed until the day of surgery and afterwards are single housed to avoid
146 injuries from other mice. Mice are housed in individual ventilated cage (IVC) racks in TypI-L cages
147 for single housed mice. The light-dark cycle follows a 12:12 h rhythm, the light phase starting at
148 10 AM. All behavioral experiments are performed in the dark phase, which resembles the active
149 phase of rodents. Water and standard food pellets are available ad libitum. After two weeks of
150 recovery, which ensures a sufficient expression of ChR2 in pyramidal neurons, mice are used for
151 behavioral experiments.
152
153 The Open Field (OF) is a 50 cm x 50 cm squared maze with sandblasted 40 cm high walls. The
154 ground is divided in 16 squares where the inner 4 represent the center. The measured behavior
155 is: 1) time spent in the center, 2) number of center entries, and 3) total distance moved. During
156 this experiment, there are 4 trials totaling 20 minutes. In trials 1 and 3, no light stimulation occurs,
157 and in trials 2 and 4, a 20 Hz stimulation with 5 ms light pulse and 1 mW light intensity of 473 nm
158 is performed (**Figure 2A**). In the later trials, habituation to the testing area was taken into
159 account, but the use of sham-injected control animals indicate how habituation is expressed.
160
161 The Barnes Maze is an experiment for learning and memory. It is a circular platform that is 92 cm
162 in diameter and contains 20 equidistant holes around the circumference. 19 of the holes are
163 closed and under one hole an escape box is presented. For 4 consecutive days, mice have 4
164 training trials to learn the location of the escape box. On the 5th day, the escape box is removed,
165 and mice are tested on how much time they need to find the correct hole. The measured behavior
166 is: 1) Time until the escape box/correct hole is found, 2) Number of target visits and errors, and
167 3) Distance moved until in the escape box. The light stimulation in different groups is done either
168 during acquisition or consolidation, which take place on the training days 1-4, or during retrieval
169 on the testing day, which is day 5 (**Figure 2D**).
170
171 [Figure 2]
172
173 For optogenetic stimulation, the light intensity and frequency have to be adapted to the
174 optogenetic tool and neuronal type that is under investigation. The lowest possible light intensity
175 should be used in order to avoid damage to the tissue, as several studies have shown that there
176 are possible heating effects due to strong light intensity[8,20]. For ChR2, a 20 Hz stimulation with a

177 5 ms light pulse is commonly used[2]. As ChR2 is quite light sensitive, 1 mW light intensity is
178 sufficient. The light stimulation protocol alternates between light off and on trials to directly
179 measure behavioral changes. The external room conditions for behavioral experiments should
180 remain stable for the whole group of animals. Important conditions to consider are the noise
181 (keep in mind that devices themselves might make noise), the smell (always clean the behavioral
182 setups with ethanol), the light intensity, and the experimenter. The experimenter should always
183 be the same person. Additionally, the time of day of the experiments should be the same for all
184 animals in one group, a few hours after the start of the dark phase in the facility is preferred.
185
186 The goal of this experiment is to increase the excitation/inhibition (E/I) ratio in the IL region
187 through strong activation of excitatory pyramidal neurons. An enhanced E/I ratio in this special
188 cortex region is known to increase anxiety levels in mice[40,42–44].
189
190 **PROTOCOL**
191
192 Procedures involving animal subjects have been approved by the institutional animal research
193 facility and the "Senatorin für Wissenschaft, Gesundheit und Verbraucherschutz" at the
194 University of Bremen (#146)
195
196 **1.   Preparation of the optical implant[9] (Figure 1C)**
197
198 1.1.   Place a ceramic ferrule flat side up in a bench vise.
199
200 1.2.   Strip the coat of a 200 µm diameter glass fiber with a fiber stripping tool and cut 2-3 cm
201 long pieces with a ceramic fiber scribe.
202
203 1.3.   Place the piece of glass fiber into the ceramic ferrule with an even overhang on both sides.
204
205 1.4.   Place a drop of superglue at the flat side of the ceramic ferrule with an injection canula.
206
207 NOTE: The protocol can be paused here.
208
209 1.5.   Take the pre-implant out of the bench vise and on the round side of the ceramic ferrule,
210 cut the glass fiber as short as possible with the ceramic fiber scribe.
211
212 1.6.   Place the pre-implant at a ferrule polishing puck and polish the round side on 4 different
213 polishing papers, by drawing an eight 20 times per paper (30 µm grit, 6 µm grit, 1 µm grit, and at
214 last 0.02 µm grit).
215
216 1.7.   Take the pre-implant out of the ferrule polishing puck and cut the glass fiber on the flat
217 side of the ceramic ferrule to the length needed for implantation. Begin measuring the length
218 behind protruding superglue.
219
220 1.7.1. For an even cutting surface just scratch the glass fiber 2-3 times and then break it.

221

222 NOTE: Use the mouse brain atlas from Paxinos and Franklin[45] to calculate the length of the
223 implant. The implant must end directly above the region of interest and the thickness of the skull
224 should be included in the length calculation. To stimulate the IL region, the glass fiber has a length
225 of 1.8 mm (**Figure 3**).

226

227 [Figure 3]

228

229 1.8.   Disinfect the finished implant for 10 minutes in ethanol and let it air dry before
230 implantation.

231

232 **2.   Injection and implantation**

233

234 2.1.   Transport a single mouse to the surgical room and weigh it. Apply anesthesia with an
235 intraperitoneal (i.p.) injection of Ketamine/Xylazine (Ketamine 0.12 mg/g, Xylazine 0.01 mg/g).

236

237 2.1.1. Fix the mouse with the left hand and turn it on its back with the head held low.

238

239 2.1.2. Target the left lower quadrant of the abdomen with the syringe and enter the injection
240 canula 1 cm under the skin.

241

242 2.1.3. Inject anesthesia in a slow and constant motion into the abdominal cavity.

243

244 2.1.4. Place the mouse back into its cage and wait until it reaches a deep state of anesthesia.

245

246 NOTE: The depth of anesthesia can be determined by the absence of blinking and between-toes
247 reflexes.

248

249 2.2.   Place the mouse on a heating plate and fix the head in a stereotactic frame. Fix the nose
250 and teeth in the front, and the ears on both sides.

251

252 NOTE: The head must be straight on the left-right and rostral-caudal axis to ensure correct
253 stereotactic coordinates.

254

255 2.3.   Apply analgesia with 2 mg/kg Carprofen subcutaneously into the back of the mouse and
256 apply opaque eye ointment on both eyes to protect them from drying.

257

258 2.4.   Moisten the hair on the scalp with a wet paper towel and then cut it off using scissors.
259 Make sure to remove all the loose hair with the wet paper towel. To disinfect the scalp, use a
260 cotton stick and take up 0.5 mL of a tincture containing iodine (Betaisodona 100 mg/mL Povidon
261 iodine and 11 mg/mL iodine) and let it air dry.

262

263 NOTE: Instead of scissors, also an electric clipper can be used for proper hair removal.

264

265 2.5.   Raise the scalp above the region of interest with a tweezer and cut 1 cm along the midline.
266 Use two tweezers to push the skin aside to expose the skull. Make sure to also remove the thin
267 skin above the skull and let the exposed skull dry.
268
269 2.6.   Roughen the skull for later implantation.
270
271 2.6.1. Apply a 2 mm x 2 mm drop of phosphoric acid (37%) from the adhesive kit (e.g., Optibond)
272 on the skull, distribute it with the tip of the syringe and let it take effect for 15 s.
273
274 2.6.2. Remove all acid with a cotton stick and rinse the skull with 1 mL of 0.9% NaCl twice.
275
276 2.6.3. Dry the skull with a cotton stick and compressed air.
277
278 Caution: Phosphoric acid is dangerous and must be removed completely to avoid tissue damage.
279
280 2.7.   Calculate the F-Factor for individual coordinates.
281
282 2.7.1. Place a glass canula in the stereotactic frame and locate it directly above bregma.
283
284 2.7.2. Zero the coordinate system and move the glass canula to lambda.
285
286 2.7.3. Calculate the F-factor[46] with the following formula:
287

288
$$\frac{Bregma - Lambda}{4.2} = F - Factor$$

289
290 2.7.4. Multiply the F-Factor with the coordinates from the mouse brain atlas to adjust them to
291 the individual mouse.
292
293 2.8.   Drill a hole in the skull for injection.
294
295 2.8.1. Use the adjusted coordinates to find the location on the skull directly above the structure
296 of interest and mark it using the tip of an injection canula by scratching it above the bone surface.
297
298 2.8.2. Use the injection canula to drill a hole into the skull at the marked location by rotating
299 the canula on the spot. If blood leaks out of the burr hole, rinse with 1 mL of 0.9% NaCl and dry
300 the skull afterwards.
301
302 2.9.   Take up the virus solution into the glass canula.
303
304 2.9.1. Place a drop of 100 µL of 0.9% NaCl onto the skull and a piece of parafilm (1 cm x 1 cm)
305 on top, sterile side up.
306
307 2.9.2. Place 1-2 µL of virus solution onto the parafilm and lower the tip of the glass canula into

308 it.

309

310 2.9.3. Connect the glass canula to a syringe, apply minimal negative pressure and wait until the
311 virus solution is taken up by the cannula (within seconds).

312

313 NOTE: It is important to stop the application of negative pressure, before air is picked up into the
314 canula. Therefore, there will always be a small remnant of the virus solution.

315

316 2.10. Inject the virus solution into the region of interest.

317

318 2.10.1. Place the virus filled glass canula above the burr hole.

319

320 2.10.2. Slowly lower the canula into the burr hole and zero the z-coordinate when the tip of the
321 canula is at the level of the skull.

322

323 2.10.3. Lower the canula carefully to the lowest position of the injection site.

324

325 2.10.4. Focus the binocular at the meniscus of virus solution within the canula.

326

327 2.10.5. Apply a small amount of positive pressure with the syringe until the meniscus is lowered
328 marginally.

329

330 2.10.6. Let the virus spread for 2-3 min before moving the glass canula upwards to the next
331 position.

332

333 2.10.7. Apply the virus solution every 200-300 µm throughout the region of interest.

334

335 2.10.8. Remove the glass canula very slowly and discard it after the final injection.

336

337 2.11. Prepare the skull for implantation with the adhesion kit (e.g., Optibond^{TM}FL).

338

339 2.11.1. Dry the skull with compressed air.

340

341 2.11.2. Apply 5 µL of primer (e.g., Optibond, 1-30% (Ethanol, Silicic acid,
342 Glycerinphosphatdimethacrylat, 2-(2-(Methacryloyloxy)ethoxycarbonyl)benzoesäure, 2-
343 Hydroxyethylmethacrylat)) with the corresponding stick and let it dry for 15 s.

344

345 2.11.3. Apply 5 µL of bond (e.g., Optibond, 15-20% 2-Hydroxyethylmethacrylat + 1-2%
346 Alkalihexafluorosilikat(Na)) with the same stick and cure it for 20 s with UV light (420-480 nm).

347

348 NOTE: It is essential that the skull is dry and that the primer and bond are applied in a very thin
349 layer.

350

351 Caution: Do not directly look into the UV light, as UV light may harm eyes.

2.12. Position the implant directly above the region of interest.

2.12.1. Fix the implant in the corresponding holder.

2.12.2. Dry the skull with compressed air.

2.12.3. Position the tip of the glass fiber directly above the burr hole and lower it carefully.

2.12.4. Stop lowering the implant when the remaining bulb of superglue touches the skull. Do not exert pressure on the skull!

NOTE: If the Injection and implantation are being done in different regions (e.g., *dorsal raphe and hippocampus*), drill all the necessary holes after applying phosphoric acid, but before the 2-component adhesion, then follow the instructions as previously described (step 2.8-2.14).

2.13. Fix the implant.

2.13.1. Check if the skull is still completely dry.

2.13.2. Apply fluid dental cement (e.g., Gradia direct flo) around the implant and in the surrounding area and cure for 20 s with UV light (420-480 nm).

Note: The amount of dental cement depends on the free skull area. The whole skull should be covered by dental cement.

2.13.3. Apply two more layers of cement and completely fill the free and dried skull area. Cure every layer with UV light (420-480 nm).

2.14. Finish the surgery.

2.14.1. Apply 0.5 g iodine ointment (Betaisodona 100 mg/mL povidone iodine and 11 mg/mL iodine) at the whole wound.

2.14.2. Inject 0.1 mL of glucose dissolved in 0.9% NaCl subcutaneously into the neck for quick recovery.

2.14.3. Release the nose and ear fixation, bring the mouse into a fresh cage and place it under a heating lamp to avoid the loss of body heat.

2.14.4. When the mouse wakes up, bring it back into the facility.

2.14.5. Check its health status at least once a day. Take appropriate action if mice display any bad constitutions (e.g., ensure post-operative analgesia with Carprofen up to 3 days if mice display

396 any signs of pain).

397

398 NOTE: After two weeks of recovery, mice can be used for behavioral experiments.

399

400 **3.   Setting up a new experiment (Example ChR2 stimulation and Open Field)**

401

402 3.1.   Pulser

403

404 3.1.1. Program the pulser (e.g., Prizmatix) for light stimulation.

405

406 3.1.2. Open the software and select the USB COM port that the light source is plugged into.

407

408 3.1.3. Choose **Select Operation Mode (3) | Execute pulse sequence after trigger HIGH, then**
409 **stop when LOW** to allow an external software to control the light source.

410

411 3.1.4. Program the light protocol. For a 20 Hz stimulation with 5 ms light pulse: choose TI = 23
412 ms, P1D = 5 ms, P1I = 22 ms and P2D = 0 ms.

413

414 3.1.5. Press **Start Sequence**. This status will remain until experiments are finished.

415

416 NOTE: The pulser software (Prizmatix Pulser) must be launched before the video tracking
417 software; otherwise video tracking software will not be able to recognize the device.

418

419 3.2.   Video tracking software (e.g., Ethovision XT)

420

421 3.2.1. Create a new experiment from a pre-defined template.

422

423 3.2.1.1.   Open the software, go to **File**, choose **New from Template**. Select **Apply a pre-**
424 **defined template**.

425

426 3.2.1.2.   Choose **Live tracking** and select the camera by pressing on **Source** and confirm the
427 connected Basler GenICam.

428

429 NOTE: The live image of the camera will now be displayed in the window on the upper right.

430

431 3.2.1.3.   Press **Next** and choose the animal which should be recorded (**Rodents**, **Mouse**).

432

433 3.2.1.4.   Press **Next** and select the arena template **Open Field, square**. Select the zone
434 template **Center, Border, Corners** and confirm with **Next**.

435

436 3.2.1.5.   Confirm **1 subject** that should be tracked with **Next**.

437

438 3.2.1.6.   Select **Center-point, nose-point and tail-base** and confirm the animal color
439 compared to the background as darker with **Next**.

440

441   3.2.1.7.   Confirm the recommended sample rate of 12.5 with **Next** and finish the step.

442

443   3.2.1.8.   Name the experiment appropriate and choose a location to save.

444

445   3.2.2.   Define the experimental settings.

446

447   3.2.2.1.   Go to **Setup** and **Experimental Settings**. Choose **Center-point, nose-point and tail-**
448   **base detection** as Tracked features.

449

450   3.2.2.2.   Select **Use of Trial Control Hardware** and go to **Settings**.

451

452   3.2.2.3.   Select **Noldus USB-IO box** and confirm with **Ok**.

453

454   3.2.2.4.   Choose **Custom Hardware** as Device Type at the TTL Port, which has been
455   connected to the pulser device, and confirm with **Ok**.

456

457   3.2.3.   Define the arena settings.

458

459   3.2.3.1.   Go to **Arena Settings** and select **Arena Settings 1**.

460

461   NOTE: The camera will now automatically open a background image.

462

463   3.2.3.2.   Confirm the image with **Grab**.

464

465   3.2.3.3.   Adapt the pre-defined zones to the real arena by changing their size. Use the
466   arrow and the two symbols on its right. If some zones are unnecessary, delete them.

467

468   3.2.3.4.   Press **1. Draw Scale to Calibrate** and pull a line from one corner of the maze to the
469   other. Enter the length of the real distance in cm.

470

471   3.2.3.5.   Repeat that for the other axis.

472

473   3.2.4.   Test if the light stimulation is working.

474

475   3.2.4.1.   Go to **Arena - Hardware Mapping** and select **Test** on the grey bar.

476

477   3.2.4.2.   Select Command **Output 1 High** and press **Test**.

478

479   NOTE: There should be light emitting from the end of the optical fiber. When selecting **Output 1**
480   **Low** and **Test**, the stimulation should stop.

481

482   3.2.5.   Define the trial control settings for 20 minutes of experiment. Set trials Off1, On1, Off2
483   and On2 to each be 5 minutes long.

484

3.2.5.1. Go to **Trial Control Setting** and select **Track duration 30 minutes**.

3.2.5.2. Prepare the main rule by adjusting the **Condition: Time** to 20 minutes by Selecting **Settings** and change 30 to 20 minutes. Confirm with **Ok**.

NOTE: The condition for **start track** should be **when subject is in arena for 2 seconds**. That way the system will automatically start tracking when the mouse is in the arena.

3.2.5.3. Create a sub-rule for the light stimulation: Go to **Structures**, **more** and select **Sub-rule**.

3.2.5.4. Give it a name such as **light stimulation protocol**.

3.2.5.5. Place it below the main rule and spread out the two boxes by selecting the blue area with the mouse courser.

3.2.5.6. Go to **Conditions**, **Time** and give it a name like **light on 1**.

3.2.5.7. Adjust **Condition is met** with **after 5 minutes**. Confirm with **Ok**.

3.2.5.8. Place the box directly behind the **Rule Begin** box of the sub-rule by pulling it to the black line.

3.2.5.9. Go to **Action | Custom Hardware** and name it: **light ON 1**.

3.2.5.10. Select **Action to perform** as **Output 1 High** and confirm with **Ok**.

3.2.5.11. Place the box directly behind the **Condition** box.

NOTE: Now after 5 minutes of the experiment the light stimulation should start.

3.2.5.12. Repeat the steps to define the time condition **After 5 minutes** and the action Output 1 Low to stop the light stimulation after another 5 minutes.

3.2.5.13. Repeat the steps again to program another light Off and light On trial.

3.2.5.14. Go to **Structures | Sub-rule reference** and check that the reference belongs to the correct sub-rule.

3.2.5.15. Choose **Start conditions** as **Without delay** and **Stop conditions** as **Execute once per start condition**. Confirm with **Ok**.

3.2.5.16. Place the reference box between action box 1 and condition box 2 of the main rule

528   and draw a line from **Action - start track** to the **Reference**.

530   NOTE: Now the main rule directly starts the sub-rule after starting the track.

532   3.2.6.   Define the detection settings to show the system what it should track.

534   3.2.6.1.   Go to **Detection settings** and select **Detection settings 1**.

536   3.2.6.2.   Place a test mouse into the arena and select **Automated Setup**.

538   3.2.6.3.   Choose **Rodent** as animal type and use the mouse curser to draw a box around
539   the mouse in the arena. Confirm the **Results OK?** question with **Yes**.

541   3.2.7.   Define the trial list for all experimental animals that should be tracked.

543   3.2.7.1.   Go to **Trial List** and plan all animals to record today: Select **add trials** and select a
544   number.

546   3.2.7.2.   Select all the conditions defined before for every mouse.

548   3.2.7.3.   Name the **Animal-ID** and the **Treatment** correctly to later simplify the analysis.

550   NOTE: The Animal-ID is irrelevant for the system and only important for later data analyzation by
551   the experimenter. The grouping in Treatment and Control group is important for the system to
552   know how to group and how to compare all the tracks in later analyzing steps.

554   3.2.8.   Go to **Acquisition** and start with the experiment.

556   **4.   Open Field experiment (anxiety)**

558   4.1.   Bring the experimental mouse to the behavioral room right before the experiment to
559   ensure a proper level of anxiety.

561   NOTE: The behavioral experiments should be performed during the dark phase when mice are
562   awake, and always in the same time slot to ensure comparability.

564   4.2.   Couple the mouse via a sleeve to the light source by pressing it gently onto the grid of the
565   cage.

567   4.3.   Place it into a waiting cage with fresh litter for 10 min to get acclimatized to the light
568   cable.

570   4.4.   Start acquisition by pressing the **Start** button in the video tracking software (e.g.,
571   Ethovision XT).

4.5.   Transfer the mouse from the waiting cage into the left upper corner of the open field. Remove the arm within 2 seconds to avoid tracking an arm instead of the mouse.

4.6.   Leave the visual field of the mouse during the experiment and keep calm.

4.7.   After 20 minutes, when the experiment is finished, remove the mouse from the maze, disconnect the light cable and put it back into its home cage.

4.8.   Bring the mouse back into the facility.

5.   Barnes Maze (learning)

5.1.   Bring all experimental mice into the behavioral room around 1 hour before the experiment.

5.2.   Prepare the Barnes Maze by closing all holes except one, under which an escape box is placed. Place a wall of carton in the middle of the platform, which is the starting area for the mouse.

5.3.   Connect one mouse to the light source (sleeve on a light cable) at both implants.

5.4.   Place the mouse directly into the middle of the Barnes Maze into the wall of carton, which prevents the mouse from running around before the experiment starts.

5.5.   Press **Start** in the video tracking software (e.g., Ethovision XT) and remove the carton.

NOTE: The software tracks the mouse until the correct hole is reached but be prepared to stop the trial manually just in case the software does not recognize the hole transition.

5.6.   Take the mouse out of the maze and remove the connection to the light cable.

NOTE: If this is a training day with several trials per mouse, leave the mouse in the waiting room next to the behavioral room until the next training session starts. If this was the testing day with only one testing trial per mouse, bring the mouse back to the facility.

6.   Data analysis (Example Open Field data with 4 distinguishable trials)

6.1.   Video tracking software (e.g., Ethovision XT)

6.1.1.   Define the experimental groups and trials in the data profile.

6.1.1.1.   Go to **Data Profiles** on the left and choose **Treated vs. Control**.

616   6.1.1.2.   Go to **Nesting** in the new window on the middle-left and select **Trial control state**.

617

618   6.1.1.3.   Choose the state interval from the Element **Action: start Track** to the Element

619 **Action: light goes ON 1**.

620

621   6.1.1.4.   Place the Nesting box between the Filter box **Treatment** and the corresponding

622 result box.

623

624 NOTE: This defined interval is Off1, which describes the first 2.5 minutes of the experiment where

625 no light stimulation is present.

626

627   6.1.1.5.   Repeat the steps for intervals On1 (from element **Action: light goes on 1** to the

628 element **Action: light goes out 1**), Off2 (from the element **Action: light goes out 1** to the element

629 **light goes on 2**) and On2 (from the element **Action: light goes on 2** to the element **Action: stop**

630 **track**).

631

632   6.1.1.6.   Repeat the 4 intervals for the control filter group.

633

634 NOTE: Every nesting box needs its own result box with the names Off1, On1, Off2, On2. Now both

635 the treatment and control group are divided into 4 different light stimulation trials which are

636 analyzed separately.

637

638   6.1.2.   Define the parameters to analyze in the analysis profile.

639

640   6.1.2.1.   Go to **Analysis Profiles** on the left and select **In zones**.

641

642   6.1.2.2.   Select the Dependent Variable **In Zone** and select **Center** as zone.

643

644   6.1.2.3.   Double click on **In center** and choose any of selected points and select only **in**

645 **center**.

646

647   6.1.2.4.   Before leaving the window go to **Trial Statistics** and select **Frequency**, **Cumulative**

648 **duration** and **Latency to first**.

649

650   6.1.2.5.   Add the Dependent Variable **Distance moved**.

651

652 NOTE: In **Group Statistics**, choose whether to use the standard error or standard deviation as

653 error. With this profile, the data for **Time spent in the center**, **Center entries** and **Total distance**

654 **moved** is available.

655

656   6.1.3.   Extract data

657

658   6.1.3.1.   Go to **Results** and select **Statistics and Charts**.

659

660 6.1.3.2.   Press **Calculate** to see the analyzed data.

661

662 NOTE: The trial statistics gives information about every single mouse and group statistics analyzes
663 the mean and error for both groups, divided into 4 trials with the corresponding bar plot.

664

665 6.1.3.3.   Press **Export data** and select the trial statistics and the location to save.

666

667 NOTE: The exported data is saved as an Excel file and with individual values for every mouse. In
668 this Excel file the Animal-ID helps to identify the mice.

669

670 6.1.3.4.   Go to **Heatmap Visualization** and press **Plot Heatmaps**.

671

672 6.1.3.5.   Select **Trials** on the right to see individual heatmaps for every mouse and trial.

673

674 6.1.3.6.   Do a right click on the mouse and export heatmaps as images.

675

676 6.2.   Plotting

677

678 6.2.1.   Open the spreadsheet file at the computer and calculate the means and standard errors
679 (SEM) for all 4 trials in every measured condition and group.

680

681 6.2.2.   Generate graphs in a statistics program (e.g., Sigma Plot).

682

683 6.2.2.1.   Copy the means and SEM into the correct order from the spreadsheet file to Sigma
684 Plot. The rows have to contain the data for Off1, On1 etc. and the columns contain trial, mean
685 and SEM as heads.

686

687 6.2.2.2.   Select all three columns and go to **Create Graph**.

688

689 6.2.2.3.   Select the **Bar** box and choose ungrouped bars with error (upper row, third box).

690

691 6.2.2.4.   Confirm with **Finish** to open a new graph page.

692

693 6.2.2.5.   Label the whole graph, then go to **Home**, select the **Graph** box on the left and
694 press **Export**. Select a destination folder and choose **MetaFile (*.wmf)** as format.

695

696 NOTE: The .wmf format can be processed later in a graphical software like CorelDraw.

697

698 6.2.3.   Calculate statistics for obtained data.

699

700 6.2.3.1.   Copy raw data from the spreadsheet (Off1, On1 etc.) into single columns of Sigma
701 Plot.

702

703 6.2.3.2.   Mark the columns to compare and go to **Analysis**, choose **t-test** and press **Run**.

704

705   6.2.3.3.   Confirm the data format **Raw** with **Next** and run the test with **Finish**.

706

707   **REPRESENTATIVE RESULTS**
708   The aim of this protocol is to measure changes in the behavior of genetically modified mice during
709   an optogenetic experiment. Optogenetic manipulation is done by injection of an adeno
710   associated viral vector. Light stimulation in freely moving mice is possible via implantation of a
711   light fiber directly above the region of interest.

712

713   In **Figure 4**, the results of an optogenetic experiment are presented. A strong activation of
714   excitatory pyramidal neurons in the IL region via ChR2 increased anxiety-related behavior in the
715   Open Field. ChR2 was injected in the IL region of the mPFC in Nex-Cre mice for expression in
716   pyramidal neurons (**Figure 4A**). During two anxiety tests, the Open Field (**Figure 4B,C**) and the
717   Novelty-Suppressed Feeding test (**Figure 4F,G**), ChR2 is stimulated with blue light and activates
718   pyramidal neurons. As control, another group of mice received an injection of the fluorophore
719   tdTomato instead of ChR2 (**Figure 4D,G**). In such experiment, anxiety is defined as avoidance of
720   the brighter central area. Mice show an intrinsic avoidance of open areas because they are
721   anxious of predators.

722

723   In the Open Field experiment, shown in **Figure 4B**, mice executed 4 trials of 5 minutes each. In
724   trials 1 and 3 no light stimulation occurred (Off1,2) and in trials 2 and 4, blue light stimulation
725   with 20 Hz (5 ms light pulse) and 1 mW intensity was performed (On1,2). The heatmaps show
726   that, in the experimental group, the center duration differed between Off and On trials. During
727   light stimulation, mice preferentially stay in the border zone. Control animals also prefer the
728   border, but do not change their behavior upon light stimulation. In **Figure 4C**, the main behavioral
729   measurements during the Open Field experiment are shown for the experimental group. If the
730   data passed the Shapiro-Wilk test for normality, statistics were done with an independent two-
731   tailed t-test. If the normality test failed, the Mann-Whitney-Rank Sum test was used as non-
732   parametric alternative. For these kinds of experiments, a within group comparison was chosen
733   to investigate if light stimulation can directly change anxiety behavior over time, independent of
734   the baseline anxiety of experimental and control animals. The center duration decreased
735   significantly during both light stimulation trials, indicating increased anxiety levels. The total
736   distance moved was not altered, showing that locomotor behavior was not affected. The number
737   of center entries was increased, although not significantly. In **Figure 4D**, the data of the control
738   group is shown. Control animals did not display any behavioral changes between Off and On trials
739   in any of the analyzed parameters, showing that light stimulation or implantation did not cause
740   the observed effects. In sum, this test shows increased anxiety during light stimulation of IL
741   pyramidal neurons via ChR2.

742

743   In **Figure 5**, the data of an unsuccessful optogenetic experiment is shown for the Elevated-Plus
744   Maze. During the Elevated-Plus Maze experiment, which is presented in **Figure 5A,** mice
745   completed 6 trials of 3 minutes each. In trials 1, 3 and 5 no light stimulation was performed (Off1,
746   Off2, Off3) and in trials 2, 4 and 6, blue light stimulation with 20 Hz (5 ms light pulse) and 1 mW
747   intensity was performed (On1, On2, On3). In these exemplary results, the length of the

748 optogenetic protocol and the construction of the maze itself were not suitable for the transgenic
749 mouse line. In **Figure 5B,** it can be seen, that several mice slipped off the maze with their back
750 paws or even fell down. When this happened, mice got a second chance to perform the EPM one
751 day later. If they fell down again, they were excluded from analysis. When mice slipped off several
752 times but managed to stay on the maze, data was analyzed normally. Nevertheless, the data has
753 to be interpreted very carefully and control animals get greater importance. Nex-Cre mice had
754 motor difficulties to stay on the narrow open arms. To avoid this, little walls, with a height of 1
755 cm, would have helped for a secure hold of the back paws on the arms of the maze. Both the
756 heatmaps and the graphs show that experimental, as well as control mice, started to avoid the
757 open arms from trial 2 (On1) on (**Figure 5C-E**). The time on the open arms is significantly
758 decreased for both groups, as are the open arm entries. Analyzing the experimental group only
759 obtained data implicating a large anxiogenic effect of the light stimulation, as time on the open
760 arm and open arm entries are significantly decreased during the On1 trial. However, when
761 comparing this data to the control group, which show the same behavior, it becomes clear that
762 the observed behavior is not mediated by the optogenetic stimulation, but by avoidance of the
763 open arms in general due to habituation to the maze. This data underlines the importance of a
764 proper control group to distinguish between behavioral effects mediated by optogenetic
765 stimulation and possible behavioral adaption. Also, this data sheds light on the importance of
766 properly adapting an experimental setup to suit the specific mousse line and experimental
767 question.
768
769 To validate and strengthen gathered behavioral data, brains of mice are removed after the last
770 experiment to control for the correct injection and implantation (**Figure 6**). Brains are fixed in 4%
771 paraformaldehyde and removed from the skull. Brains are dehydrated in 30% sucrose for 1-2
772 days and cryosliced afterwards. The 40 µm thick coronal brain slices are washed and mounted on
773 superfrost objective slides with a mounting medium containing DAPI, which stains cell nuclei.
774 This enables the identification of target areas in the coronal slices. Fluorescence of the YFP-tag
775 or tdTomato itself indicates the location of the virus injection. In **Figure 6B** the exemplary
776 injection sites of ChR2-YFP on the left (yellow), and tdTomato on the right (red) are presented.
777 With the help of a template, adapted from the Paxinos and Franklin[45] mouse brain atlas, the IL
778 region can be identified. In both slides, the optogenetic tool is expressed in the IL region, but also
779 in adjacent brain regions. For a proper interpretation, the spreading of blue light in brain tissue
780 is consulted[8] (**Figure 1D,E**). It can be seen that the blue light will reach the DP region below the
781 IL with only less than 5% of the initial 1 mW light intensity at the fiber tip (Blue line in Figure 1D)[8].
782 Additionally, small amount of light can go upward to the PrL region due to back-scattering[47].
783 Consequently one can say that the IL region is illuminated the most strongly, however adjacent
784 regions like the DP and PrL region may also be slightly stimulated. Therefore, IL-cell specific
785 stimulation is not guaranteed and immunohistochemical analysis of the adjacent regions should
786 be performed, to see if the activity of PrL and DP cells is modulated via light. In **Figure 6C,** another
787 important control is shown: the specificity of the Nex-Cre mouse line. Via antibody staining
788 against the two cell types in the IL region, glutamatergic principle neurons and GABAergic
789 interneurons, it can be seen, that the ChR2-YFP expression only occurs in glutamatergic neurons
790 and not with GABAergic ones.
791

792 All in all, our experiments show that with optogenetic manipulation during behavioral testing,
793 changes in anxiety-related behavior could be observed. By using more than one test for the same
794 behavior, a reliable conclusion can be drawn. In addition, immunohistochemical analysis confirms
795 the obtained data. Our experiments suggest, that the specific activation of pyramidal neurons in
796 the infralimbic cortex increased anxiety-related behavior in certain assays.
797
798 **FIGURE AND TABLE LEGENDS:**
799 **Figure 1: Injection and implantation. A**) Cre-loxP system for ChR2-YFP. Double floxed
800 optogenetic tool is packed in an adeno associated virus (AAV) for injection into the brain tissue.
801 **B**) Sagittal view of the virus injection and implantation of an optical neuronal interface into/above
802 the IL region of the mPFC. Injection and implantation were done from above. All regions of
803 interest, IL, BLA and DRN, are shown. **C**) Detailed view of the implanted optical fiber, sleeve and
804 light source. **D**) Spreading of blue and red laser light stimulation in grey matter brain tissue from
805 a 200 µm light fiber (Yizhar et al. 2011). Blue light spreads, at maximum, 0.5 mm into the tissue,
806 red light about 1 mm. Color coding: red 50%, yellow 10%, green 5%, blue 1% if light reaches this
807 area. **E**) Coronal view of the unilateral implantation directly above the left IL with a 200 µm optical
808 fiber. The IL region has a width of 0.25 mm in each hemisphere and a depth of 0.2 mm. Blue and
809 red light bulbs are the boarder of 5% light spreading and are transferred from Yizhar et al to the
810 right size. LoxP: locus of X-over P1; ChR2: Channelrhodopsin; YFP: yellow fluorescent protein;
811 dflox: double floxed; IL: infralimbic cortex; BLA: basolateral amygdala; DRN: dorsal raphe nuclei;
812 PrL: prelimbic region. This figure has been modified from Berg 2019[48].
813
814 **Figure 2: Behavioral experiments with optogenetic protocols. A**) Schematic drawing of the Open
815 Field experiment with the corresponding light stimulation protocol. **C**) Schematic drawing of the
816 Elevated-Plus Maze experiment with the corresponding light stimulation protocol. **D**) Schematic
817 drawing of the Barnes Maze experiment with the corresponding light stimulation protocol. EPM:
818 Elevated-Plus Maze; OF: Open Field; BM: Barnes Maze Test. This figure has been modified from
819 Berg 2019[48].
820
821 **Figure 3: Mouse brain atlas (Paxinos and Franklin) with representative length of the implant to**
822 **reach the IL region**
823
824 **Figure 4: Optogenetic activation of IL pyramidal neurons increases anxiety behavior.** Light
825 stimulation during experiments: 473 nm, 1 mW, 20 Hz stimulation. **A**) Schematic drawing of
826 injection and implantation site for ChR2-YFP or tdTomato into the IL. During the experiment,
827 pyramidal neurons in the IL region of the mPFC are activated by ChR2. Saggital brain slices
828 adapted from the Paxinos and Franklin mouse brain atlas, saggital: lateral o,6. **B**) Open Field maze
829 with light stimulation protocol (20 min with 4x5 min alternating Off and On trials; left) and heat
830 maps of exemplary ChR2-injected(EXP) and tdTomato-injected (CT) mice in all 4 trials of the
831 experiment (right). EXP animals spend less time in the center of the OF when stimulated with
832 blue laser light. For CT animals, time spent in the center does not differ between light Off and On
833 trials. **C**) Group data for EXP animals in the OF, n=11. Mice spend significantly less time in the
834 center of the OF when stimulated with blue light (Off1 39.49±6.9 s, On1 19.87±4.47 s, Off2
835 28.13±8.55 s, On2 23.42±9.32 s, Off1:On1, t-test, p = 0.033, *; Off1:On2, MWRS, p=0,049, *).

836   Distance moved is not affected (Off1 2703.09±292.65 cm, On1 3113.4±491.15 cm, Off2 3331.86
837   ±482.62 cm, On2 3082.17±658,61 cm). # of center entries decrease with time, but show no
838   significant differences (Off 1 22.36±3.78, On1 18.45±3.95, Off2 17.36±1.99, On2 13.27±2.64). **D**)
839   Group data for CT animals in the OF, n=15. Time mice spend in the center of the OF, the distance
840   moved, # of center entries does not change between light On and Off trials (Time in center
841   Off116.73±2.65 s, On1 16.02±1.89 s, Off2 12.02±1.76 s, On2 13.04±2.58 s; Distance Off1
842   3399.69±296.77 cm, On1 3210.6±446.9 cm, Off2 3030.28±513.83 cm, On2 2955±617.7 cm; # of
843   center entries Off1 14.2±1.98, On1 13.6±2.02, Off2 10.8±1.88, On2 11.67±2.5). CT mice show
844   significantly higher baseline anxiety (Off1 EXP:CT, MWRS, p=0.005, **). Values are mean±S.E.M.
845   * indicate significant differences (p≤0.05), ** indicate significant differences (p≤0.01). t-test
846   always two-tailed, MWRS: Mann-Whitney Rank Sum test; IL: infralimbic cortex; BLA: basolateral
847   amygdala; DRN: dorsal raphe nuclei; OF: Open Field; CT: control animals; EXP: experimental
848   animal; L: light. This figure has been modified from Berg et al. 2019, PLoS One[43] and from Berg
849   2019[48].
850

851   **Figure 5: EPM experiment failed to show behavioral effects in Nex-Cre mice.** Light stimulation
852   during experiments: 473 nm, 1 mW, 20 Hz stimulation. **A**) Elevated-Plus Maze with light
853   stimulation protocol (18 min, 6x3 min, alternating Off and On trials). **B**) Group data for the mice
854   that "slip off" is included into the data, total n=23. Nex-Cre mice had a tendency to slip off of the
855   open arm with their back paws, independent of the experimental group (left). Only mice that
856   stayed on the maze for all 6 trials were considered in later analyses. Slip off's in the first Off1
857   phase are reason for later avoidance of the open arms (Off1 EXP 1.63±0.6, CT 2.2±0.79, Off2+3
858   EXP 0.125±0.125, CT 0±0, On1+2+3 EXP 0.625±0.26, CT 0.1±0.1). Pie chart (right) shows mice
859   falling from the maze during the 18 min with 42.42%. Only 57.57% finished the experiment. **C**)
860   Heat maps of exemplary EXP and CT mice in all 6 trials of the experiment. Both groups show a
861   decrease in open arm duration after the Off1 trial. **D**) Group data for EXP animals in the EPM,
862   n=12. Time spent in the open arms decreased significantly during the first two trials and
863   constantly afterwards (Off1 73.91±12.22 s, On1 36.15±14.65 s, Off2 15.61±6.23 s, On2
864   19.49±7.51 s, Off3 9.36±4.44 s, On3 7.96±3.47 s. Off1:On1, t-test, p=0,041, *). The distance
865   moved is not affected (Off1 679.96±71.63 cm, On1 712.24±112.82 cm, Off2 717.49±97.39 cm,
866   On2 782.51±81.11 cm, Off3 722.11±68.60 cm, On3 663.90±106.57 cm). The amount of open arm
867   entries decreases significantly from Off1 to On1 and then stays constant (Off1 8.08±1.08, On1
868   3.33±0.76, Off2 2.16±0.69, On2 2.91±1.09, Off3 1.73±0.75, On3 1.73±0.66. Off1:On1, t-test,
869   p=0.002, **). The time spent in the center of the EPM decreases along trials but shows no
870   significant difference from Off to On trial (Off1 41.71±5.34 s, On1 31.2±4.59 s, Off2 19.8±3.44 s,
871   On2 24.49±3.38 s, Off3 20.37±4.77 s, On3 18.85±4.07 s). **E**) Group data for CT animals in the EPM,
872   n=11. CT data shows the same significant decreases as EXP data, indicating the experiment has
873   not worked properly (Time in open arms Off1 86.92±12.74 s, On1 33.78±14.38 s, Off2
874   18.01±11.61 s, On2 16.41±9.61 s, Off3 11.36±4.01 s, On3 5.43±2.07 s. Off1:On1, MWRS, p=0.009,
875   **; Distance Off1 705.11±88.36 cm, On1 789.45±77.53 cm, Off2 724.74±80.49 cm, On2
876   676.57±111.99 cm, Off3 716.99±132.47 cm, On3 663.03±132.46 cm; Open arm entries Off1
877   7.09±1, On1 3.72±1.17, Off2 1.45±0.47, On2 1.36±0.58, Off3 0.91±0.43, Off3 1.64±0.59.
878   Off1:On1, MWRS, p=0.01, *; Time spend in the center Off1 35.89 s, On1 29.25±3.96 s, Off2
879   22.17±3.58 s, On2 15.9±2.57 s, Off3 13.86±4.2 s, On3 16.89±5.75 s). Values are mean ± S.E.M. *

880 indicate significant differences (p≤0.05), ** indicate significant differences (p≤0.01). t-test is
881 always two-tailed, MWRS: Mann-Whitney Rank Sum test; EPM: Elevated-Plus Maze; CT: control
882 animals; EXP: experimental animal; OA: open arms. This figure has been modified from Berg et
883 al. 2019, PLoS One[43] and from Berg 2019[48].

884

885 **Figure 6: Injection side of ChR2 and tdTomato in the IL and Nex-Cre specificity. A**) Schematic
886 drawing of the implantation site on coronal brain slices at AP + 1.66 mm, mL 0.3 mm, DV -1.8
887 mm, with unilateral injection and implantation (adapted from mouse brain atlas, Paxinos and
888 Franklin, Bregma +1.54 mm). **B**) Exemplary injection sites of ChR2-YFP (left, yellow) and tdTomato
889 (right, red) merged with DAPI stained cell nuclei (blue) in Nex-Cre mice. Scale bar 1 mm. Insets
890 show high magnification of IL region. Scale bar 150 μm. White boxes indicate location of insets**.**
891 **C**) Top row: confocal images of the left IL region of a Nex-Cre mouse stained with CamKII as a
892 marker for glutamatergic neurons (blue), and ChR2-YFP (yellow) or Gad67 as a marker for
893 GABAergic neurons (green), of a Nex-Cre mouse. Bottom row: colocalization of ChR2-YFP (yellow)
894 with CamKII (left, blue), but not with Gad67 (right, green), showing specificity of Nex-Cre mice
895 for glutamatergic neurons. Scale bar 50 μm. PrL: prelimbic cortex; IL: infralimbic cortex; DP:
896 dorsal peduncular cortex. This figure has been modified from Berg et al. 2019, PLoS One[43] and
897 from Berg 2019[48].

898

899 **DISCUSSION**
900 Using light to manipulate neuronal signaling has been the method of choice for nearly one decade
901 now. Since 2005, the number of published articles about the development of new optogenetic
902 tools[4,6,8,14,49–51] and studies where such tools are utilized to investigate brain circuits[21,23,40,43,52],
903 highly increased. On one hand, with the enormous diversity of injectable optogenetic tools,
904 implantation variants, transgenic mouse lines and behavioral experiments, the possibility for
905 experiments is manifold and unlimited. On the other hand, the possibility to make faults in
906 choosing experimental conditions is very high and the experiments are so specific, that often the
907 comparability to other studies is difficult.

908

909 **Critical steps**
910 One important critical step of this protocol is proper planning. The choice of the optogenetic tool
911 should match the scientific question. Is it only necessary to manipulate the overall activity of a
912 neuron or synapse? Then commercially provided tools like ChR2[21,25,27] and Arch[37] are a good
913 choice. But apart from that, if one special neurotransmitter system or even a single receptor
914 should be manipulated, an individual receptor chimera is often the better choice[3,6]. Several
915 receptor chimeras with GPCRs, the so called Opto-XRs, and guidelines to produce them are
916 already available[4,50]. Other than the choice of optogenetic tools, the mouse line in combination
917 with the behavioral experiment is also critical. Different background strains, like for example
918 C57Bl/6 and BALB/cByJ, display different behavioral phenotypes in some respects[53,54]. C57Bl/6
919 mice have a low baseline anxiety and can be used for anxiogenic manipulation, whereas
920 BALB/cByJ show higher anxiety levels and are therefore more sensitive to anxiolytic drugs.
921 Additionally, the transgenic variants of these background strains may also vary in their
922 phenotype[48]. With a proper combination of specific promoters in conjunction with an
923 optogenetic tool and transgenic mouse line, nearly every desired cell population can be targeted.

924

925   A critical step during surgery is targeting the correct location. With the help of the mouse brain
926   atlas, proper coordinates for the anterior-posterior axis, and medial-lateral axis, and depth of the
927   structure can be established[45]. In reality, every skull has a slightly different form and size. Thus,
928   the F-factor[46] to adjust the stereotactic coordinates is quite important, as is the correct nose and
929   ear fixation during stereotactic surgery. If the head of the mouse is tilted, the injection canula
930   will fail to target the desired region of interest.

931

932   Additionally, the diameter of the injection canula is also critical. If it is too small, no virus can be
933   released into the tissue, if it is too wide, the canula will leak virus solution on its way to the region
934   of interest. If the implanted optical fiber terminates directly above the target region, the virus
935   expression in the cortex regions above does not matter. But if the implant is placed above other
936   regions to stimulate axon terminals, the axons of upper cortex regions will also be activated by
937   light and falsify obtained data. As an example: The IL region and the prelimbic (PrL) region both
938   project to the basal amygdala[55,56] but have completely different functions and roles in the
939   modulation of anxiety[26,57]. If the implant is placed above the amygdala to activate axon terminals
940   from the IL region, and during the injection virus solution was also placed into the PrL due to the
941   wrong injection canula, the risk of also activating axon terminals from the PrL is very high.

942

943   During the preparation of the skull for the fixation of the implant, the sparse usage of primer and
944   bond is crucial for a reliable and durable fixation. If the 2-component adhesion system is not
945   applied thinly, the dental cement might detach from the skull after a couple of days or weeks. In
946   addition, the skull also has to be completely dried before fixing the implant, as otherwise the
947   cement will not attach properly to the skull.

948

949   Critical steps also exist in the behavioral part of this protocol. First, the construction of the maze
950   is very important. In every behavioral setup, several variants exist in the literature regarding size
951   and form, as well as for the procedure itself[58–60]. It is important to choose a variant that makes
952   the data comparable and reproducible. Also, special requirements for utilized mouse lines should
953   be taken into account[43,48]. In the representative data for the EPM it can be seen that several Nex-
954   Cre mice fell from the maze or slipped off several times (**Figure 2b**). For these mice, a maze with
955   a small wall around the open arms would have been a better alternative.

956

957   Second, it is critical to keep all external room conditions constant[61], otherwise different groups
958   of mice would not be comparable at all. In this regard, it is very important to choose the time of
959   the experiment as one where the experimental setup is vacant and the experimenter is always
960   present. Furthermore, events in the building, such as construction work, testing of any systems
961   (fire alarm) or the cleaning day of the mouse facility, should be considered in order to avoid
962   interference with the obtained data.

963

964   Finally, handling and housing conditions are critical for behavioral experiments. When an
965   implantation is performed, mice need to be single housed because of the risk of injury from other
966   mice. To ensure good comparability between groups and a low error within one group, every
967   mouse needs to have the same cage size and enrichment. For anxiety-related experiments, single

968 housing has some advantages as singe housed male mice show a lower baseline anxiety level,
969 less variation in their anxiety level, and less depressive-like symptoms [15,16]. Group housed male
970 mice might strongly differ in their anxiety level because of hierarchy among the mice. Besides the
971 housing, a constant and equal handling of all mice and groups is also important. Grabbing the
972 mouse in order to connect the light fiber on the implant is very stressful. Therefore, this
973 procedure has to be the same for every mouse, meaning the same technique and the same
974 experimenter. Furthermore, the habituation time in the waiting cage, which is meant to calm the
975 mouse down from the stressful connecting procedure, also needs to have equal conditions in
976 duration, litter and position to the maze. The handling within the mouse facility is also critical for
977 later behavioral performance. Experimental and control animals should not be cleaned on
978 different days or by different people, as this is also stressful for mice. Additionally, the cleaning
979 day should not be the experimental day to avoid differences in behavior.
980
981 **Troubleshooting**
982 There are several problems which might occur during the protocol. For example, drilling a whole
983 in the skull during the stereotactic surgery could damage blood vessels. Usually, strong bleeding
984 occurs, especially above bregma and lambda. If this happens, do not try to stop the bleeding with
985 cotton sticks as they tend to extend even more bleeding out of the vessel because of their
986 absorbency, instead, directly rinse with NaCl.
987
988 It can also happen that the pressure injection of the virus solution is not working. In this case, it
989 could be that parafilm, a scab from the burr hole or brain tissue, is clogging the tip of the canula.
990 In this case, remove the canula slowly out of the brain without changing the x- or y-axis and use
991 a tweezer to remove 1-2 mm of the front part of the canula tip. Before lowering the canula again,
992 test for functionality by applying small amount of pressure to see if virus comes out of the canula
993 tip. To avoid constipation, lower the canula with a constant speed and do not stop the movement
994 until the deepest depth of the injection side is reached. If too much of the canula tip is removed
995 and the diameter is too large, the canula will damage tissue and the risk of applying the virus all
996 at once will be increased. Thus, make sure that only the clogged part of the tip is carefully
997 removed.
998
999 During the behavioral experiment, the setup of the experiment in the video tracking software
1000 (e.g., Ethovision XT) might cause problems. If, for example, the light output is not working
1001 properly, this can be due to several reasons. The Pulser has to be opened, programmed and
1002 started before Ethovision XT is opened. The hardware needs to be selected correctly in the
1003 "Experimental setup" (step 3.2.2.4). If the wrong IO-Box, or anything other than "Costume
1004 Hardware" is selected, the Pulser device cannot be controlled by Ethovision. If the test of the
1005 light output is successful, but the programmed light protocol in "Trial control settings" does not
1006 work during acquisition, the sub-rule or sub-rule reference might be located incorrectly or the
1007 conditions and actions are unclear. For example: does the reference belong to the correct sub-
1008 rule? Is the reference programmed correctly (e.g., how often is the sub-rule executed)?
1009
1010 Additionally, it might happen that during "detection settings" the animal is adequately tracked,
1011 but during acquisition there are samples where the subject is not found. In this case, check if the

1012 illumination in the experimental room was changed, or if anything produced unwanted shadows
1013 within the maze. The entire bottom of the maze has to have the same color, as the setting will
1014 only work for one specific combination. If for whatever reasons different bottom colors or
1015 shadows can not be avoided, define the detection setting in the darkest part of the maze.
1016
1017 To change any settings after the acquisition of the first animals, do not apply these changes in
1018 the already used settings. Duplicate them to adjust them. This also means that the already
1019 recorded trial is not valid anymore for data analysis. In such a case, record all animals for this
1020 experimental group with the original settings, and create a new experiment afterwards where
1021 the recorded videos are analyzed instead of live tracking. In this "from video" experiment, several
1022 settings can be used for analysis without losing comparability between animals or even data.
1023

1024 Limitations and future applications
1025 This method of manipulating behavior with optogenetics in freely moving animals also includes
1026 limitations. During the surgery, the proximity of the two implants is restricted. For double
1027 implantation, the distance between the two implants must minimally be the width of the
1028 apparatus to hold the implant. The apparatus needs to lower the second implant into the burr
1029 hole, while the first implants is already fixed. A solution for this might be an angled implantation,
1030 where the tips of the glass fiber can be very close while the ceramic ferules above the skull have
1031 larger distance[23,55–57,62,63]. A disadvantage of an angled implantation is the light spreading. When
1032 the fiber tip is slanted instead of from straight above, the stimulated area is different. In case of
1033 two target regions in close proximity, the changed position of the light stimulation needs to be
1034 considered.
1035
1036 During the behavioral experiment, the construction of the maze might interfere with the optical
1037 cable connected to the animal. Some behavioral tests, such as the light-dark box, contain an
1038 indoor area[64,65], and other mazes contain compartments which the mouse needs to enter. Such
1039 experiments cannot be performed with this setup. Alternatively, a wireless system might be an
1040 option[22,26,66]. But luckily some mazes, such as the Barnes Maze, can be arranged in such a way,
1041 that the mice are able to enter the relevant compartments[67].
1042
1043 Besides those with closed zones, mazes that are too wide can cause also problems. The larger
1044 the area of the maze, the longer the cable has to be to allow the animal to go to every position
1045 in the maze. Care has to be taken that the animal is not able to step on the cable or grab it and
1046 bite it. A solution for that might be a construction that rolls up the redundant cable. A
1047 disadvantage is that the drag to unroll the cable is hard for mice. This solution would better suit
1048 suited for rats. Another possible option could be to do the light stimulation in advance, instead
1049 of during the experiment, of course this is only feasible if a long-term effect due to the light
1050 stimulation occurs[23].
1051

1052 Comparison to existing/alternative methods
1053 Alternative methods would be chemical or electrical stimulation during behavior[8,18]. Chemical
1054 agonists or antagonists are able to activate or silence neurons via specific receptors and can also
1055 manipulate single neurotransmitter systems[38,68]. On one hand, the receptor-specificity is quite

1056 high for chemicals, because specific agonist or antagonist only activate certain receptors [39]. On
1057 the other hand, the specificity for receptor subtypes of the same neurotransmitter group is often
1058 insufficient. Most chemicals bind to at least two sub-types with different probabilities[69].
1059 Additionally, chemicals cannot distinguish between neuronal cell types as long as they possess
1060 the same receptor types. Beyond this, temporal and spatial resolution is poor for chemical
1061 manipulations in comparison to optogenetics. Agonists or antagonists are often administered
1062 orally[35] or via systemic injections[57,70]. If the infusion of the chemical is done directly in the brain
1063 tissue, effects appears faster than with oral applications, but still on a slower timescale than with
1064 light stimulation. As the administered chemicals diffuse in the brain and are not specific for
1065 neuronal types or brain regions, manipulation of specific brain circuitries it not possible.
1066
1067 Electrical stimulation has a higher temporal resolution than chemical stimulation[9,14]. The spread
1068 within in neuronal tissue is less than with chemical stimulation and the spatial resolution is better
1069 than with chemical stimulation. However, electrical stimulation lacks the possibility to specifically
1070 address different neuronal cell types or receptor types, as every neuron in proximity to the
1071 electrode will respond to the electrical stimulation.
1072
1073 Alternative methods to the behavior in freely moving mice are for example electrophysiological
1074 recordings in brain slices, where single neurons or axons can be modulated with optogenetics
1075 and elicited effects can be measured via recording electrodes[6,71]. *In vitro* experiments offer the
1076 possibility to investigate the molecular and cellular basis of optogenetic stimulations but have
1077 the limitation that intrinsic connectivity and input from other brain regions is missing. Another
1078 option is to use optogenetic in conjunction with multiphoton imaging[1,72]. In this case, mice have
1079 their head fixed and can be anesthetized or be awake to solve simple tasks.
1080
1081 To perform a successful optogenetic experiment, a wide range of tools and applications are
1082 available nowadays. The selection of optogenetic tools and the behavioral set-up is critical to
1083 answer specific research questions. If the right combination of tools and experiments is chosen,
1084 optogenetics allows an unprecedented, in-depth investigation of neuronal circuitries with high
1085 temporal and spatial resolution. This will help to understand and develop new therapeutic
1086 strategies for psychiatric diseases and cognition.
1087

1098

1099 **DISCLOSURES**

1100   The authors have nothing to disclose.
1101
1102   **REFERENCES**
1103   1.   Chow, B. Y. et al. High-performance genetically targetable optical neural silencing by light-
1104   driven proton pumps. *Nature Letters* **463**, 98–102 (2010).
1105   2.   Zhao, S. et al. Cell type–specific channelrhodopsin-2 transgenic mice for optogenetic
1106   dissection of neural circuitry function. *Nature Methods* **8**, 745–752 (2011).
1107   3.   Spoida, K., Masseck, O. A., Deneris, E. S. & Herlitze, S. Gq/5-HT2c receptor signals activate
1108   a local GABAergic inhibitory feedback circuit to modulate serotonergic firing and anxiety in mice.
1109   *Proceedings of the National Academy of Science of the United States of America* **111**, 6479–6484
1110   (2014).
1111   4.   Kleinlogel, S. Optogenetic user ' s guide to Opto-GPCRs modified GPCRs. *Frontiers in
1112   Bioscience* **21**, 794–805 (2016).
1113   5.   Mahn, M., Prigge, M., Ron, S., Levy, R. & Yizhar, O. Biophysical constraints of optogenetic
1114   inhibition at presynaptic terminals. *Nature Neuroscience* **19**, 554–556 (2016).
1115   6.   Masseck, O. A. et al. Vertebrate Cone Opsins Enable Sustained and Highly Sensitive Rapid
1116   Control of Gi/o Signaling in Anxiety Circuitry. *Neuron* **81**, 1263–1273 (2014).
1117   7.   Oh, E., Maejima, T., Liu, C., Deneris, E. & Herlitze, S. Substitution of 5-HT 1A Receptor
1118   signaling by a light-activated G protein-coupled receptor. *Journal of Biological Chemistry* **285**,
1119   30825–30836 (2010).
1120   8.   Yizhar, O., Fenno, L. E., Davidson, T. J., Mogri, M. & Deisseroth, K. Optogenetics in Neural
1121   Systems. *Neuron Primer* **71**, 9–34 (2011).
1122   9.   Masseck, O. A. A Guide to Optogenetic Applications, With special Focus on Behavioral and
1123   In Vivo Electrophysiological Experiments. in *HandboOk of In Vivo Neural Plasticity Techniques - A
1124   Systems Neuroscheince Approach to the Neural Basis of Memory and Cognition* (ed. Manahan-
1125   Vaughan, D.) 557 (Academic Press, 2019).
1126   10.   Goebbels, S. et al. Genetic Targeting of Principal Neurons in Neocortex and Hippocampus
1127   of NEX-Cre Mice. *Genesis* 611–621 (2006) doi:10.1002/dvg.
1128   11.   Yang, Y. S. & Hughes, T. E. Cre Stoplight: A red/green fluorescent reporter of Cre
1129   recombinase expression in living cells. *Biotechniques* **31**, 1036–1041 (2001).
1130   12.   Schnütgen, F. et al. A directional strategy for monitoring Cre-mediated recombination at
1131   the cellular level in the mouse. *Nature Biotechnology* **21**, 562–565 (2003).
1132   13.   Taniguchi, H. et al. A Resource of Cre Driver Lines for Genetic Targeting of GABAergic
1133   Neurons in Cerebral Cortex. *Neuron* **71**, 995–1013 (2011).
1134   14.   Deisseroth, K. Optogenetics. *Nature Methods* **8**, 268–273 (2011).
1135   15.   Palanza, P., Gioiosa, L. & Parmigiani, S. Social stress in mice: Gender differences and
1136   effects of estrous cycle and social dominance. *Physiology and Behavior* **73**, 411–420 (2001).
1137   16.   Karolewicz, B. & Paul, I. A. Group housing of mice increases immobility and antidepressant
1138   sensitivity in the forced swim and tail suspension tests. *European Journal of Pharmacology* **415**,
1139   197–201 (2001).
1140   17.   Masseck, O. A., Rubelowski, J. M., Spoida, K. & Herlitze, S. Light- and drug-activated G-
1141   protein-coupled receptors to control intracellular signalling. *Experimental Physiology* **96**, 51–56
1142   (2011).
1143   18.   Aravanis, A. M. et al. An optical neural interface: in vivo control of rodent motor cortex

1144 with integrated fiberoptic and optogenetic technology. *Journal of Neural Engineering* **4**, (2007).

1145 19. Zhang, F. et al. Multimodal fast optical interrogation of neural circuitry. *Nature Article*
1146 **446**, 633–639 (2007).

1147 20. Owen, S. F., Liu, M. H. & Kreitzer, A. C. Thermal constraints on in vivo optogenetic
1148 manipulations. *Nature Neuroscience* **22**, 1061–1065 (2019).

1149 21. Hare, B. D. et al. Optogenetic stimulation of medial prefrontal cortex Drd1 neurons
1150 produces rapid and long-lasting antidepressant effects. *Nature Communication* **10**, 1–12 (2019).

1151 22. Allsop, S. A., Vander Weele, C. M., Wichmann, R. & Tye, K. M. Optogenetic insights on the
1152 relationship between anxiety-related behaviors and social deficits. *Frontiers in Behavioral*
1153 *Neuroscience* **8**, 1–14 (2014).

1154 23. Fuchikami, M. et al. Optogenetic stimulation of infralimbic PFC reproduces ketamine's
1155 rapid and sustained antidepressant actions. *Proceedings of the National Academy of Science of*
1156 *the United States of America* **112**, 8106–8111 (2015).

1157 24. Correia, P. A. et al. Transient inhibition and long-term facilitation of locomotion by phasic
1158 optogenetic activation of serotonin neurons. *Elife* **6**, 1–26 (2017).

1159 25. Felix-Ortiz, A. C., Burgos-Robles, A., Bhagat, N. D., Leppla, C. A. & Tye, K. M. Bidirectional
1160 modulation of anxiety-related and social behaviors by amygdala projections to the medial
1161 prefrontal cortex. *Neuroscience* **321**, 197–209 (2016).

1162 26. Marek, R., Xu, L., Sullivan, R. K. P. & Sah, P. Excitatory connections between the prelimbic
1163 and infralimbic medial prefrontal cortex show a role for the prelimbic cortex in fear extinction.
1164 *Nature Brief Communication* (2018).

1165 27. Parfitt, G. M. et al. Bidirectional Control of Anxiety-Related Behaviors in Mice: Role of
1166 Inputs Arising from the Ventral Hippocampus to the Lateral Septum and Medial Prefrontal Cortex.
1167 *Neuropsychopharmacology* **42**, 1715–1728 (2017).

1168 28. Bandelow, B. & Michaelis, S. Epidemiology of anxiety disorders in the 21st century.
1169 *Dialogues in Clinical Neuroscience* **17**, 327–335 (2015).

1170 29. Kessler, R. C. et al. Lifetime Prevalence and Age-of-Onset Distributions of DSM-IV
1171 Disorders in the National Comorbidity Survey Replication. *Archives of General Psychiatry* **62**, 593–
1172 602 (2005).

1173 30. Kessler, R. C., Petukhova, M., Sampson, N. A., Zaslavsky, A. M. & Wittchen, H.-U. Twelve-
1174 month and lifetime prevalence and lifetime morbid risk of anxiety and mood disorders in the
1175 United States. *International Journal of Methods Psychiatric Research* **21**, 169–184 (2014).

1176 31. Andlin-Sobocki, P. & Wittchen, H. U. Cost of anxiety disorders in Europe. *European Journal*
1177 *of Neurology* **12**, 39–44 (2005).

1178 32. Forster, G. L., Novick, A. M., Scholl, J. L. & Wall, M. J. The Role of the Amygdala in Anxiety
1179 Disorders. *Intech* 61–102 (2012).

1180 33. Liberzon, I. Neural circuits in anxiety and stress disorders : a focused review. *Therapeutics*
1181 *and Clinical Risk Management* **11**, 115–126 (2015).

1182 34. Sylvers, P., Lilienfeld, S. O. & LaPrairie, J. L. Differences between trait fear and trait anxiety:
1183 Implications for psychopathology. *Clinical Psychology Review* **31**, 122–137 (2011).

1184 35. Daws, L. C., Koek, W. & Mitchell, N. C. Revisiting Serotonin Reuptake Inhibitors and the
1185 Therapeutic Potential of 'Uptake-2' in Psychiatric Disorders. *ACS Chemical Neuroscience* **4**, 16–21
1186 (2013).

1187 36. Felix-Ortiz, A. C. et al. BLA to vHPC inputs modulate anxiety-related behaviors. *Neuron*

1188 *Report* **79**, 658–664 (2013).

37.   Padilla-Coreano, N. et al. Direct Ventral Hippocampal-Prefrontal Input Is Required for Anxiety-Related Neural Activity and Behavior. *Neuron Article* **89**, 857–866 (2016).

38.   Lisboa, S. F., Stecchini, M. F., Corrêa, F. M. A., Guimarães, F. S. & Resstel, L. B. M. Different role of the ventral medial prefrontal cortex on modulation of innate and associative learned fear. *Neuroscience* **171**, 760–768 (2010).

39.   Bi, L.-L. et al. Enhanced excitability in the infralimbic cortex produces anxiety-like behaviors. *Neuropharmacology* **72**, 148–156 (2013).

40.   Yizhar, O. et al. Neocortical excitation/inhibition balance in information processing and social dysfunction. *Nature Article* **477**, 171–178 (2011).

41.   Goebbels, S. et al. Genetic Targeting of Principal Neurons in Neocortex and Hippocampus of NEX-Cre Mice. *Genesis* **44**, 611–621 (2006).

42.   Rubenstein, J. L. R. & Merzenich, M. M. Model of autism: increased ratio of excitation/ inhibition in key neural systems. *Genes, Brain and Behavior* **2**, 255–267 (2003).

43.   Berg, L., Eckardt, J. & A., M. O. Enhanced activity of pyramidal neurons in the infralimbic cortex drives anxiety behavior. *PLoS One* **14**, 1–19 (2019).

44.   Meunier, C. N. J., Amar, M., Lanfumey, L., Hamon, M. & Fossier, P. 5-HT1A receptors direct the orientation of plasticity in layer 5 pyramidal neurons of the mouse prefrontal cortex. *Neuropharmacology* **71**, 37–45 (2013).

45.   Paxinos, G., Franklin, K. B. J., Paxinos, G and Franklin, K. B. J., Paxinos, G. & Franklin, K. B. J. *Mouse Brain in Stereotaxic Coordinates*. *Academic Press* volume 2nd (2004).

46.   Gore, B. B., Soden, M. E. & Zweifel, L. S. Manipulating gene expression in projection-specific neuronal populations using combinatorial viral approaches. *Current Protocols in Neuroscience* **435**, 1–6 (2014).

47.   Stujenske, J. M., Spellman, T. & Gordon, J. A. Modeling the Spatiotemporal Dynamics of Light and Heat Propagation for InVivo Optogenetics. *Cell Report* **12**, 525–534 (2015).

48.   Berg, L. Imbalance of excitation and inhibition within the prefrontal cortex supports anxiety behavior. Doctoral dissertation. Ruhr-University Bochum (2019).

49.   Boyden, E. S. A history of optogenetics: The development of tools for controlling brain circuits with light. *F1000 Biology Reports* **3**, 1–12 (2011).

50.   Airan, R. D., Thompson, K. R., Fenno, L. E., Bernstein, H. & Deisseroth, K. Temporally precise in vivo control of intracellular signalling. *Nature* **458**, 1025–1029 (2009).

51.   Sparta, D. R. et al. Construction of implantable optical fibers for long-term optogenetic manipulation of neural circuits. *Nature Protocol* **7**, 12–23 (2012).

52.   Covington, H. E. et al. Antidepressant Effect of Optogenetic Stimulation of the Medial Prefrontal Cortex. *Journal of Neuroscience* **30**, 16082–16090 (2010).
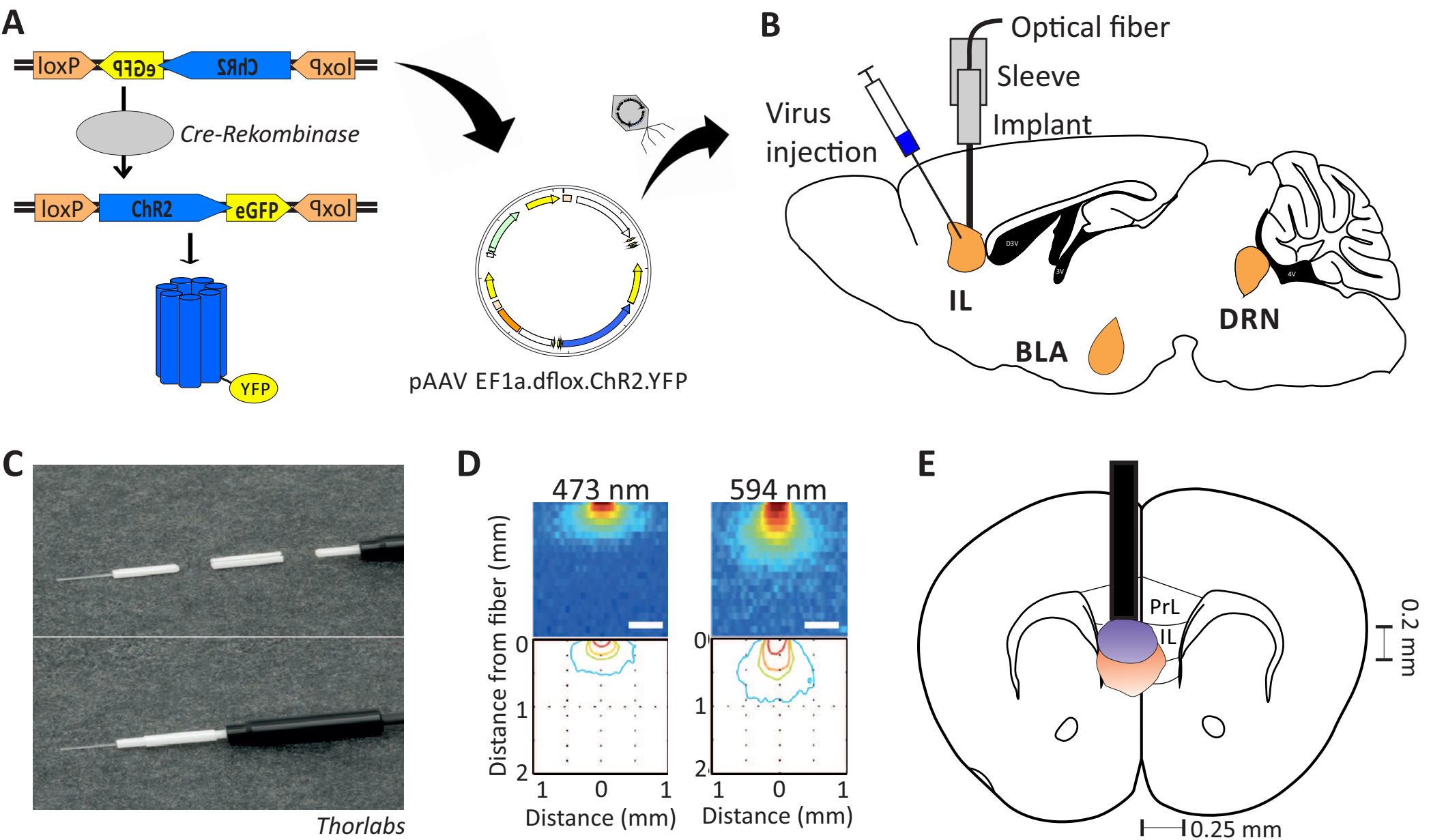
53.   Lepicard, E. M., Joubert, C., Hagneau, I., Perez-Diaz, F. & Chapouthier, G. Differences in anxiety-related behavior and response to diazepam in BALB/cByJ and C57BL/6J strains of mice. *Pharmacology, Biochemistry and Behavior* **67**, 739–748 (2000).

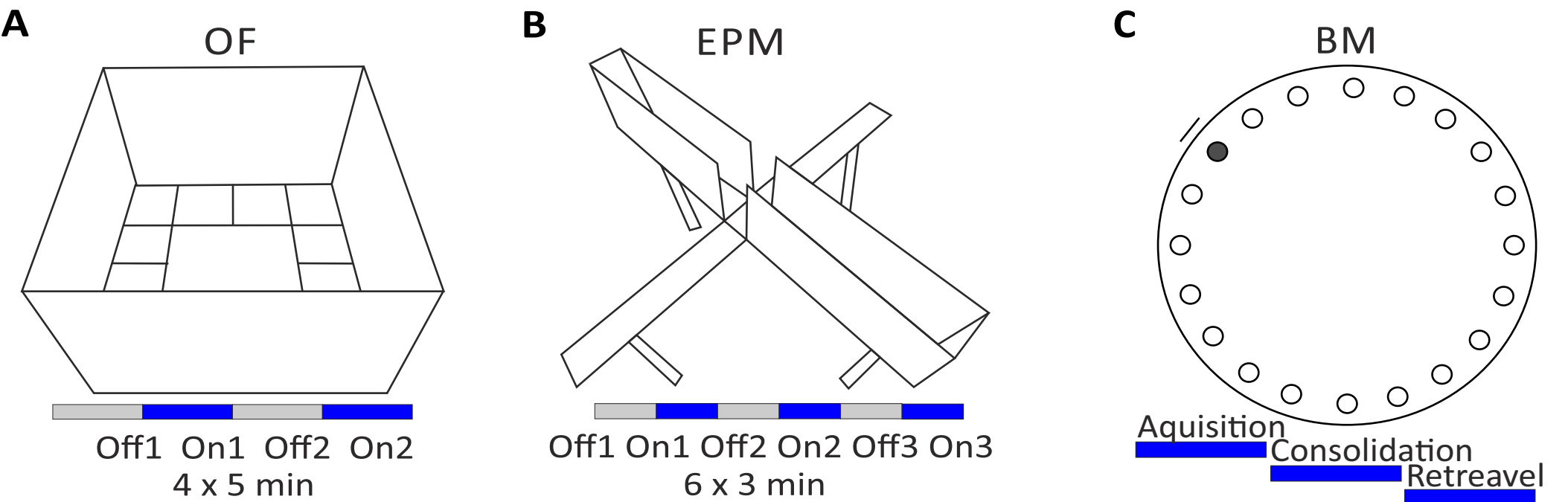54.   Schmidt, M. V. & Müller, M. B. Animal models of anxiety. *Elsevier* **3**, 369–374 (2006).

55.   Cho, J. H., Deisseroth, K. & Bolshakov, V. Y. Synaptic Encoding of Fear Extinction in mPFC-amygdala Circuits. *Neuron Article* **80**, 1491–1507 (2013).

56.   Adhikari, A. et al. Basomedial amygdala mediates top-down control of anxiety and fear. *Nature* **527**, 179–185 (2015).
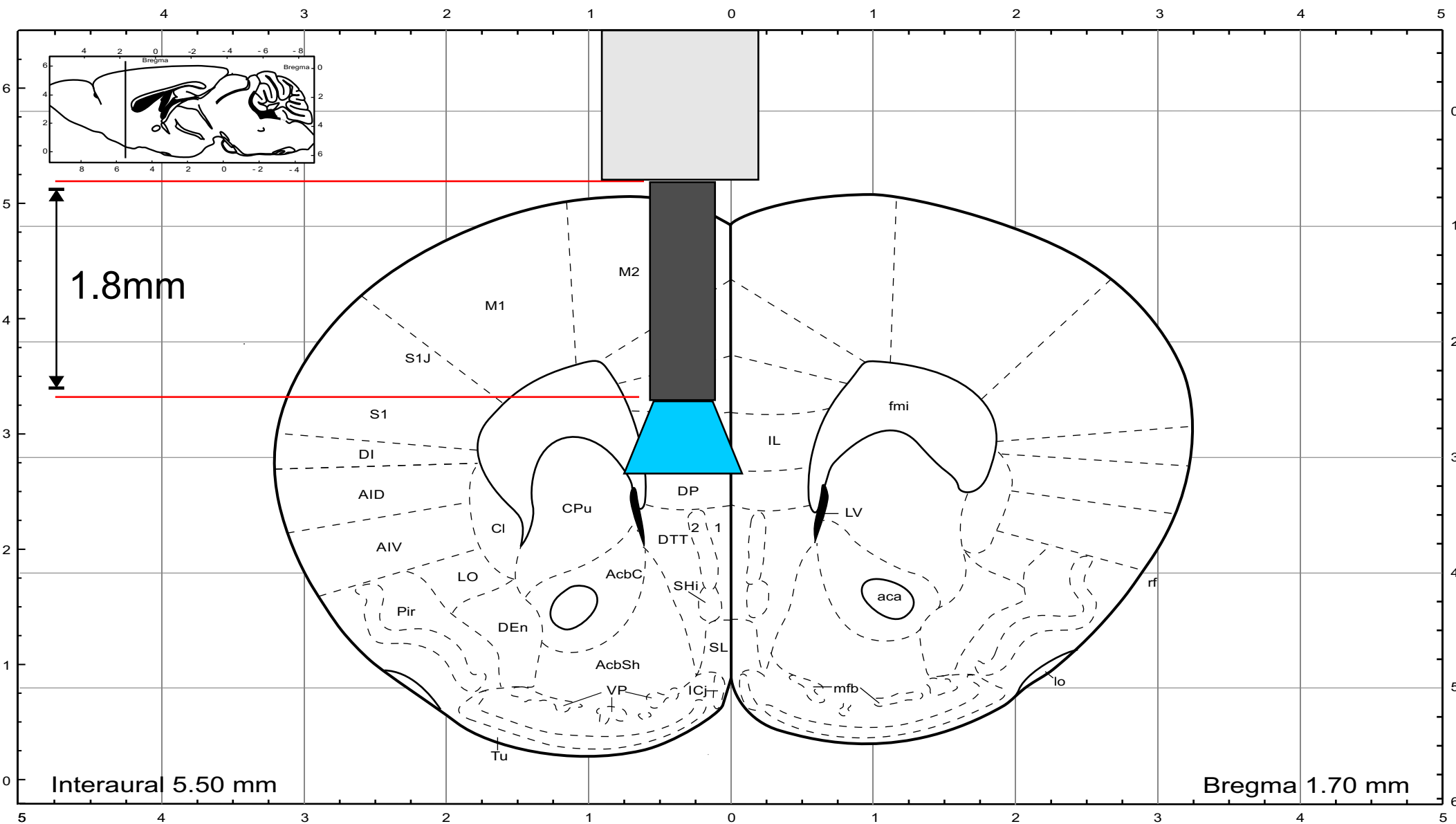
1232 57. Suzuki, S. et al. The infralimbic and prelimbic medial prefrontal cortices have differential
1233 functions in the expression of anxiety-like behaviors in mice. *Behavioural Brain Research* **304**,
1234 120–124 (2016).
1235 58. Carola, V., D'Olimpio, F., Brunamonti, E., Mangia, F. & Renzi, P. Evaluation of the elevated
1236 plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice.
1237 *Behavioural Brain Research* **134**, 49–57 (2002).
1238 59. Prut, L. & Belzung, C. The open field as a paradigm to measure the effects of drugs on
1239 anxiety-like behaviors: A review. *European Journal of Pharmacology* **463**, 3–33 (2003).
1240 60. Tye, K. M. et al. Amygdala circuitry mediating reversible and bidirectional control of
1241 anxiety. *Nature Letter* **471**, 358–362 (2011).
1242 61. Bouwknecht, J. A. et al. Differential effects of exposure to low-light or high-light open-
1243 field on anxiety-related behaviors: Relationship to c-Fos expression in serotonergic and non-
1244 serotonergic neurons in the dorsal raphe nucleus. *Brain Research Bulletin* **72**, 32–43 (2007).
1245 62. Overstreet, D. H., Knapp, D. J., Angel, R. A., Navarro, M. & Breese, G. R. Reduction in
1246 repeated ethanol-withdrawal-induced anxiety-like behavior by site-selective injections of 5-HT1A
1247 and 5-HT2C ligands. *Psychopharmacology (Berlin)* **187**, 1–12 (2006).
1248 63. Takahashi, A. et al. Glutamate Input in the Dorsal Raphe Nucleus As a Determinant of
1249 Escalated Aggression in Male Mice. *Journal of Neuroscience* **35**, 6452–63 (2015).
1250 64. Klemenhagen, K. C., Gordon, J. A., David, D. J., Hen, R. & Gross, C. T. Increased Fear
1251 Response to Contextual Cues in Mice Lacking the 5-HT1A Receptor. *Neuropsychopharmacology*
1252 **31**, 101–111 (2006).
1253 65. Ramos, A. Animal models of anxiety: do I need multiple tests? *Trends in Pharmacological*
1254 *Science* **29**, 493–498 (2008).
1255 66. Isosaka, T. et al. Htr2a-Expressing Cells in the Central Amygdala Control the Hierarchy
1256 between Innate and Learned Fear. *Cell* **163**, 1153–1164 (2015).
1257 67. Regev, L. & Goshen, I. Employing Optogenetics in Memory Research. in *Optogenetics: A*
1258 *Roadmap* 219–256 (2017).
1259 68. Shah, A. A., Sjovold, T. & Treit, D. Inactivation of the medial prefrontal cortex with the
1260 GABA A receptor agonist muscimol increases open-arm activity in the elevated plus-maze and
1261 attenuates shock-probe burying in rats. *Brain Research* **1028**, 112–115 (2004).
1262 69. Knight, A. R. et al. Pharmacological characterisation of the agonist radioligand binding site
1263 of 5-HT2A, 5-HT2B and 5-HT2C receptors. *Naunyn-Schmiedebergs Archiv of Pharmacology* **370**,
1264 114–123 (2004).
1265 70. Graeff, F. G., Viana, M. B. & Mora, P. O. Dual Role of 5-HT in Defense and Anxiety.
1266 *Neuroscience and Biobehavioral Reviews* **21**, 791–799 (1997).
1267 71. Cheriyan, J. & Sheets, P. L. Altered Excitability and Local Connectivity of mPFC-PAG
1268 Neurons in a Mouse Model of Neuropathic Pain. *Journal of Neuroscience* **38**, 4829–4839 (2018).
1269 72. Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G. & Deisseroth, K. Millisecond-timescale,
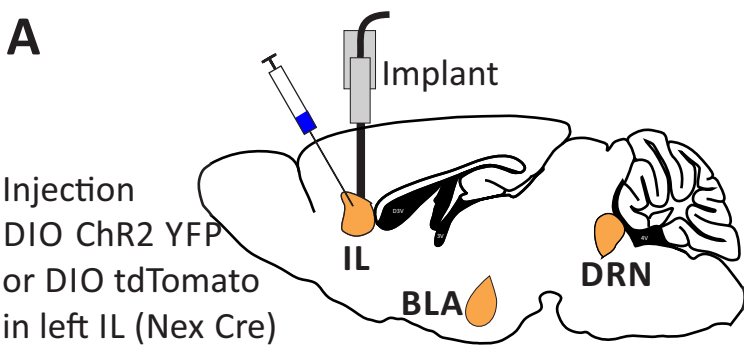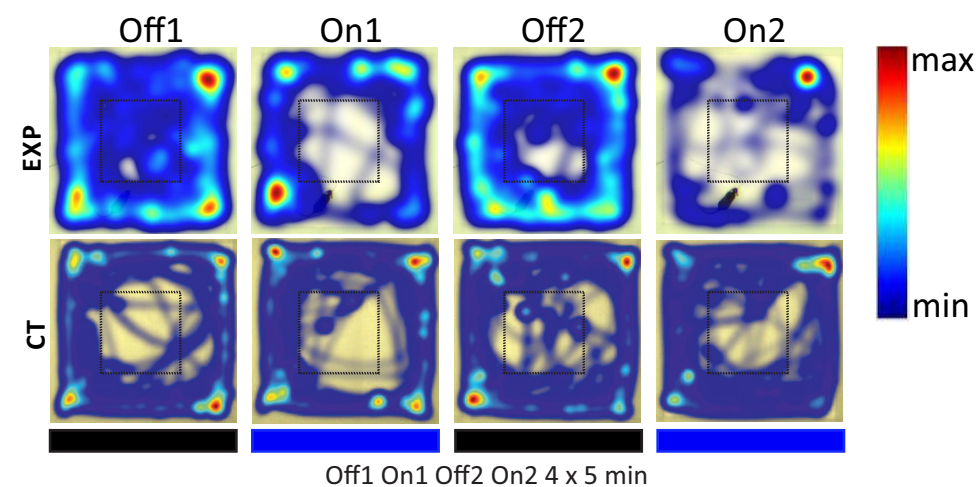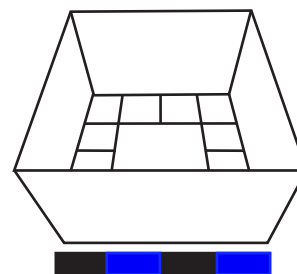1270 genetically targeted optical control of neural activity. *Nature Neuroscience* **8**, 1263–1268 (2005).
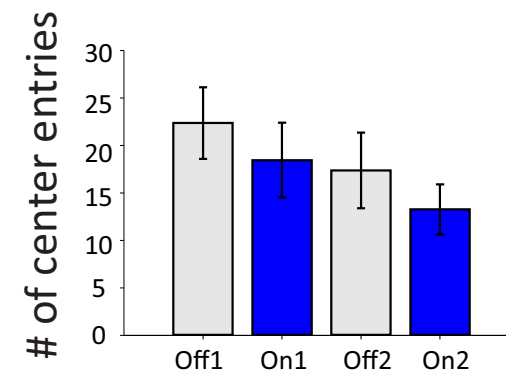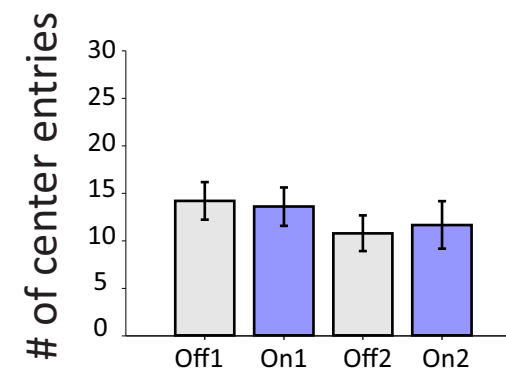1271

This figure has been modified from *Berg et al. 2019* PLoS One

A OF

Off1 On1 Off2 On2
4 x 5 min

B EPM

Off1 On1 Off2 On2 Off3 On3
6 x 3 min

C BM

Aquisition
Consolidation
Retreavel

This figure has been modified from *Berg et al. 2019* PLoS One

1.8mm

M2

M1

S1J

S1

DI

AID

AIV

LO

Pir

DEn

CI

CPu

AcbC

AcbSh

VP

Tu

DTT

DP

SHi

SL

ICj

2 1

IL

fmi

LV

aca

mfb

rf

lo

Interaural 5.50 mm

Bregma 1.70 mm

A Implant

Injection
DIO ChR2 YFP
or DIO tdTomato
in left IL (Nex Cre)

IL BLA DRN

B

Off1 On1 Off2 On2

EXP

CT

max

min

Off1 On1 Off2 On2 4 x 5 min

C OF: ChR2 YFP (EXP)

no Light
Light

Time in center [s]

Off1 On1 Off2 On2

Distance [cm]

Off1 On1 Off2 On2

# of center entries

Off1 On1 Off2 On2

D OF: tdTomato (CT)

no Light
Light

Time in center [s]

Off1 On1 Off2 On2

Distance [cm]

Off1 On1 Off2 On2

# of center entries

Off1 On1 Off2 On2
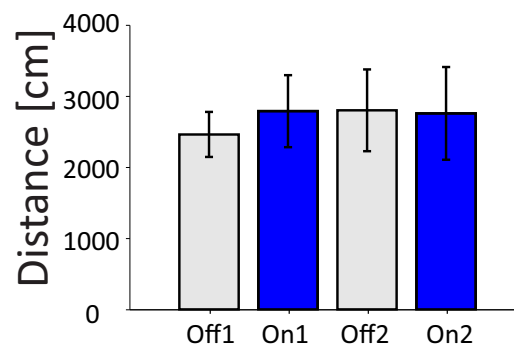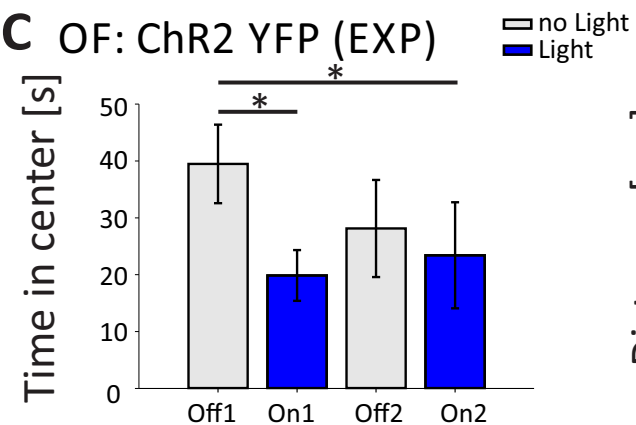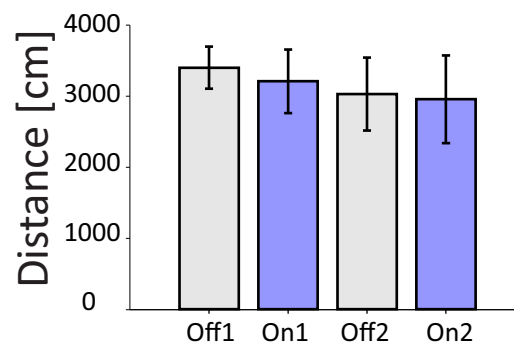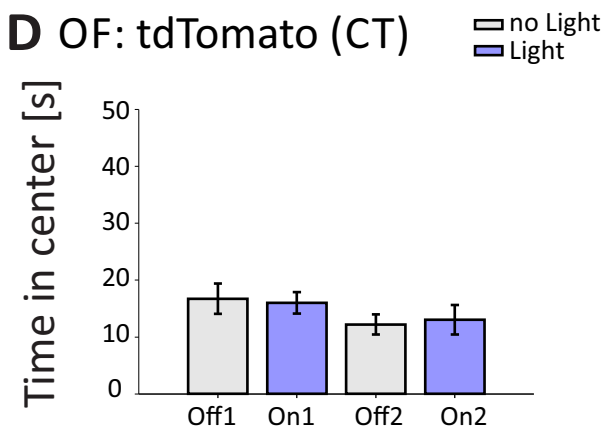
This figure has been modified from *Berg et al. 2019* PLoS One
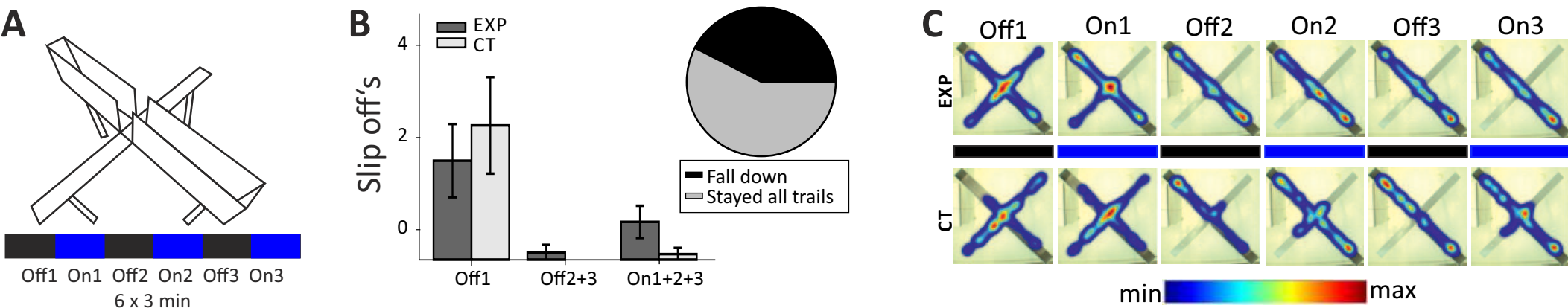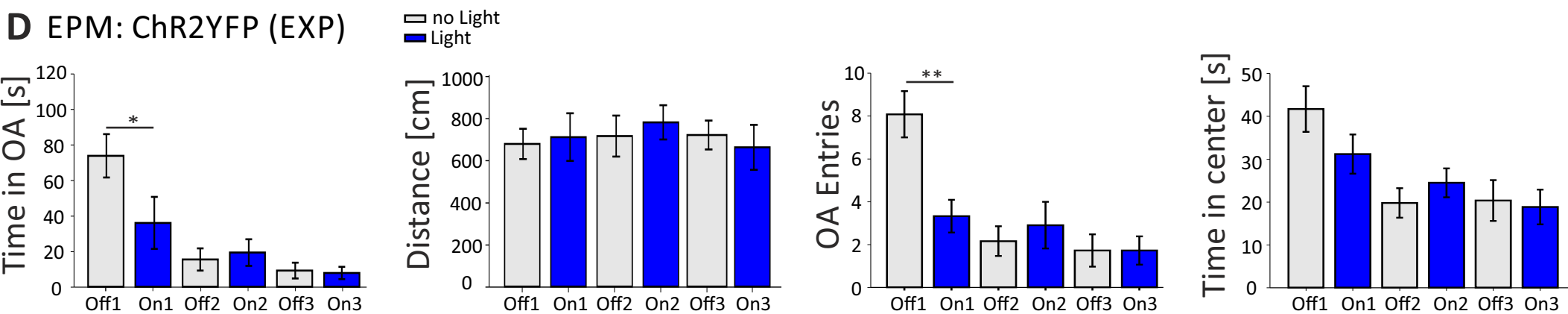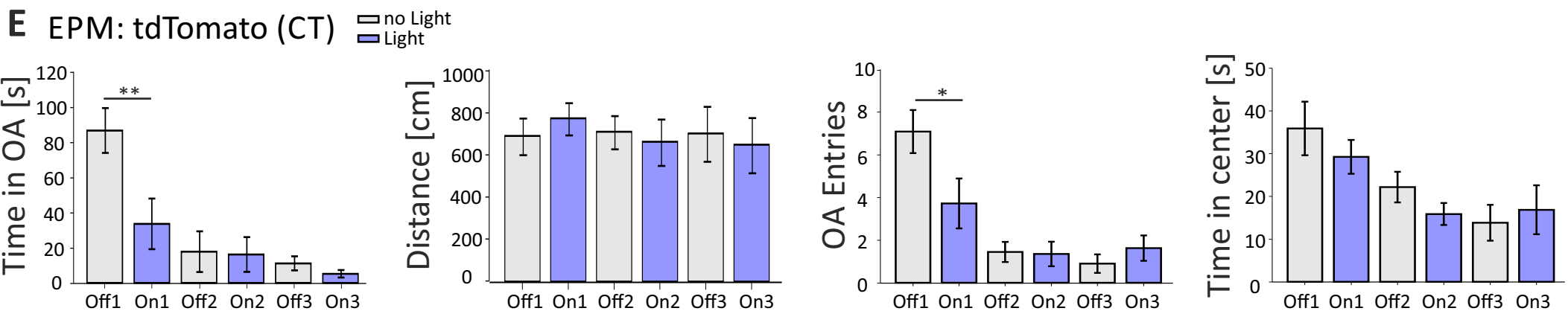
This figure has been modified from *Berg et al. 2019* PLoS One
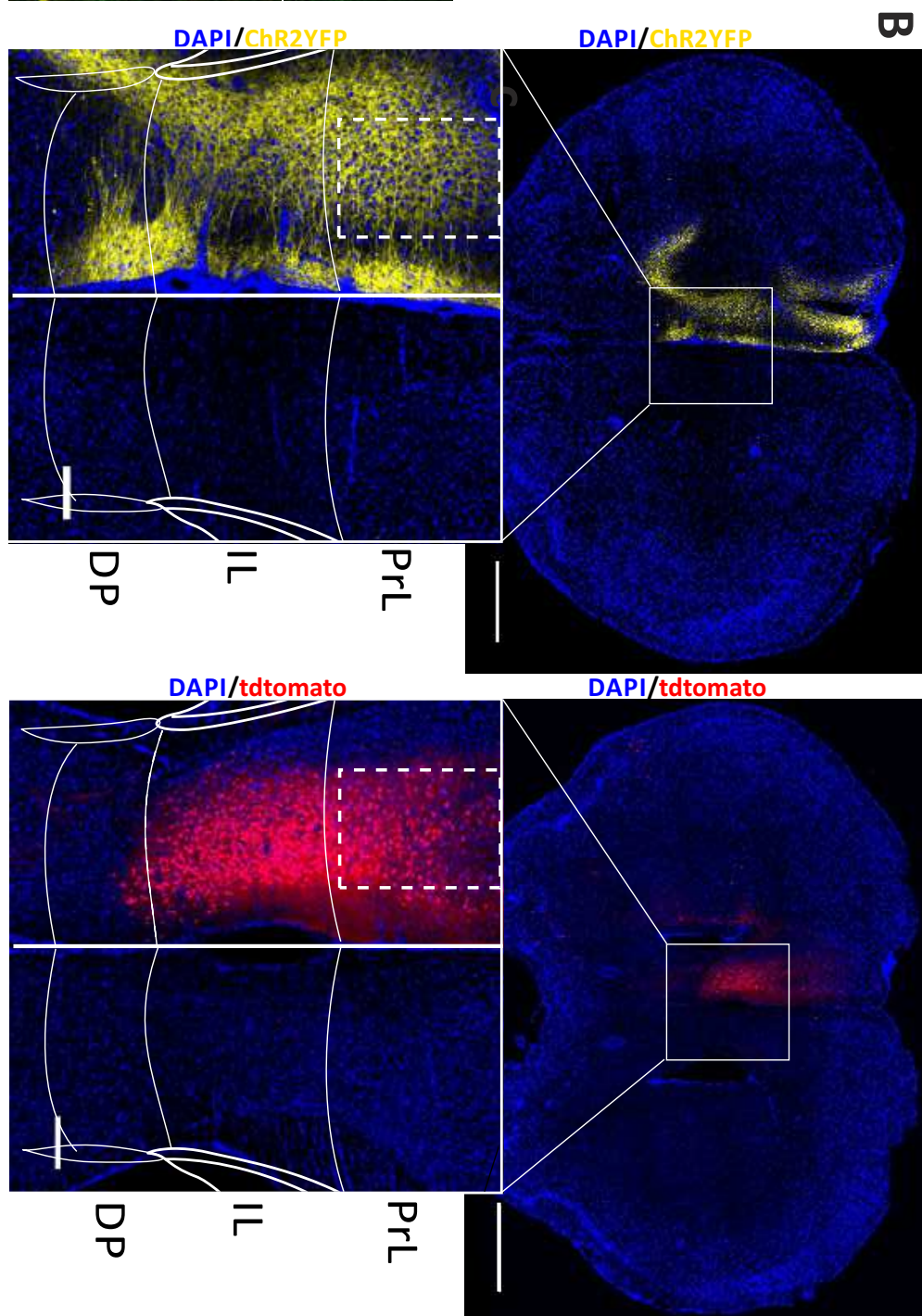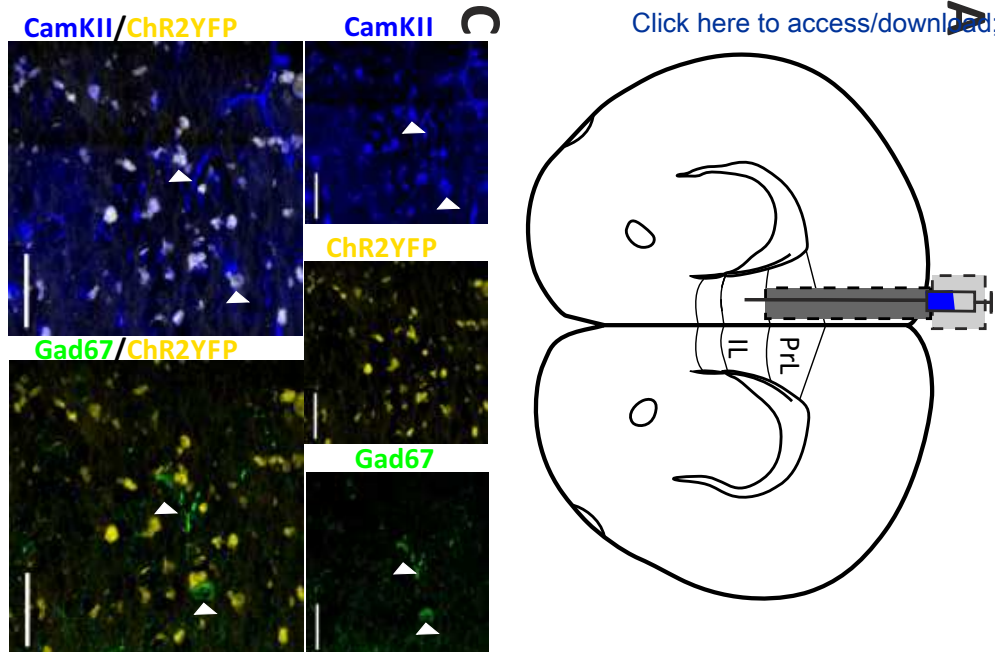
This figure has been modified from *Berg et al. 2019 PLoS One*

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Ketamin	Sigma-Aldrich	K2753-64	Anestasia
20 % Glucose	AlleMan Pharma		Injection s.c. for fast recovery
Behavioral mazes	Costum made		Measure anxiety
Bepanthen	Bayer		Ophthalmic oinment
Betaisodona	Monodipharma		Sterilant containing iodine
Betaisodona	Monodipharma		Iodine oinment
Binocular	Olympus	SZ52, 110AL0.62x WD160	Surgery
Ceramic ferrules	Thorlabs	CFLC230-10	Implant
Ceramic Fiber Scribe	Thorlabs	CSW12.5	Cutting of the glass fiber
Channelrhodopsin2-YFP virus	Penn Vector Core	Addgene 20298	Optogenetic tool
Compressed air	Kontakt Chemie	Druckluft 67	Drying of the skull
Coordinate system	Stoelting		Stereotactic coordinates for the surge
Correl Draw			Graphical software version 13
Cryoslicer	MICROM	HM500OM	Production of brain slices for staining
Ethovision XT 14	Noldus		Software for behavioral tracking
Exel			Statistical Software
Ferrule Polishing Puck	Thorlabs	D50-F	Polishing implants round side
Fiber Patch Cord dual	Prizmatix	Optogenetics-Fiber 500, 1,20 m, Ferrule core 1,25 mm	Cables, which are connected with the
Fiber Patch Cord single	Prizmatix	Optogenetics-Fiber 500, 1,20 m, Ferrule core 1,25 mm	Cable, which is connected with the im
Fiber Stripping Tool	Thorlabs	T06S13	Stripping glass fiber for implant
Filter paper	VWR European	516-0300	Cut into pieces for the Novelty-Suppr
Food pellets	Mühle Levers	Höveler Nagerfutter	Nutrition for the mice
Glass pipettes	Harvard Apparatus	GC150-10	Injection pipettes
Gradia direct-Flo	Henry Schein	103322	Fluid dental cementum
Heating lamp	efbe-Schott/Phillips	R95E	Prevent the mice from cooling after tl
Heating plate	Stoelting		Integrated into coordinate system
Injection canula	Braun	100 Sterican, 0,4 x 20 mm, Gr. 20	All injections and to bore hole into th
Litter		T 1350	Grounding for the Novelty-Supressed

Mouse cages	Zoonlab	405 cm^2	Single housing for experiments
Optibond FL	Kerr	26684E	Preparation of the skull for implantati
Optical glass fiber	Thorlabs	FT200EMT	Light fiber for implant
Optogenetics-LED.STSI	Prizmatix		Optogenetic toolbox for light stimulat
Paraformaldehyde	Sigma-Aldrich	16005-1KG-R	Perfusion of mice to remove the brain
Polishing sheet 0.02 µm grit	Thorlabs	LFCF	Polishing implants round side
Polishing sheet 1 µm grit	Thorlabs	LF1D	Polishing implants round side
Polishing sheet 30 µm grit	Thorlabs	LF30D	Polishing implants round side
Polishing sheet 6 µm grit	Thorlabs	LF6D	Polishing implants round side
Pulser Software	Prizmatix		Software for light device control
Rimadyl-Carprofen	Zoetis		Analgesia
Sigma Plot			Software for statistics
Sleeve	Thorlabs	FT200EMT	Connection of implant and light cable
SodiumCloride (NaCl)	Braun	3570410	Rinsing of the skull
Superglue	Pattex Henkel		To Fix the glass fiber in the ferrule
td-Tomato virus	Penn Vector Core	Addgene 51503	Optogenetic tool
UV light	KoQGHJ	wireless, 1200 mW/cm^2	Polymeration lamp for dental cement
Xylavet-Xylazin	cp pharma		Anesthesia

ery

two implants of a bilateral implantation

plant via a sleeve
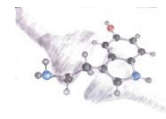
essed Feeding test

he surgery

e skull
 Feeding test

ion

tion during behavioral experiments
ns

um

⬡ Universität Bremen

⬡ Universität Bremen · **Fachbereich 2** · Postfach 33 04 40 · 28334 Bremen

Institut für Hirnforschung
Synthetische Biologie
FB2 Biologie und Chemie

Submission of Review (61023_R2)

Prof. Dr.
Olivia, Masseck

Date: 17.07.2020

Leobener Str.5
NW2, Raum 4115
28359 Bremen

Telefon   (0049421) 218 - 62965
eMail   masseck@
           uni-bremen.de
www   www.uni-bremen.de/synbio

Dear JoVE Team, dear Nam Nguyen,

We would like to thank the editor for the chance to improve some further details in the manuscript and also for adjusting the numbering and format to publication standards. We have tried to adjust our manuscript "61023_R2-Optogenetic manipulation of neuronal circuitries to modulate anxiety-related behavior or cognitive functions in freely moving mice" accordingly.

In the following section we will comment on each editorial suggestion and explain how we integrated it into the manuscript or the video.
All changes in the manuscript are tracked to identify all edits.

Editorial comments to the written manuscript:
1. Some additional minor details are needed in the written protocol. Please see the comments in the attached manuscript.

All comments in the attached manuscript were addressed and the requirements are added as detailed as possible.

2. Please avoid the use of personal pronouns (you, us, etc.)

All personal pronouns were exchanged with other words or phrases, instead of one „we" in the acknowledgements in line 1111.

3. I have formatted and renumbered the protocol steps to meet our publications standard. As such, please review the protocol step references in the Discussion.

We want to thank the editor for formatting the manuscript and numbers protocol. All protocol step references in the discussion are renewed and now fit the new numbering.

Editorial comments to the video:

1. On-Screen Text Cards:
• 12:24 Consider reformatting this a bit to have all the words from "6. Data analysis (example Open Field)" on a single line

The On-Screen Text card at 12:24 is formatted and all words from „6." are in one row.

2. Video Editing Feedback:
• 02:52 Convert this jump cut to a dissolve
• 02:57 After the fade out there is a very brief fade back in before moving on to stereotaxic assembly with the settings in the lower left. This brief fade in is probably an error and should be removed.
• 03:37 Convert this jump cut to a dissolve
• 03:48 Convert this jump cut to a dissolve
• 03:50 Convert this jump cut to a dissolve
• 04:03 Convert this jump cut to a dissolve
• 04:03-04:45 Please convert this series of jump cuts to dissolves
• 05:20-05:52 Please convert this series of jump cuts to dissolves

All jump cuts which were mentioned here are now changed into a dissolve. The speed of the video was not changed by that, because we had to be careful concerning the maximal length of 15 minutes for the video.

• 06:35 The end of this chapter feels a bit rushed. Consider holding on the Prizmatix Pulser window until the narration completes. Then go to the next chapter title card and wait on that for a moment so it can be read, then go on.

We thank the editor for this suggestion and changed the video accordingly. Now the narration for the Prizmatix Pulser is completes with the Pulser window and goes on after showing the next title card for a moment without narration.

• 10:36 Consider holding for one more half-second and giving the speaker half a second of screen time before she speaks, the pacing here is a little too fast. Also, when she says ""retrieval"" @10:50 allow for one more second before fading out as part of the word is cut-off.

We changed the interview accordingly. The on-screen time before and after the speaker gives his interview was elongated.

3. Audio Track Feedback:
• The audio levels should be consistent throughout. The interview segments seem quieter than the narration. Use the volume levels of the narration as a target and adjust accordingly. Avoid over amplifying and allow audio peaks to reach between -6 and -12 dB.

We adapted the audio level of the interviews for a consistent level throughout interview and narration segments. We hope we met your requirements.

4. Please avoid commercial branding whenever possible in the video: Please use generic terms for EthoVision (Video tracking software) at 6:08. I have changed some of this language in the revised manuscript attached here as well.

We thank the editor for the already changed phrases in the manuscript. We tried to avoid every commercial branding in the text and the video title cards and changed them to more generic terms. I hope we met the editor's idea.

Jill Gerdey

Doctoral student

Biology and Chemistry, Synthetic Biology, University of Bremen, Leobenerstraße 5, D-28359 Bremen

Tel: 0421 218 62966
E-Mail: Jill.Baumann@rub.de

Jill Gerdey was trained as veterinary assistant and studied afterwards Biology at the Ruhr-University Bochum until she successfully finished her Master of Science in 2017. Afterwards, she started a graduation program at the International Graduate School in Neuroscience at the Ruhr-University Bochum in 2017 and is currently working on her dissertation till now.

During her dissertation, Jill is working in the group of Prof. Dr. Olivia Masseck in Bochum and later in Bremen in the department of "Synthetic Biology". She is investigating the influence of the neurotransmitter serotonin on learning and memory. Within this project, she supervised several students during their master or bachelor theses.

Jill visited several conferences actively, as for example the SfN in San Diego, California, in 2018. She successfully finished the FELASA category B and C course at the Ruhr-University.

Olivia Masseck

Professor

Biology and Chemistry, Synthetic Biology, University of Bremen, Leobenerstraße 5, D-28359 Bremen

Tel: 0421 218 62965
E-Mail: masseck@uni-bremen.de

In October 2018, Olivia Masseck was appointed by the University of Bremen as professor of Synthetic Biology in the Faculty of Biology and Chemistry.

Following her studies in biology and mathematics, she received a doctorate in neuroscience from the International Graduate School of Neuroscience at Ruhr-Universität Bochum. After a research stay at the laboratory of neurobiology of sensorimotor networks ("Laboratoire de Neurobiologie des Reseaux Sensorimoteurs") at Université Paris Descartes (France), she worked as a research assistant and working group leader at the Department of General Zoology and Neurobiology at Ruhr-Universität Bochum, which was one of the first research groups worldwide to establish optogenetic methods.

From 2016 to 2018, she held the junior professorship for high-resolution fluorescence microscopy at Ruhr-University Bochum. Her research focuses on the molecular mechanisms of serotonin, which is involved in anxiety, depression, decision making, learning and memory. The main focus is how serotonin is modulating behavior. In Bremen, she is now working on setting up a working group that will use modern methods, such as optogenetics, to investigate the serotonergic system.

Dr. Laura Berg

Postdoctoral student

Biology and Chemistry, Synthetic Biology, University of Bremen, Leobenerstraße 5, D-28359 Bremen

Tel: 0421 218 62966
E-Mail: Laura.Berg@rub.de

Laura Berg studied "Biological Science" at the Westfälische Wilhelms-University in Münster from 2011 to 2014 and afterwards Biology with the main emphasis in Neurobiology at the Ruhr-University Bochum until 2016. With the Master of Science Laura started a graduation program at the International Graduate School in Neuroscience at the Ruhr-University Bochum in 2016. The dissertation with the title "Imbalance of excitation and inhibition within the prefrontal cortex supports anxiety behavior" is expected to be successfully finished in December 2019.

During her dissertation, Laura was working in the group of Prof. Dr. Olivia Masseck in Bochum and later in Bremen in the department of "Synthetic Biology". She is investigating the influence of the neurotransmitter serotonin on the development and manifestation of anxiety disorders in the mouse model. Within this project, she supervised several students during their master or bachelor theses.

Laura visited several conferences actively, as for example the Optogen 2017 in Lecce, Italy, where she won the poster prize from Elsevier. Afterwards she presented her work as a poster at the SfN in San Diego, California in 2018. Since 2019 she works as a volunteer for the Commission for animal ethics at the LANUV NRW. Laura successfully finished the FELASA category B and C course at the Ruhr-University.
In December 2019, Laura finished her PhD with the thesis "Imbalance of excitation and inhibition within the prefrontal cortex supports anxiety behavior" at the Ruhr-University in Bochum.

In 2019 she published her first article in the journal PLoS One:
Berg, L., Eckardt, J. Masseck, O. M. 2019. „Enhanced activity of pyramidal neurons in the infralimbic cortex drives anxiety behavior". *PLoS ONE 14(1): e0210949*

License link PLoS One: https://journals.plos.org/plosone/s/licenses-and-copyright

Licenses and Copyright

The following policy applies to all PLOS journals, unless otherwise noted.

Reuse of PLOS Article Content

PLOS applies the Creative Commons Attribution (CC BY) license to articles and other works we publish. If you submit your paper for publication by PLOS, you agree to have the CC BY license applied to your work. Under this Open Access license, you as the author agree that anyone can reuse your article in whole or part for any purpose, for free, even for commercial purposes. Anyone may copy, distribute, or reuse the content as long as the author and original source are properly cited. This facilitates freedom in re-use and also ensures that PLOS content can be mined without barriers for the needs of research.

Content Owned by Someone Else

If you have written permission to do so, yes. If your manuscript contains content such as photos, images, figures, tables, audio files, videos, etc., that you or your co-authors do not own, we will require you to provide us with proof that the owner of that content (a) has given you written permission to use it, and (b) has approved of the CC BY license being applied to their content. We provide a form you can use to ask for and obtain permission from the owner. Download the form (PDF).

If you do not have owner permission, we will ask you to remove that content and/or replace it with other content that you own or have such permission to use.

Don't assume that you can use any content you find on the Internet, or that the content is fair game just because it isn't clear who the owner is or what license applies. It's up to you to ascertain what rights you have—if any—to use that content.

Using Article Content Previously Published in Another Journal

Many authors assume that if they previously published a paper through another publisher, they own the rights to that content and they can freely use that content in their PLOS paper, but that's not necessarily the case – it depends on the license that covers the other paper. Some publishers allow free and unrestricted re-use of article content they own, such as under the CC BY license. Other publishers use licenses that allow re-use only if the same license is applied by the person or publisher re-using the content.

If the paper was published under a CC BY license or another license that allows free and unrestricted use, you may use the content in your PLOS paper provided that you give proper attribution, as explained above.

If the content was published under a more restrictive license, you must ascertain what rights you have under that license. At a minimum, review the license to make sure you can use the content. Contact that publisher if you have any questions about the license terms – PLOS staff cannot give you legal advice about your rights to use third-party content. If the license does not permit you to use the content in a paper that will be covered by an unrestricted license, you must obtain written permission from the publisher to use the content in your PLOS paper. Please do not include any content in your PLOS paper which you do not have rights to use, and always give proper attribution.

Acceptable Licenses for Data Repositories

If any relevant accompanying data is submitted to repositories with stated licensing policies, the policies should not be more restrictive than CC BY.

Removal of Content Used Without Clear Rights

PLOS reserves the right to remove any photos, captures, images, figures, tables, illustrations, audio and video files, and the like, from any paper, whether before or after publication, if we have reason to believe that the content was included in your paper without permission from the owner of the content.

Guidelines for Trademarks

Ensure that any reference to a trademark (such as a brand name) is used as an adjective, and not a noun or verb. The trademark should be immediately followed by the generic term for the object that it modifies. Note that because a trademark cannot be used as a noun, it cannot be presented in the possessive or plural form. Please see the following example for reference:

> INCORRECT: The stimuli were presented on 12 MacBook Pros®.

> CORRECT: The stimuli were presented on 12 Macbook Pro® computers.

Giving Proper Attribution for Use of Content

When citing a PLOS research article, use the "Vancouver style", as outlined in our Submission Guidelines. For example:

Kaltenbach LS et al. (2007) Huntingtin Interacting Proteins Are Genetic Modifiers of Neurodegeneration. *PLOS Genet* 3(5): e82. doi:10.1371/journal.pgen.0030082.

When citing non-article content from a PLOS website (e.g., blog content), provide a link to the content, and cite the title and author(s) of that content.

For examples of proper attribution to other types of content, see websites such as Open.Michigan.

jove
JOURNAL OF VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Author(s):

| Optogenetic manipulation of neuronaal circuitries to modulate anxiety-related behavior or cognitive functions in freely moving mice. |
| Laura Berg, Jill Gerdey, Olivia Andrea Masseck |

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access   ☑ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1.   **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion

of the Article, and in which the Author may or may not appear.

2.   **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3.   **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

![jove logo] JOURNAL OF VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4.   **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5.   **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6.   **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7.   **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum

rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8.   **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9.   **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10.   **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11.   **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12.   **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13.   **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14.   **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.