Submission ID #: 61493
Scriptwriter Name: Anastasia Gomez
Project Page Link: https://www.jove.com/account/file-uploader?src=18755413

Title: Unraveling the Role of Discrete Areas of the Rat Brain in the Regulation of Ovulation Through Reversible Inactivation by Tetrodotoxin Microinjections

Authors and Affiliations:

Carlos-Camilo Silva[1,2*], Montserrat Bolaños-Hurtado[1*], Cinthia Juárez-Tapia[4], Angélica Flores[1,2], Isabel Arrieta-Cruz[3,5], María-Esther Cruz[3], Roberto Domínguez[1,2,3]

[1]Chronobiology of Reproduction Research Lab, Biology of Reproduction Research Unit, Facultad de Estudios Superiores Zaragoza, Universidad Nacional Autónoma de México, México City, México
[2]Developmental Biology Lab, Biology of Reproduction Research Unit, Facultad de Estudios Superiores Zaragoza, Universidad Nacional Autónoma de México, México City, México
[3]Neuroendocrinology Lab, Biology of Reproduction Research Unit, Facultad de Estudios Superiores Zaragoza, Universidad Nacional Autónoma de México, México City, México
[4]Facultad de Ciencias, Universidad Nacional Autónoma de México, México City, México
[5]Department of Basic Research, Instituto Nacional de Geriatría, Ministry of Health, México City, México

*These authors contributed equally

Corresponding Authors:

Roberto Domínguez (rdcasala@hotmail.com)

Email Addresses for All Authors:
ccsmbr@gmail.com
montserratbh@gmail.com
cinth@ciencias.unam.mx
angy1414@gmail.com
iarrieta@inger.gob.mx
mecbloy@yahoo.com.mx
rdcasala@hotmail.com

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   No

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

   ZEISS, STEMI DV4 STEREO MICROSCOPE

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 25
Number of Shots: 58

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Prof. Carlos-Camilo Silva:** This protocol allows researchers to explore the nuclei of the neuronal circuit that regulates ovulation in female mammals at specific times of the estrous cycle.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Dr. Cinthia Juárez-Tapia:** This technique can be adapted to study any process under regulation of the brain in any species. Also, the cannulas are fabricated in the lab, lowering the overall cost.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. Videographer NOTE: The last two takes within the shot did not work out. Use the previous one.

Introduction of Demonstrator on Camera

1.3. **Prof. Carlos Camilo Silva:** Demonstrating the procedure will be Montserrat Bolaños-Hurtado, an undergraduate student from my laboratory.

1.3.1. INTERVIEW: Author saying the above.

1.3.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Ethics Committee of Facultad de Estudios Superiores Zaragoza, UNAM.

Protocol

Videographer NOTE: Several shots are filmed in 4K (UHD) definition, for the editor to be able to crop into more extreme closeups. Added '4K" to start of those filenames.
Several 4K items were not slated: some because of filming straight into the microscope, others, because 4K has a tiny longer delay before it starts recording, and the slate was already away by then. The shot names have added into the filenames.

2. Construction of Bilateral Cannulas, Obturators, and Caps

2.1. Begin by extracting the stainless-steel shaft from the hub of two 23-gauge hypodermic needles using pressure tweezers **[1]**, then remove any remaining glue with a scalpel blade **[2]**.

2.1.1. Talent extracting the shaft from a needle.

2.1.2. Talent removing glue with a scalpel.

2.2. Draw a line 15 millimeters apart from the blunt end of the shafts with a fine permanent marker **[1]** and use cutting tweezers to remove the beveled ends **[2]**. Hold the 15-millimeter segments with fine hemostats and press them perpendicularly with a cut-off disc attached to a rotatory tool to obtain 14-millimeter segments of tubing **[3]**.

2.2.1. Talent drawing the line.

2.2.2. Talent cutting off the beveled end.

2.2.3. Talent pressing the segments with fine hemostats.

2.3. Use moldering clay to attach the two segments to a microscope slide, ensuring that both are at the same horizontal level **[1]**. Observe the segments through an ocular micrometer and adjust the segments with fine tweezers until the desired distance is obtained **[2-TXT]**. Solder both segments with a single point **[3]**.

2.3.1. Talent attaching the segments to a slide.

2.3.2. SCOPE: Talent adjusting the segments with tweezers. **TEXT: 10 X** Videographer NOTE: Unslated. Added shot to the filename.

2.3.3. Talent soldering the segments.

2.4. Create a support to attach the cannula to the stereotaxic holder by cutting a 10-millimeter segment of resilient 0.3-millimeter stainless steel wire **[1]**. Use moldering clay to place 2 millimeters of the wire in contact with the previous solder point and lay the rest above the blunt end of the cannulas **[2]**, then solder them together **[3]**.

2.4.0 Added Shot: measuring 10 mm

2.4.1. Talent cutting the wire.

2.4.2. Talent placing the wire in contact with the solder point. 2.4.2 and 2.4.3 shot together

2.4.3. Talent soldering the wire to the cannula.

2.5. To build the obturators, cut two 16-millimeter segments of round stainless-steel soft wire **[1-TXT]**. Hold a bilateral cannula with hemostats, perpendicular to a lab bench, and insert one of these segments into each cannula until they reach the bench **[2]**, then bend the remnant at a 90-degree angle **[3]**.

2.5.1. Talent cutting the wire. **TEXT: 0.35 mm diameter**

2.5.2. Talent inserting a segment into a cannula. Videographer NOTE: Unslated. Added shot to the filename.

2.5.3. Talent bending the rest of the wire. Videographer NOTE: Unslated. Added shot to the filename.

2.6. To construct the caps, cut two 2-millimeter segments of silicone tubing **[1-TXT]** and apply a drop of silicon glue at one of the ends of each segment, making sure that the glue does not enter the tubing **[2]**. Let it dry for at least 24 hours **[3]**.

2.6.1. Talent cutting silicone tubing. **TEXT: 0.76 mm inner diameter** Videographer NOTE: Unslated. Added shot to the filename.

2.6.2. Talent applying glue to a segment. Author NOTE: 2.6.2 and 2.6.3 shot together

2.6.3. Talent leaving the tubing to dry. Videographer NOTE: Stretch last seconds or frames to desired length for 'drying'

3. Construction of Microinjectors

3.1. Extract the stainless-steel shaft from the hub of two 30-gauge hypodermic needles as previously demonstrated **[1]**, then draw a line 18.5 millimeters apart from the blunt end of the shafts **[2]** and remove the remnant of the beveled ends with cutting tweezers **[3]**.

3.1.1. Talent extracting the shaft.

3.1.2. Talent drawing the line.

3.1.3. Talent removing the beveled end.

3.2. Repeat the process with a single 23-gauge needle to build adaptors by cutting two 6-millimeter-long segments starting from the blunt end **[1]**. Eliminate the occluded portions of the 6-millimeter segments by pressing them perpendicularly against the cut-off disc until two 4-millimeter adaptors are obtained **[2]**.

3.2.1. Talent cutting segments from the 23-gauge needle.

3.2.2. Talent pressing the segments against the cut-off disk. Videographer NOTE: Slated as 3.2.1

3.3. Insert the beveled end of the 30-gauge segments into the adaptors **[1]**, then look through a stereomicroscope to ensure that the ends of the segments and adaptors are at the same level **[2]**. Apply cyanoacrylate glue to the distal joint using a cotton swab and allow it to dry for 15 minutes **[3]**.

3.3.1. Talent inserting the segment into the adaptor. Videographer NOTE: At end of shot: refocus for CU result

3.3.2. SCOPE: Segment and adaptor ends at the same level. Videographer NOTE: Changed shot to ECU

3.3.3. Talent applying glue to the distal joint. Videographer NOTE: Unslated. Added shot to the filename.

3.4. Soak two PTFE tubing connectors in 70% ethanol for 5 minutes **[1]**, then attach them to the microinjectors through the adaptors **[2]**. Wait until the diameter of the connectors shrinks for at least 24 hours before using it **[3]**.

3.4.1. Talent putting the connectors to soak in ethanol.

3.4.2. Talent attaching the microinjectors through the adaptors. Videographer NOTE: Unslated. Added shot to the filename.

3.4.3. Connector diameter after shrinking. Author NOTE: Not filmed

4. Animal Maintenance and Vaginal Smears

4.1. House the rats in groups of four in standard polypropylene cages in a room with a 14 to 10 light–dark photoperiod. Set the temperature to 22 plus or minus 2 degrees Celsius and the humidity to 40%. Take vaginal smears every day **[1]**.

4.1.1. Rats in cages.

4.2. Sterilize a modified bacteriological loop with an inner diameter of 1-millimeter using an alcohol lamp, then cool it with sterile water **[1]**. Hold the rat with a secure grip and introduce 5 millimeters of the bacteriological loop into the vagina, touching its internal walls, then remove the loop **[2]**. *Videographer: This step is important!*

4.2.1. Talent sterilizing the loop and then cooling it.

4.2.2. Talent inserting the loop into the rat and then removing it. 4.2.2 and 4.3.1 shot together

4.3. If successful, a cloudy drop will be seen at the tip. Place this drop on a microscope slide. Repeat this process for each rat, sterilizing and cooling the loop between each animal **[1]**.

4.3.1. Talent placing the drop on the microscope slide.

4.4. After the drops dry off, stain the samples with hematoxylin-eosin and observe them under a microscope at 10 X magnification **[1]**.

4.4.1. Talent placing the slide with the samples on the microscope.

4.5. Determine the proportion of leukocytes, epithelial nucleated cells, and keratinized cells on each smear **[1]** and classify it according to the criteria of estrous cycle stages **[2-TXT]**.

4.5.1. Talent recording observations.

4.5.2. LAB MEDIA: Figure 1. *Video Editor: Label A "***Estrus***", B "***Metestrus***", C "***Diestrus***", and D "***Proestrus***".*

5. Microinjection of TTX or Vehicle Solutions into Freely Moving Rats

5.1. Configure the microinjection pump with the infusion rate and total time of injection **[1]**. Then, fill two 10-microliter Hamilton syringes with sterile distilled water **[2]** and insert a piece of PTFE tubing into the tubing connector of each microinjector, ensuring that the length of the tubing does not constrain the movement of the rat **[3]**.

5.1.1. Talent configuring the microinjection pump.

5.1.2. Talent filling syringes with water.

5.1.3. Talent inserting the PTFE tubing into the connector.

5.2. Connect the tubing to the Hamilton syringes **[1]** and press the plunger until a drop of water is visible at the tip of the microinjector **[2]**. Retract the plunger to create a 2-centimeter air pocket in the tubing **[3]**. *Videographer: This step is important!*

5.2.1. Talent connecting the tubing to the syringe.

5.2.2. Talent pressing the plunger and a drop of water coming out.

5.2.3. Talent retracting the plunger.

5.3. Place a pad that is soaked with the inactivating substance and a 2 by 2-centimeter square of paraffin film above it **[1]**. Pipette a sufficient amount of TTX to fill the needle of the injector and 1 centimeter of the tubing above the film **[2]**. *Videographer: This step is difficult!*

5.3.1. Talent placing the pad and paraffin film.

5.3.2. Talent pipetting TTX to fill the needle.

5.4. Absorb the TTX drop by gently retracting the plunger **[1]**. Mount the syringes in the microinjection pump **[2]** and use its controls to manipulate the plunger until a drop of TTX can be seen at the tip of each microinjector **[3]**. *Videographer: This step is difficult!*

5.4.1. Talent retracting the plunger.

5.4.2. Talent mounting the syringes in the microinjection pump.

5.4.3. Drop of TTX at the tip of the microinjector.

5.5. Use only rats that showed at least three consecutive cycles after the surgery, taking into consideration their stage of the cycle and the time of the day. Hold the rat with a firm grip **[1]**, remove the cap and obturators from the cannulas **[2]**, insert the microinjectors into the guide cannulas, and return the animal to the cage **[3]**.

5.5.1. Talent taking a rat out of the cage.

5.5.2. Talent removing the cap and obturators from the cannulas. Videographer NOTE: Removed just one. Look for closeup of obturator coming out

5.5.3. Talent inserting the microinjectors into the guide cannulas.

5.6. Turn on the pump and wait until the microinjection finishes **[1]**. Leave the microinjectors in place for two additional minutes to avoid reflux of the solution, then remove them **[2]**. Replace the obturators, put on the cap, and return the animal to the colony room **[3]**. *Videographer: This step is important!*

5.6.1. Talent turning on the pump.

5.6.2. Talent removing the microinjectors.

5.6.3. Talent replacing the obturators and putting on the cap. Videographer NOTE: Use part where it is pushed in with one finger. Putting on cap was not successful

6. Tissue Processing

6.1. After euthanizing the rat, use fine iris scissors to dissect each ovary, cutting at the utero-tubal junction **[1]**. Remove the oviduct from each ovary by cutting as far as possible from the ampulla region **[2]**.

6.1.1. Talent dissecting an ovary. Videographer NOTE: Two takes, slated as Take 1

6.1.2. Talent removing the oviduct from the ovary.

6.2. Gently dry the oviducts with an absorbent paper towel **[1]** and search for the ampulla under the stereomicroscope. With the non-dominant hand, hold the oviduct in place by pinching far from the ampulla with a 23-gauge needle, then use another needle in the dominant hand to make a 1-millimeter incision in the middle region of the ampulla **[2]**. *Videographer: This step is important!*

6.2.1. Talent drying the oviduct.

6.2.2. SCOPE: Talent holding the oviduct in place and making an incision in the ampulla. NOTE: Unslated. 6.2.2 and 6.3.1 shot together. 6.3.1 starts approximately at 00:36 sec.

6.3. The cumulus-oocyte complexes will protrude from the incision as a drop of viscous fluid. Use the needle in the dominant hand to gently and slowly pull the drop far from the oviduct **[1]**. *Videographer: This step is important!*

6.3.1. SCOPE: Talent pulling the COC from the oviduct.

6.4. Stain the sample with hematoxylin-eosin, add mounting medium, and apply a coverslip **[1]**. Observe it under the microscope to determine the number of oocytes shed by each ovary. Then, process the animal's brain as described in the text manuscript to determine the final position of the cannulas **[2]**. *Videographer: This step is important!*

6.4.1. Talent putting mounting medium on the sample.

6.4.1.1. Added shot: (6.4.1 Take 2) Removing air bubbles

6.4.2. SCOPE: Sample.

Results

7. Results: Percentage of Ovulating Animals and Median of the Number of Ova Shed after Microinjection with Artificial Cerebrospinal Fluid (ACSF) or Tetrodotoxin (TTX)

7.1. A total of 30 female rats were implanted with guide cannulas aiming at one of the two target areas **[1]**. All the animals were cyclic before the surgery **[2]** but only 7 did not show alterations of the estrous cycle after the procedure **[3]**. By the fifth cycle 28 rats were considered cyclic and the remaining two were discarded from the experiment **[4]**.

7.1.1. LAB MEDIA: Figure 3.

7.1.2. LAB MEDIA: Figure 3. *Video Editor: Emphasize the -1 cycles data point.*

7.1.3. LAB MEDIA: Figure 3. *Video Editor: Emphasize the 0 and 1 cycles data point.*

7.1.4. LAB MEDIA: Figure 3. *Video Editor: Emphasize the 5 cycles data point.*

7.2. The percentage of ovulating animals and the number of ova shed by intact animals and by the groups treated with either ACSF or TTX into the suprachiasmatic nucleus are shown here **[1]**. All the intact and ACSF-treated rats ovulated **[2]**, while all of the animals microinjected with TTX did not **[3]**.

7.2.1. LAB MEDIA: Figure 4 A and B.

7.2.2. LAB MEDIA: Figure 4 A. *Video Editor: Emphasize the intact and ACSF bars.*

7.2.3. LAB MEDIA: Figure 4 A. *Video Editor: Emphasize the TTX bar.*

7.3. Similar results can be observed for rats microinjected in the arcuate nucleus **[1]**.

7.3.1. LAB MEDIA: Figure 4 C and D.

7.4. The ovulatory outcome of animals that were treated with ACSF or TTX outside the target areas is demonstrated here **[1]**.

7.4.1. LAB MEDIA: Figure 5.

7.5. As determined after histological confirmation, most of their cannulas were placed in the anterior commissure or the retrochiasmatic area, two areas that do not contribute to the regulation of ovulation. Hence, ovulation was not altered **[1]**.

7.5.1. LAB MEDIA: Figure 5.

Conclusion

8. Conclusion Interview Statements

8.1. **Montserrat Bolaños-Hurtado:** When attempting this protocol, remember that the recording of the estrous cycle is critical since the activity of the brain nuclei involved in the regulation of ovulation changes depending on the stage of the cycle.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: Any shots between 4.2.2 – 4.5.1.*

8.2. **Dr. Cinthia Juarez-Tapia:** This method can be combined with hormonal measurements and molecular and histological approaches to assess alterations in the physiology of reproductive glands and the activity of genes and proteins.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Videographer: This statement is optional. If you don't have time, skip it.*

8.3. **Prof. Carlos-Camilo Silva:** This technique has been used in studies attempting to demonstrate the participation of specific brain areas in the regulation of processes like learning and memory, drug abuse and sexual behavior.

8.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Videographer: This statement is optional. If you don't have time, skip it.*