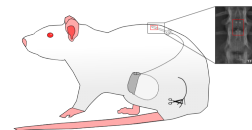


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Chronic testing of temporal patterns of spinal cord stimulation in the rat

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We use this protocol and it's working

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Abstract

This protocol outlines the surgical processes and paw withdrawal threshold testing of spinal cord stimulation patterns in the spared nerve injury model of chronic pain in the rat.

Materials

Spared Nerve Injury

1. Solutions and Drugs

- 70% ethanol
- Alcohol wipes
- Chlorohexidine wipes
- Sevoflurane
- Triple antibiotic

2. Surgical Tools

- Fur clippers
- #11 blade scalpel
- Small, straight hemostat
- Retractor
- Curved forceps (blunt and sharp)
- Microscissors

3. Disposable Materials

- Sterile gauze squares
- Sterile cotton swabs
- Sterile 5-0 silk suture
- Sterile 4-0 absorbable Vicryl suture

4. Miscellaneous

- Sharpie
- Clean cage for animals after surgery
- Heated pad
- Rectal thermometer
- SpO₂/heart rate monitor
- Bead sterilizer

Electrode + IPG Surgery

1. Solutions and Drugs

- 70% Ethanol
- Chlorohexidine scrub
- Triple antibiotic
- 0.25% bupivavaine
- Carprofen diluted to 2.5 mg/mL, injected at 5mg/kg
- Sevoflurane
- Gentamicin
- Puralube petroleum jelly (or similar, for eyes)
- Injectable saline in case of fluid + heat loss
- 100 mL sterile saline vial - wound/equipment wash

2. Surgical Tools

- Fur clippers
- IPG kit (screwdriver, IPG, blockers for unused lead locations)
- Electrode + lead
- #11 blade scalpel
- Four mosquito clamps
- Small, straight hemostate
- Periosteum scraper
- Rat-tooth forceps
- Curved forceps (sharp and blunt tips)
- Microscissors
- Rongeurs - smallest available, make sure tips are intact
- Periosteal elevator
- Trocar

3. Disposable Materials

- 2 × 1 mL sterile syringe
- Sterile needle for injection
- 2x sterile drape, with aperture
- 2 pairs sterile gloves
- Absorbable 4-0 Vicryl suture (x3)
- 5-0 silk suture
- Gauze squares (2" + 4")
- Cotton swabs
- Aluminum foil
- Blue drape material (to weigh implanted materials, help with adding sevoflurane, additional place for tools)

4. Miscellaneous

- Heated pad
- Rectal thermometer
- SpO₂/heart rate monitor
- Bead sterilizer

Paw Withdrawal Threshold Testing

1. Wavewriter Alpha IPG (SC-1232, Boston Scientific)
2. Clinician programmer (NM-7164, Boston Scientific)
3. Electronic von Frey (IITC Life Sciences)
4. Rigid testing tips (IITC Life Sciences)
5. Electronic von Frey Mesh stand
6. Acrylic animal enclosures

Baseline Paw Withdrawal Threshold Testing

- 1 Paw withdrawal threshold testing is performed with an electronic von Frey, and is a modified version of measuring mechanical sensitivity to that described in the foundational literature [Chaplan SR 1994, Bradman MJG 2015]

Room Preparation:

- Bring animals in their home cages into the testing room. Wait 30 minutes.
- While waiting, calibrate aesthesiometer (Electronic von Frey, IITC Life Science). Place rigid tip tester on aesthesiometer.
- Place animals into individual Plexiglas containers over metal grid flooring. Wait 15 minutes. Blinded tester and record keeper remain in the room.

- 2 Paw Withdrawal Testing:

- Make sure that limbs to be tested are weight-bearing. Do not test when animals are laying on their sides, stomachs, or are otherwise asleep. Use small noises or gently brushing the underside of the animal if necessary to ensure a calm, awake position.
- Present tip on aesthesiometer perpendicularly to lateral 1/3 of the plantar surface of the left hindpaw, aiming for the underside of the paw, not the digits, and avoiding the raised tori on the surface. Increase pressure until animals removes hindpaw. Filament application should be smooth and avoid bouncing. Repeat after 30 seconds if not.
- Mark down maximum force applied (from aesthesiometer).
- If ambulation is observed, consider stimulus ambiguous. Wait at least 30 seconds and repeat stimulus.
- Repeat for left hindpaw of all animals.
- Repeat for right hindpaw of all animals.
- Repeat this left, then right progression for all animals for a total of 6 measurements from each hindpaw.
- Use the average of the 6 technical replicates as the paw withdrawal threshold, in grams, for the given paw of the animal.

Important!

Experimenters should remain in the room for the entire duration of testing without external interruption and with minimal talking.

Use Sharpie to mark the base of the animal's tail to distinguish between cagemates.

Spared Nerve Injury

- 3 This experimental step is performed in accordance with the previously published spared nerve injury model of the rat [Decosterd 2000].

Set-up:

- Autoclave surgical instruments, swabs, and gauze
- Cover workspace with absorbent sheet
- Pre-flush gas chamber with oxygen before opening
- Line gas chamber with absorbent sheet
- Make sure gas line is connected to induction chamber

Induction:

- Obtain animal and weigh, then place into induction box
- Open the gas tank and turn on oxygen to 2L/min
- Turn on sevoflurane to 4% to fill chamber
- When animal is sedated take out of chamber and fit into standalone face mask - be sure to change air flow to the facemask from the induction chamber.
- Turn gas down to 2% in 1.5-2 L/min oxygen, or lower as needed if breathing is too slow (goal is ~60 breaths per minute)
- Use clippers to shave left hindlimb in the curve of the femur/hip and position on side so that shaved limb is facing up.

Pre-incision:

- Place heart rate monitor clip on the foot (if using monitor)
- Insert rectal temperature probe
- Check for toe pinch reflex - wait if there is a response. Recheck every 2 minutes, increase sevoflurane if there continues to be a response after 3 rechecks. Only proceed once there is no response.
- Place sterile drape over animal
- Sterilize left hindlimb with chlorhexidine scrub followed by alcohol wipe repeated 3x.

Surgery:

- Use a #11 scalpel to make an incision along the left hindlimb. Aim for the muscle inside the curve of the femur/hip. The sciatic nerve branch point usually lies under a point on the line from the knee to the base of the tail.
- Make a small score on the muscle overlaying the sciatic nerve then use the hemostat or scissors to blunt dissect through the muscle, taking care not to stretch any part of the nerve. Do not stretch once an opening in the muscle has been made because the nerves underneath could be stretched.
- Expose the branch point of the sciatic nerve without stretching or impinging the nerve.
- Identify the common peroneal, tibial, and sural branches of the sciatic nerve. The CP branch is usually the most superficial and runs most rostral. The tibial is usually in the middle and dives ventrally. The sural branch is the smallest, and loops back toward the tail region.
- Use 5-0 silk suture with a curved needle to loop under the common peroneal branch. Ligate tightly with a double suture knot. Perform an axotomy by removing 2-4 mm of

the nerve branch *distal* to the ligation using microscissors.

- Repeat the ligation and axotomy on the tibial branch. Leave the sural branch intact.
- Close the muscle and skin in two layers using 4-0 Vicryl absorbable suture.
- Turn off the gas. Place animal into a clean cage to recover with heat support underneath the cage.

Injury Confirmation Paw Withdrawal Threshold Testing

- 4 Steps are repeated as in Section 1&2 of Baseline Paw Withdrawal Threshold Testing with the following considerations:

- Animal order in testing cages is maintained between baseline testing and spared nerve injury. In case of animal loss, the cage is left empty.
- The average of the 6 technical replicates is the post-surgical paw withdrawal threshold, in grams, for the given paw of the animal.

Spinal Cord Stimulation Electrode and Implantable Pulse Generator Surgery

- 5 Set-up:
- At least 1-2 days prior to surgery, sterilize implantable pulse generator (IPG) and electrode/lead with EtO
 - Weigh animal
 - Inject animal with 5 mg/kg of carprofen at least 30 minutes prior to surgery start time.
 - Autoclave surgical instruments, swabs, and gauze
 - Cover heat pad with absorbent sheets, orient heat pad so rat is left/right on the table
 - Pre-flush gas chamber with oxygen before opening
 - Line gas chamber with absorbent sheet
 - Make sure gas line control valve is connected to standalone face mask and not stereotax
 - Fill 1 mL with 0.25% bupivacaine. Maximum amount is not to exceed volumes described previously (<https://animal.research.uiowa.edu/iacuc-guidelines-analgesia>):
 - Fill 3 mL syringe with injectable normal saline, for hydration support.
 - Turn on bead sterilizer

Induction:

- Turn on oxygen to 2L/min
- Turn on sevoflurane to 4% to fill chamber - allow 30 seconds for chamber to fill
- Place animal inside chamber
- When animal is sedated take out of chamber and fit into standalone face mask.
- Turn gas down to 3% in 1.5-2L/min oxygen, or lower as needed if breathing is too slow (goal is about 60 breaths per minute)
- Use clippers to shave the abdomen on the right side from bottom of the rib cage down to thigh, and the thoracic spine from about T9 to L3

- Flip gas control valve to divert gase to stereotax and move rat to nose cone in stereotax.

Pre-incision:

- Cover eyes with Puralub petroleum jelly
- Turn on heating pad
- Place heart rate monitor clip on foot
- Insert rectal temperature probe
- Place rat on covered heat pad, double check nosecone placement
- Place sterile drape over the animal
- Check for toe pinch reflex - wait if there is a response. Recheck every 2 minutes, increase sevoflurane if there continues to be a response after 3 rechecks. Only proceed once there is no response.
- Sterilize animal's thoracic spine with 3x chlorohexidine/alcohol
- Create sterile area for tools/equipment, cover light handle, sevoflurane vaporizer, microscope adjustments, and pen with sterile foil
- Put gob of 3x antibiotic onto sterile field

SCS Implant Surgery:

- Use a #11 scalpel to make an incision along the midline of the thoracic spine from roughly T12 to L2
- Use bupivacaine syringe to drop ~0.25 mL over the incision site. Wait a minute for absorption.
- Scrape tissue away from the spinous processes using periosteal scraper or another scraper tool. Avoid cutting and removing muscle as much as possible.
- Use the electrode to be implanted to determine the size of the epidural space required (1-1.5 spinal levels most likely).
- Beginning at the caudal side of L1, remove just enough bone to insert the electrode epidurally. Insert the electrode rostrally so that the electrode contacts end up at about T12-T13.
- Thread suture (5-0 silk) through the silicone wings on the side of the electrode, and secure to muscle or ligament on each side of the spinal column. Thread a third suture loosely around the wires at the back of the electrode and through the muscle. Thread a fourth suture as needed, depending on where the lead is tunneled.
- Place sterile gauze over open area + exposed lead, and gently turn the rat onto its side.

IPG + electrode check

- Connect the IPG to the lead. Place a piece of sterile gauze onto the top of the IPG.
- Connect to the clinician programmer. Test & record impedances. Readjust if impedances are not in the green (<8000 Ohms).
- Test lower/lower single frequency stimulation motor threshold and higher/higher single frequency stimulation motor threshold. Do this by increasing stimulation

amplitude in 250 μ A intervals until a visible twitch or contraction is seen in the thigh. Record for both the rostral-caudal and medial-lateral orientations.

- Disconnect IPG.

Lead tunneling

- Sterilize the animal's abdomen with 3x chlorohexidine/alcohol.
- Open the skin with a single incision from just below the rib cage to the top of the thigh on the right hand side.
- Drop ~0.25 mL of bupivacaine along the site of the incision. Allow to absorb for ~1 minute.
- Use needle hemostats to gently separate the skin from the muscle, creating a subcutaneous pocket that spans the entire abdomen below the rib cage.
- Feed a trocar from the abdomen under the skin to the opening on the back, taking care not to puncture into the abdominal cavity.
- Remove the inner piece of the trocar, and feed the electrode lead through the remaining tube.
- Remove the rest of the trocar.
- Re-cover lead with sterile gauze, cover the abdominal opening with sterile gauze, and gently turn the animal back onto its stomach.

Finishing Procedure for the Back

- Close muscle with 4-0 Vicryl sutures, wash with saline if it has dried out some.
- Close skin with 4-0 Vicryl sutures
- Place triple antibiotic onto the surface of the incision.
- Gently turn the animal back onto its back.

IPG Placement

- Reconnect the IPG to the lead. Wash with saline.
- Loop lead underneath the IPG, and place the IPG centrally subcutaneous over the abdomen.
- Secure the IPG to the abdominal wall using sutures and the loops in the IPG.
- Secure lead to the abdominal wall if needed.

Finishing Procedure for the Abdomen

- Close the skin with 4-0 Vicryl sutures.
- Place triple antibiotic along the cut edges of the skin.
- Turn off sevoflurane.
- Inject 3mL of subcutaneous saline for hydration support.
- Allow rat to breath oxygen until it begins to stir. Then return animal to a clean cage to recover. Place clean cage over heat pad for supplemental warmth. Monitor animal closely until sternal recumbency is regained. Keep on heat with access to mash food, hydration pack, and water for at least 2 hours before returning to vivarium.

Paw Withdrawal Threshold Testing for Spinal Cord Stimulation

- 6 6 stimulation patterns were delivered in two different configurations, for a total of 12 different stimulation conditions (sham, 2-2 Hz, 54-54 Hz, 25-40 Hz, 54-2 Hz, 2-54 Hz across medial-lateral and rostral-caudal contact pairs).

The 6 stimulation patterns for a given contact pair orientation were delivered in a random order across 6 days of testing at the same time of day (morning or afternoon, start times separated by 5 hours). In each cohort of up to 4 animals, half of the cohort received medial-lateral stimulation in the morning and rostral-caudal stimulation in the afternoon. The other half of the cohort received the opposite.

6.1 Room Preparation:

- In the morning, at least 1 hour after lights on in the 12/12 h light cycle, bring animals in their home cages into the testing room. Wait 30 minutes.
- While waiting, calibrate aesthesiometer (Electronic von Frey, IITC Life Science). Place rigid tip tester on aesthesiometer.
- While waiting, hold magnet up to the underside of the cage to put the IPG into pairing mode. Connect to IPG, and record impedances for each connected electrode contact. Test motor threshold. Repeat for each animal.
- After 30 minutes, place animals into Plexiglas chambers. Animal order in chambers is preserved each day of testing.

6.2 Motor Threshold Testing:

- Select desired single frequency program (2/2 Hz, 54/54 Hz in medial-lateral or rostral-caudal configuration).
- Increase amplitude in 25 μ A intervals until you see a visible muscle contraction (54/54 Hz) or rhythmic twitch (2/2 Hz) in the lower half of the animal. This is typically in the leg, but occasionally is first visible in the lower abdomen or the sacrum, depending on electrode placement.
- Turn off stimulation. Step amplitude down one interval. Turn on stimulation, verify no twitch.
- Increase amplitude one interval, verify twitch. Step up again to verify that there is a clear and strong noticeable twitch. Record the first amplitude with visible twitch.
- Repeat for all 4 single frequency programs.
- If lowest frequency motor threshold is marked different than highest frequency, used lowest frequency threshold for 2/2 Hz stimulation. If not, use highest frequency threshold for highest single frequency and all dual-frequency conditions.
- Stimulation will be at 80% of motor threshold for the rat.

6.3 Paw Withdrawal Threshold Testing for dfSCS

Baseline Testing

- After placing animals into Plexiglas chambers, wait 15 minutes for acclimation. Animals should be in a restful, awake state.
- Make sure that limbs to be tested are weight-bearing. Do not test when animals are laying on their sides, stomachs, or are otherwise asleep. Use small noises or gently brushing the underside of the animal if necessary to ensure a calm, awake position.
- Present tip on aesthesiometer perpendicularly to lateral 1/3 of the plantar surface of the left hindpaw, aiming for the underside of the paw, not the digits, and avoiding the raised tori on the surface. Increase pressure until animals removes hindpaw. Filament application should be smooth and avoid bouncing. Repeat after 30 seconds if not.
- Mark down maximum force applied (from aesthesiometer).
- If ambulation is observed, consider stimulus ambiguous. Wait at least 30 seconds and repeat stimulus.
- Repeat for left hindpaw of all animals.
- Repeat for right hindpaw of all animals.
- Repeat this left, then right progression for all animals for a total of 3 measurements from each hindpaw.

Stimulation Testing

- Turn on desired stimulation pattern/configuration at 80% of respective motor threshold amplitude (see 6.2). Wait 10 minutes.
- Record 3 paw withdrawal thresholds of the left hindpaw and 3 of the right in an alternating manner as described in baseline testing.
- Wait 24 minutes from stimulation onset of the final animal.
- Record 3 paw withdrawal thresholds of the left hindpaw and 3 of the right in an alternative manner.

Post-Stimulation Testing

- Turn off stimulation
- Perform 3 repeats of left and 3 repeats of right hindpaw 10 minutes after turning off stimulation
- Perform 3 repeats of left and 3 repeats of right hindpaw starting 24 minutes after turning off stimulation.
- Animals are returned to their homecage with *ad libitum* access to food and water. 4.5 hours after the start of the morning testing, motor thresholds are retested. They are returned to the testing chambers 5 hours after the start of the morning test for the afternoon tests and the steps outlined in baseline, stimulation, and post-stimulation testing are repeated for another stimulation condition.

Important!

Experimenters should remain in the room for the entire duration of each testing block without interruption and with minimal talking. The same experimenters should be



available for each day of testing. Care should be taken to keep daily start time consistent (within 1 hour) across test days.

Protocol references

Spared nerve injury model:

Decosterd I, Woolf CJ (2000) Spared nerve injury: An animal model of persistent peripheral neuropathic pain. *Pain* 87:149-158.

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