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6–OHDA ICR injections in Medial Forebrain Bundle (MFB)

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SOX6 mDA differentiation

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We use this protocol and it's working

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Abstract

This protocol was used to unilaterally inject 6-OHDA in the medial forebrain bundle of mice using a Neurostar Robotic Stereotaxic Frame. This produced hemiparkinsonian mice that survived up to 8 months with motor impairment.

Guidelines

Section 1. Rationale (summary on page)
- 6-OHDA is a toxin incorporated into DAergic cells by DAT and NET; cannot cross BBB so must be injected into the brain.
- MFB injection used to avoid several injections (e.g., in striatum) or missing targets (more probable with SN).
- Critical pre- and post-surgical care designed to avoid loss of animals.

Section 2. Protocol (procedural steps present on these pages)

2.1 Pre-operational care
2.1.1 Habituation
- Handle the animals weeks before the procedure to habituate them to your contact.
- Make sure the animals are also habituated to new diets before the surgery.
- If behaviors are necessary for the study, make sure to have finished training them at least a week before the surgery.

2.1.2 Weight control
- Ensure animals have a minimum weight of 22 g.
- Monitor animal weight at least 2 weeks before and feed with caloric diets (Dietgel, diluted condensed milk with ddH_2O 1:1, etc.).

2.2 Intracranial injections
2.2.1 6–OHDA preparation
- For a 3.6 µg/µl of free base–6-OHDA, prepare 4.4 µg/µl of 6–OHDA-HCl.
- Dilute the 6–OHDA-HCl in sterile (by filtration) saline with 0.02% ascorbic acid.
- Protect from light and keep cold. Prepare fresh and do not use more than 4 hours after preparation (preferably 2 hours).

2.2.2 Pre-operational analgesia
- 30 minutes before cranial drilling, inject subcutaneously (SC) Carprofen (Rimadyl) and Buprenorphine (Vetergesic) at doses of 5 and 0.05 mg/kg, respectively.
- To prepare analgesics: dilute Rymadil (Rimadyl) 50 mg/ml to 5 mg/ml (1:10 dilution with saline or Benelyte) and Vetergesic 0.3 mg/ml to 0.1 mg/ml (1:3 dilution with saline or Benelyte).
- Using these diluted analgesics, the dose can be calculated as 1 µl of Rymadil 5 mg/ml and 0.5 µl of Vetergesic 0.1 mg/ml per gram of mouse weight.
- Inject SC using 30G 0.3 ml insulin syringes (BD Micro-fine).

2.2.3 Stereotaxic frame and surgical field preparation
- Ensure the robotic Neurostar frame is connected (Drill, Injector, AP, DV, ML, Heating Pad, Autostop) and the robot box is on.
- Initiate Stereodrive software and check movement of the frame (*If it doesn't move at one of the axis, check cable connection*).

- Check frame coordinates calibration, injectomate calibration and drill functioning.
- Turn on the heating pad at 38°C.
- Once calibrated and working properly, sterilize the surgical field with isopropyl alcohol (or similar).

2.2.4 Injector preparation

- Pull a glass capillary to a length of 1–1.5 cm and an outer diameter of 150–300 µm (you can insert the tip inside a 25G needle).
- Mount the glass capillary tip in the Hamilton capillary adaptors for the 5–10 µl Hamilton needle (#701RN) and prime the injector by filling sterile water/saline/oil (*preferably sterile saline, oil stains too much although it is very effective at avoiding air bubbles*) using a 27G and injecting on the plunger hole.
- Fill until some drops fall from the tip, then close with the plunger.
- Mount on the frame and position both the drill and glass tip at approximately the same level. Clean with chlorhexidine and sterile saline.

2.2.5 Anesthesia

- Isoflurane (Attane) is used as anesthetic gas.
- For induction, use 4% Isoflurane concentration at a flow of 400 ml/min directed to the clear plexiglass box.
- Recommendation: While the animal is anesthetized, shave the head for surgery (AWAY FROM SURGICAL FIELD) and finish induction.
- Once fully asleep, direct the flow to mouse mask and position the mouse in mask while securing the teeth bar. Immediately turn the flow down to 200 ml/min and 1.5% isoflurane concentration.
- Position the animal so the skull can be secured using either ear bars or blunt bars (If you use blunt bars with adjustable heads, make sure that the head does not collide with the drill DV).
- Check anesthesia plane periodically by absence of pedal reflex. Change isoflurane concentration if necessary.
- Also, check breathing rate. If the mouse is breathing erratically or over ventilating, change the air flow until breathing improves (usually 200 ml/min works, but in any case, never go over 250 ml/min or under 150 ml/min).

2.2.6 Peri-surgical preparation

- Once the animal is secured and under anesthesia, inject SC in the scalp with a 30G needle 20–50 µl (a drop) of Lidocain (Marcain) and wait approximately 5 minutes.
- Sterilize equipment (either with disinfectant or glass bead sterilizer):
  - Scalpel handle (#3 for #15 blades)
  - Fine forceps
  - Blunt forceps
  - Suture forceps
  - Fine scissors
- Disinfect scalp with chlorhexidine and moisten the eyes with artificial tear drops or eye gel.

2.2.7 Surgery

- Open scalp with surgical blades. Make sure the incision is just big enough to access Bregma, Lambda and about 2 mm of one side.

- Swab with sterile cottons swabs H_2O_2 3% (in saline) until Bregma is identifiable and then clean with sterile saline.
- Coordinate drill and syringe using Bregma, and then correct for tilt and scaling with the syringe. *(Do not fully believe that drill and syringe are fully synchronized, since the syringe is much narrower than the drill tip. Check if you have to correct the drill position by marking where the syringe should go and seeing if the drill would bore into the mark).*
- Drill at the right medial forebrain bundle coordinates:
  - MFB: 1.2 mm ML, -1.2 mm AP, 4.5 to 5.0 mm DV from Bregma.

2.2.8 6–OHDA ICR injection

- Aspirate 0.5–1 µl of air (enough to generate an air barrier between the priming solution and the 6–OHDA solution).
- Aspirate 2 µl of 6–OHDA solution and inject the capillary glass tip in the deep MFB coordinates at a rate of 0.2 mm/min.
  - MFB: 1.2 mm ML, -1.2 mm AP, 5.0 mm DV from Bregma.
- Keep the tip inside for 5 minutes before injecting.
- Inject 1 µl of 6–OHDA at a rate of 0.2 µl/min and ascending 500 µm for 3 minutes (Turn ON DV movement: MOVE UP 0.5 mm, 3 minutes) and inject for a total of 5 minutes (2 minutes at 4.5 mm DV).
- Hopefully, the track generated by the upwards movement will help avoid the off-target diffusion of 6-OHDA.
- Leave the tip 5 minutes after injection. Retreat the tip at a rate of 0.2 mm/min *(You can click the upwards arrow on the left side of the software window instead of using the keyboard keys for a 5 mm continuous retreat).*

2.2.9 Post-injection care

- After carefully securing the drill/tip (Move Home), moisturize scalp with sterile saline using cotton swabs and close the wound using the blunt forceps.
- When the skin is closed, suture so no grooves or pockets are formed.
- Close isoflurane flow (or change to box for next mouse going back to 2.2.2 and 2.2.4) and release from teeth bar clamp. (Make sure that the tongue has not dried onto the clamp; if so, moisturize with saline to avoid damaging the tongue).
- Inject IP 1 ml of sterile WARMED saline or Benelyte. Place in a warm cage for anesthesia recovery. Wait until mouse is able to move with all limbs to return to the home cage.

2.3 Post-surgical care

2.3.1 Temperature control

Mice will stay 2 weeks in a controlled temperature room at 27–28°C. After that, they can go back to general care.

2.3.2 Analgesia

For 72 hours after the day of injection, analgesics are injected SC as follows. Use dose as explained in 2.2.2:

- Rymadil 5 mg/kg + Vetergesic 0.1 mg/kg in the morning
- Vetergesic 0.1 mg/kg in the evening (4–6 hours after morning dose)

2.3.3 Rehydration

1 ml of warmed (not hot, you should be able to hold the solution and it feels warm without discomfort) sterile Benelyte or saline with glucose 10% IP.

2.3.4 Special diet

Prepare Dietgel chunks on the flat lid of a small 35 mm ø petri dish, a paste of mice feed with Diluted condensed milk 1:1 on the deep side of the same petri dish and 2 caps of 50 ml falcon tubes filled with Diluted condensed milk 1:1.

2.3.5 Weight and health monitoring

For 2 weeks, check weight in the morning and general health (movement, pain signs, skin, etc.) twice a day. After that, check twice a week, with at least 3 days in-between.

If after critical care, the animals are not back to original weight or over 22 g, feed with special diet before transplantation.

Materials

- 6–OHDA-HCl (for preparing 6–OHDA solution; for 3.6 µg/µl free base use 4.4 µg/µl 6–OHDA-HCl)
- Sterile saline (by filtration)
- Ascorbic acid (0.02%)
- Carprofen (Rimadyl) 50 mg/ml (for dilution to 5 mg/ml)
- Buprenorphine (Vetergesic) 0.3 mg/ml (for dilution to 0.1 mg/ml)
- Benelyte or saline (for analgesic dilutions)
- Glass capillaries (pulled to 1–1.5 cm length, outer diameter 150–300 µm)
- 25G needle (to insert capillary tip inside)
- Hamilton capillary adaptors for 5–10 µl Hamilton needle (e.g., #701RN)
- 5–10 µl Hamilton needle (#701RN)
- 27G needle (for priming injector)
- 30G 0.3 ml insulin syringes (BD Micro-fine)
- Sterile water/saline/oil (for priming; preferably sterile saline)
- Robotic Neurostar stereotaxic frame (Drill, Injector, AP, DV, ML, Heating Pad, Autostop) and robot box
- Stereodrive software
- Heating pad (settable to 38°C)
- Isopropyl alcohol (or similar) for surgical field sterilization
- Drill, injector and related stereotaxic accessories
- Dietgel, diluted condensed milk (for weight maintenance) with ddH_2O (1:1)
- Chlorhexidine (for cleaning surgical site)
- Isoflurane (Attane) and anesthesia gas delivery setup (clear plexiglass induction box, flowmeter/regulator, mouse mask, tubing)
- Plexiglass induction box (clear) for induction
- Teeth bar (for securing mouse) and ear bars or blunt bars (with adjustable heads)
- 30G needle (for SC scalp injection of Lidocain)
- Lidocain (Marcain) (for local scalp SC injection)
- Scalpel handle (#3 for #15 blades)
- Fine forceps
- Blunt forceps
- Suture forceps
- Fine scissors
- Sutures (for skin closure)
- Artificial tear drops or eye gel
- Sterile cotton swabs
- H_2O_2 3% (in saline)
- Warm cage or heating recovery area
- Warmed sterile saline or warmed Benelyte (for IP injection/re-hydration)
- Glucose (to prepare saline with 10% glucose for IP rehydration)
- Small 35 mm petri dish (flat lid) and small petri dish for food presentation
- 50 ml Falcon tubes (for diluted condensed milk)

Protocol materials

Dietgel **ClearH20 Catalog #**72-06-5022

Rimadyl (Carprofen) **Zoetis**

Safety warnings

- 6–OHDA-HCl solution: Protect from light, maintain cold; prepare fresh and do not use more than 4 hours after preparation (preferably use within 2 hours).
- Ensure animals meet minimum weight requirement (minimum 22 g) before surgery.
- Sterilize surgical field and equipment (e.g., with isopropyl alcohol).
- Check robotic/stereotaxic equipment movement and connections before use (*If it doesn't move at one of the axis, check cable connection*).
- When priming injector, prefer sterile saline (oil can stain but may avoid air bubbles).
- Anesthesia flow limits: do not exceed 250 ml/min or go below 150 ml/min when adjusting isoflurane airflow.
- Monitor anesthesia plane by absence of pedal reflex and by respiration; adjust isoflurane concentration if necessary.
- If mouse breathing is erratic or over-ventilating, change airflow until breathing normalizes (usually ~200 ml/min).
- When securing head with blunt bars, ensure head does not collide with drill DV.
- Do not fully believe that drill and syringe are fully synchronized; check and correct drill position relative to the syringe as needed.
- After release from the clamp, ensure the tongue has not dried onto the teeth bar; if dried, moisturize with saline to avoid tongue damage.
- For IP rehydration, use warmed (not hot) solution — it should feel warm without causing discomfort.

Ethics statement

This protocol requires prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee.

In our case, all procedures were approved by the local ethical committee (Stockholms djurforsoksetiska namnd) and followed the EU directive 2010/63/EU under the ethical permit of Ernesto Arenas Cases and Sandra Gellhaar (12265-2021; 2-3143/23; 2-3595/24; 2-3147/24).

Before start

2.1 Pre-operational care
- Habituation: handle animals weeks before procedure; habituate to new diets; finish behavioral training ≥1 week before surgery if needed.
- Weight control: ensure minimum animal weight of 22 g; monitor weight ≥2 weeks before; provide caloric diets (Dietgel, diluted condensed milk with ddH_2O 1:1, etc.).

Pre-operational care

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1 Handle the animals weeks before the procedure in order to habituate them to your contact. Make sure the animals are also habituated to new diets before the surgery. If behaviors are necessary for the study, make sure to have finished training them at least a week before the surgery.

2 Make sure that animals have a minimum weight of 22 g. To achieve this weight, monitor animal weight at least 2 weeks before and feed with caloric diets (Dietgel **ClearH20 Catalog #**72-06-5022 , Dietgel Recovery, Diluted condensed milk with ddH_2O 1:1, etc.).

Intracranial injections

3 For a 3.6 µg/µl of free base–6-OHDA, you need a 4.4 µg/µl of 6-OHDA-HCl.
Dilute the 6-OHDA-HCl in sterile (by filtration) saline with 0.02% ascorbic acid. Protect from light and maintain On ice . Make sure to prepare it fresh and not to use it more than 4 hours after preparation (preferably 2 hours).

4 30 minutes before the cranial drilling, inject subcutaneously (SC) Rimadyl (Carprofen) **Zoetis** and Buprenorphine (Vetergesic) at a dose of 5 and 0.1 mg/kg respectively.
In order to prepare the analgesics, dilute the Rymadil 50 mg/ml to 5 mg/ml (1:10 dilution with saline or Benelyte) and Vetergesic 0.3 mg/ml to 0.1 mg/ml (1:3 dilution with saline or Benelyte).
Using these diluted analgesics, the dose can be calculated to 1 µl of Rymadil 5 mg/ml and 0.5 µl of Vetergesic 0.1 mg/ml per gram of weight of mouse. Inject SC using 30G 0.3 ml insulin syringes (BD Micro-fine).

5 Make sure that the robotic Neurostar frame is connected (Drill, Injector, AP, DV, ML, Heating Pad, Autostop) and the robot box is on.
Initiate Stereodrive software and check movement of frame (*If it doesn't move at one of the axis, check cable connection*). Check frame coordinates calibration, injectomate calibration and drill functioning.
Turn on the heating pad at 38°C.
Once everything is calibrated and working properly, sterilize the surgical field with isopropyl alcohol (or similar).

6 Pull a glass capillary to a length of 1–1.5 cm, and a outer diameter of 150–300 µm (you can insert the tip inside a 25G needle).
Mount the glass capillary tip in the Hamilton capillary adaptors for the 5–10 µl Hamilton needle (#701RN) and prime the injector by filling sterile water/saline/oil *(preferably sterile saline, oil stains too much although it is very effective at avoiding air bubbles)* using a 27G and injecting on the plunger hole. Fill until some drops fall from the tip, and close with the plunger.
Mount on the frame and position both the drill and glass tip at approximately the same level. Clean with chlorhexidine and sterile saline.

7 Isoflurane (Attane) is used as anesthetic gas.
For induction, use 4% Isoflurane concentration at a flow of 400 ml/min directed to the clear plexiglass box.
Recommendation: While the animal is anesthetized, shave the head for surgery (AWAY FROM SURGICAL FIELD) and finish induction.
Once fully asleep, direct the flow to mouse mask and position the mouse in mask while securing the teeth bar. Immediately turn the flow down to 200 ml/min and 1.5% isoflurane concentration. Position the animal so the skull can be secured using either ear bars or blunt bars (If you use blunt bars with adjustable heads, make sure that the head does not collide with the drill DV).
Check anesthesia plane periodically by absence of pedal reflex. Change isoflurane concentration if necessary.
Also, check breathing rate. If the mouse is breathing erratically or over ventilating, change the air flow until breathing improves (usually 200 ml/min works, but in any case, never go over 250 ml/min or under 150 ml/min).

8 Once the animal is secured and under anesthesia, inject SC in the scalp with a 30G needle 20–50 µl (a drop) of Lidocain (Marcain) and wait approximately 5 minutes.
Sterilize equipment (either with disinfectant or glass bead sterilizer):
- Scalpel handle (#3 for #15 blades)
- Fine forceps
- Blunt forceps
- Suture forceps
- Fine scissors

Disinfect scalp with chlorhexidine and moisten the eyes with artificial tear drops or eye gel.

9 Open scalp with surgical blades. Make sure the incision is just big enough to access Bregma, Lambda and about 2 mm of one side.
Swab with sterile cotton swabs H_2O_2 3% (in saline) until Bregma is identifiable and then clean with sterile saline.
Coordinate drill and syringe using Bregma, and then correct for tilt and scaling with the syringe. (Do not fully believe that drill and syringe are fully synchronized, since the

syringe is much narrower than the drill tip. Check if you have to correct the drill position by marking where the syringe should go and seeing if the drill would bore into the mark).
Drill at the right medial forebrain bundle coordinates: MFB: 1.2 mm ML, -1.2 mm AP, 4.5 to 5.0 mm DV from Bregma.

10 Aspirate 0.5–1 µl of air (enough to generate an air barrier between the priming solution and the 6–OHDA solution).
Aspirate 2 µl of 6-OHDA solution and inject the capillary glass tip in the deep MFB coordinates at a rate of 0.2 mm/min.
MFB: 1.2 mm ML, -1.2 mm AP, 5.0 mm DV from Bregma.
Keep the tip inside for 5 minutes before injecting.
Inject 1 µl of 6-OHDA at a rate of 0.2 µl/min and ascend 500 µm over 3 minutes (Turn ON DV movement: MOVE UP 0.5 mm, 3 minutes) and inject for a total of 5 minutes (2 minutes at 4.5 mm DV).
Hopefully, the track generated by the upwards movement will help avoid the off-target diffusion of 6-OHDA.
Leave the tip inside for 5 minutes after injection. Retreat the tip at a rate of 0.2 mm/min. (You can click the upwards arrow on the left side of the software window instead of using the keyboard keys for a 5 mm continuous retreat).

11 After carefully securing the drill/tip (Move Home), moisturize the scalp with sterile saline using cotton swabs and close the wound using blunt forceps.
When the skin is closed, suture so no grooves or pockets are formed.
Close isoflurane flow (or change to box for next mouse) and release from teeth bar clamp. Make sure that the tongue has not dried onto the clamp; if so, moisturize with saline to avoid damaging the tongue.
Inject IP 1 ml of sterile WARMED saline or Benelyte. Place the mouse in a warm cage for anesthesia recovery. Wait until the mouse is able to move with all limbs before returning to the home cage.

Post-surgical care

12 Mice will stay up to 1 week in a controlled temperature room at 27–28°C. After that, they can go back to general care.

13 For 72 hours after the day of injection, analgesics are injected SC as follows. Use dose as explained in 2.2.2:
- Rymadil 5 mg/kg + Vetergesic 0.1 mg/kg in the morning
- Vetergesic 0.1 mg/kg in the evening (4–6 hours after morning dose)

14 Inject 1 ml of warmed (not hot — you should be able to hold the solution and it feels warm without discomfort) sterile Benelyte or saline with 10% glucose IP.

15 Prepare Dietgel chunks on the flat lid of a small 35 mm Ø petri dish, place a paste of mice feed mixed with diluted condensed milk 1:1 on the deep side of the same petri dish, and provide 2 caps of 50 ml Falcon tubes filled with diluted condensed milk 1:1.

16 For 2 weeks, check weight in the morning and general health (movement, pain signs, skin, etc.) twice a day. After that, check twice a week, with at least 3 days in-between.