PROTOCOL FOR STEREOTAXIC SURGERY IN THE RAT

ANIMAL SURVIVAL SURGERY IS A DELICATE AND EXPENSIVE PROCEDURE THAT REQUIRES ALL YOUR ATTENTION AND CARE. PLEASE MAKE SURE TO READ ALL INSTRUCTIONS AND HAVE ALL MATERIALS PREPARED IN ADVANCE. DO NOT ALLOW ANYTHING TO DISTRACT YOU DURING SURGERY (CELLULAR PHONES: OFF!!). TAKE CARE OF YOUR ANIMALS AT ALL POINTS AND TREAT THEM GENTLY!

Materials required in advance:

- Animals:
- Lewis albino female rats, ~200g, ~12 weeks old

- Instruments:
- Stereotaxic frame (cleaned with alcohol)
- Head shaver (clean)
- 1 ml syringes and 27G (1/2") needles (sterile)
- Small scissors (sterile)
- Scalpel with #10 blade (sterile)
- Electric drill (Dremel 300) with drill bits #105 or #108 (Dremel, 0.8mm), or bits #106 or #109 (1.6mm) (sterile by alcohol)
- 10ul Hamilton syringe type #701 (sterile by alcohol)
- surgical suture thread #4 and suture needle (P3 or PS2, sterile by alcohol)
- plastic "cones" to hold the animals (clean from blood or feces)
- thermal pads to maintain animal temperature (clean with alcohol)
- "Deltaphase" thermal pads to maintain animal temperature (clean with alcohol)
- adsorbent paper to put under the animal body in the stereotaxic frame
- Q-tips (sterile)

- Materials:
- 70% isopropyl alcohol for asepsis
- povidone iodine or clorhexidine
- sterile bone wax
- cotton pads and sterile gauze
- sterile artificial tears
- 2% hydrogen peroxide solution
- triple antibiotic ointment (bacitracin, neomycin, polymixin B)
- sterile 10 mM PBS, pH 7.4

- Drugs:
- Ketamine 100mg/ml
- Xylazine 100 mg/ml
- Motrin (ibuprofen) 20 mg/ml
- Baytril (enrofloxacin) 100 mg/ml

- Surgical dressing:
- Surgical gown or lab coat
- Gloves
- Mouth mask
- Hair net

PRELIMINARY STEPS:

a) Preparation of anesthesia:
(this mixture is for RATS only)

Mix 8.0 ml Ketamine HCl 100 mg/ml
\+ 0.8 ml Xylazine 100 mg/ml
\+ 1.2 ml PBS
(this mixture lasts for three weeks, store in the dark at 4C)

Also, prepare a diluted ketamine in 10mM PBS (75 mg/ml) for anesthetic reinforcement if necessary

b) Preparation of cells: (CNS-1 glioma)
- trypsinize tumor cells and centrifuge 4min x 300g
- wash cells once (4min x 300g) using Hanks Balanced Saline Solution to remove traces of serum
- resuspend cells in HBSS and count them in hemocytometer
- centrifuge again 4min x 300g
- resuspend in HBSS: $2.5x10^4$ cells/µl (small tumors) or $1.0x10^5$ cells/µl (large tumors for survival studies)
- keep cells on ice and use within the next 4h. Vortex cells gently to prevent clumping.

c) Preparation of deltaphase pads: Microwave the pads (one at a time) at maximum power for 45sec. or until the internal gel changes from white to clear. Alternatively (preferred method), activate the pads by placing them in a water bath at 45C for 1h. After activation the pads will keep a temp slightly above 37C for several hours. These pads can be replaced with a thermal blanket to maintain animal temperature

SURGICAL PROCEDURE:

1) Bring all the animals in advance to the surgery room and weight each animal before surgery.

2) Hold the animal gently and put it into the restraining cone.

3) Restrain the animal and inject the anesthesia intra-peritoneal: 70 to 100 µl/100g body weight.

4) Wait for loss of consciousness and muscle tone (< 5min). Check cardiac rhythm and respiration. Gently open the mouth and move the tongue out of the airway to prevent choking.

5) Shave the head of the animal thoroughly (from in front of the eyes to behind the ears).

6) Place the animal in the stereotaxic frame and check loss of paw reflex with a pinch tweezer. Place the animal on top of a deltaphase heat pad (to keep body temperature). Check loss of palpebral reflex (==> deep anesthesia) with a small puff of clean air using a plastic pipette.

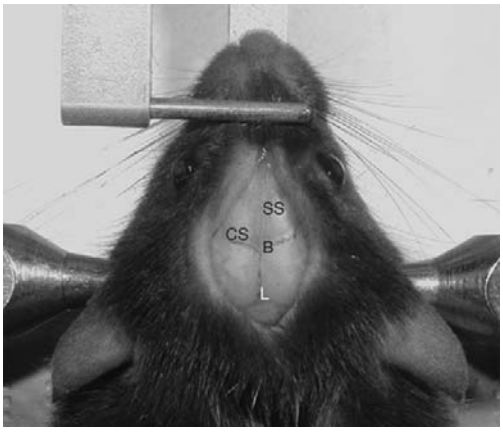
7) Open the mouth of the animal and place the head in the noseclamp, then insert the ear bars carefully. Make sure the tongue is out of the airway. Close the nose clamp and verify that the head cannot be moved. The upper surface of the head **should be horizontal** and parallel to the base of the stereotaxic frame.

8) If the temperature in the room is cold, cover the body of the animal with a cloth to prevent heat loss. This is not necessary if the animal is placed on a deltaphase heat pad. Add some artificial tears to the eyes to prevent dryness and damage of the cornea.

9) Load ~5 µl of well-dispersed cells in the Hamilton syringe (only 3 µl will be used) and secure the syringe to the stereotaxic arm, without touching the animal.

10) Clean the skin in the skull **twice**: first with q-tips with povidone iodine (or clorhexidine) and then with q-tips with isopropyl alcohol (circling movements out from the center)

11) Using the scalpel, make an incision in the midline between the eyes and cut ~10-15mm towards the back of the head. Do not cut more than necessary! Enlarge the incision to the sides using alcohol scrub or q-tips, or a clean spatula. Wipe blood from incision with q-tips. If bleeding is intense, use q-tips with 2% hydrogen peroxide but **do not use them in excess**! (they will cauterize the skin and the healing process will take much longer)



12) Localize the bregma in the skull (see representative figure with a mouse head)

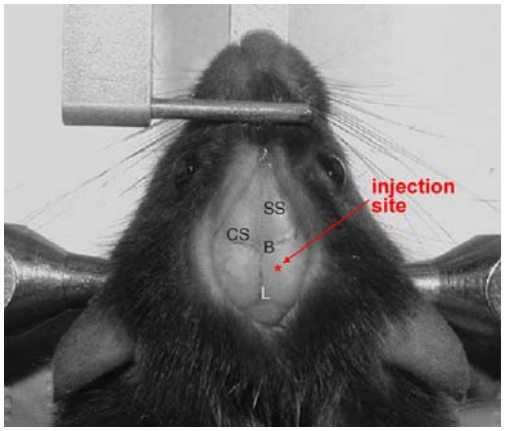
SS: sagital suture
CS: coronal suture
B: bregma
L: lambda

(notice the position of the earbars and the nose clamp.
The skull is flat horizontal)

If desired, mark the position of the bregma with a permanent marker for wet tissues.

13) Swing the stereo arm above the skull (**careful not to touch the syringe plunger!**) and carefully lower the tip of the syringe needle to measure the Z position of the lambda and bregma points. If necessary, adjust the position of the nose clamp to make sure that the lambda and bregma are at the same Z position.

14) Again using the tip of the syringe, measure the following coordinates for entry point:



2.8 mm caudal to bregma
2.5 mm lateral (right) to bregma

Mark this position with permanent tissue marker

After marking the position, swing the arm away to have space for drilling but **do not change the X,Y coordinates of the arm**.

15) Using the Dremmel drill at lowest speed and with drill-bit #105 (or 108), drill carefully a vertical hole (hold the drill steady and vertical!). Be careful **to NOT apply any pressure** or you will puncture the brain!! You need to drill until the hole just penetrates the skull.

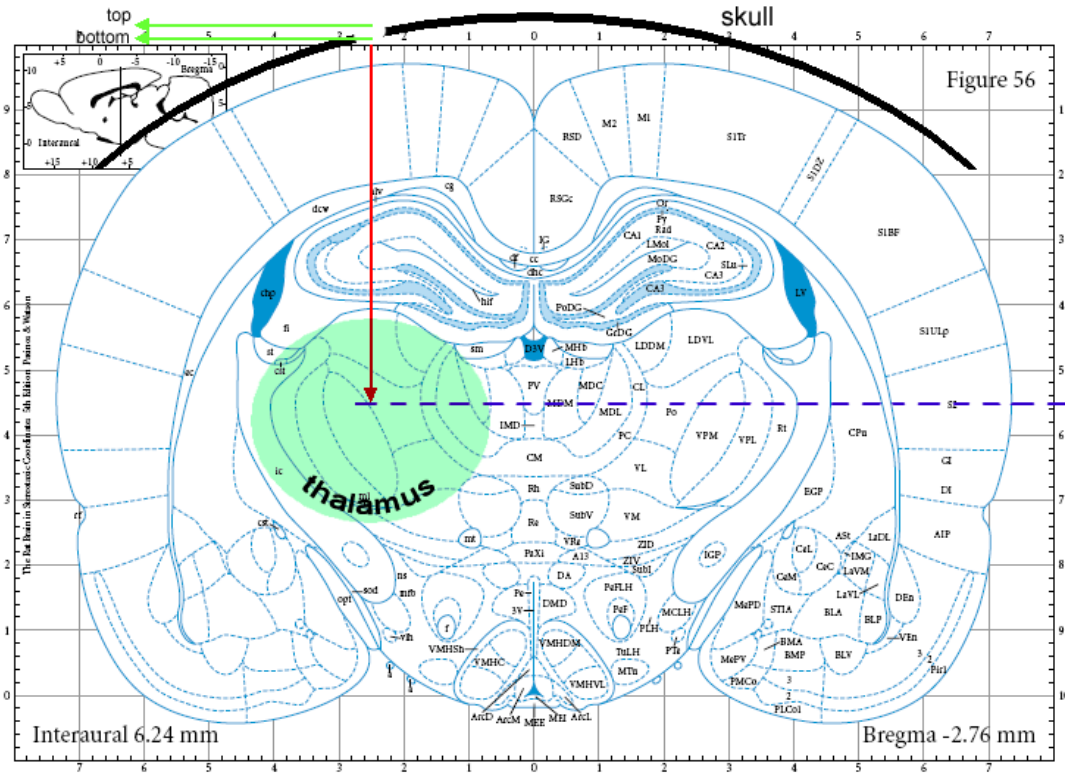
16) Bring the stereo arm over the hole and position the syringe on top of the hole. Make sure that the syringe is completely vertical (**no angle**) and that it is in the desired X,Y coordinates. The syringe **CANNOT** penetrate at an angle!!

17) Carefully lower the syringe at ~0.2 mm every 20 seconds, until it reaches a depth of 6 mm from the top of the hole or 5.5 mm from the bottom of the hole (see figure).

18) After the syringe is in the correct position, press slowly the plunger and inject the cells at 0.4 ul every 30 seconds. **BE CAREFUL** with the injection! **NEVER back up the syringe** during injection!!

19) Wait 2 minutes after injecting a total volume of 3 µl, and then retract the stereo arm at a speed of 0.2 mm every 20 seconds.

20) Check that there is no backflow of fluid after removing the syringe, clean the incision if necessary and apply a small amount of bone wax to seal the hole.



This is the position of injection in the Rat Brain Atlas (Paxinos & Watson). Notice that the position is minus 2.76mm from the Bregma and 2.50mm right from the midline (right side is on the left in the Atlas). The depth is 5.5mm if counted from the bottom of the hole or 6.0mm if counted from the top of the hole (border of the skull).

21) Suture the borders of the wound bringing the lips of the wound together and making triple-knot stitches every 4 mm. Use an FS-2, P3, or P-12 needle (cutting triangular needle, 3/8 inch) and #4 nylon thread for the stitches. Take care to **prevent exposure** of the fleshy lips of the wound and do not cut the skin with the suture! Make at least 4 to 5 stitches and make sure that there are no exposed parts of skin afterwards.

22) Apply antibiotic ointment to the lips of the wound.

23) Return the animal to the cage and place it on top of a small deltaphase heating pad. When the animals recover (~1-2h) they will immediately urinate, so have paper towels ready to clean the pads between animals.

24) Wait until all animals are conscious before bringing them back to the animal facility

25) Post-operative care:
- Check the animals daily, specially in the first three days after surgery and notify immediately any problems such as lack of movement, inflammation in the wound, distended belly, lordosis (arched back, a symptom of acute pain), removed stitches, etc. If the stitches appear removed, carefully apply a minimal amount of surgical skin glue ('VetBond', use only under supervision from a veterinarian or senior staff!).

- For the first 4 days after injection: Twice a day (9am and 5pm) feed the animals 100ul of Children's Motrin (20mg/ml) per mouth, using a small syringe without needle. Be gentle with animals that have been recently operated!!

- For the first 10 days after injection (or up to 14 days if there is any evidence of poor suture): Apply antibiotic to the drinking water: Prepare 2.1 ml Baytril (enrofloxacin, 100mg/ml) in 1000ml sterile water and fill water bottles up to ~2/3 capacity. Place the cages facing away from the lixit (licking water inlet) so the animals must drink the water in the bottles. Change the water in the bottles **every 3 days** (the Baytril degrades), and maintain the stock solutions in the dark but not refrigerated. Return animals to normal water line afterwards.