

Video Article

Stereotaxic Surgery for Excitotoxic Lesion of Specific Brain Areas in the Adult Rat

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Abstract

Many behavioral functions in mammals, including rodents and humans, are mediated principally by discrete brain regions. A common method for discerning the function of various brain regions for behavior or other experimental outcomes is to implement a localized ablation of function. In humans, patient populations with localized brain lesions are often studied for deficits, in hopes of revealing the underlying function of the damaged area. In rodents, one can experimentally induce lesions of specific brain regions.

Lesion can be accomplished in several ways. Electrolytic lesions can cause localized damage but will damage a variety of cell types as well as traversing fibers from other brain regions that happen to be near the lesion site. Inducible genetic techniques using cell-type specific promoters may also enable site-specific targeting. These techniques are complex and not always practical depending on the target brain area. Excitotoxic lesion using stereotaxic surgery, by contrast, is one of the most reliable and practical methods of lesioning excitatory neurons without damaging local glial cells or traversing fibers.

Here, we present a protocol for stereotaxic infusion of the excitotoxin, N-methyl-D-aspartate (NMDA), into the basolateral amygdala complex. Using anatomical indications, we apply stereotaxic coordinates to determine the location of our target brain region and lower an injection needle in place just above the target. We then infuse our excitotoxin into the brain, resulting in excitotoxic death of nearby neurons. While our experimental subject of choice is a rat, the same methods can be applied to other mammals, with the appropriate adjustments in equipment and coordinates.

This method can be used on a variety of brain regions, including the basolateral amygdala¹⁻⁶, other amygdala nuclei^{6,7}, hippocampus⁸, entorhinal cortex⁹ and prefrontal cortex¹⁰. It can also be used to infuse biological compounds such as viral vectors^{1,11}. The basic stereotaxic technique could also be adapted for implantation of more permanent osmotic pumps, allowing more prolonged exposure to a compound of interest.

Video Link

The video component of this article can be found at <https://www.jove.com/video/4079/>

Protocol

Anesthesia and analgesia: Thirty minutes prior to anesthesia, inject the rat with 0.05 mg/kg subcutaneous buprenorphine for analgesia. Initiate anesthesia with 30-40 mg/kg intraperitoneal sodium pentobarbital. At this point, also inject atropine to prevent respiratory failure (0.4 mg/kg, subcutaneous) and meloxicam as further analgesic (2 mg/kg subcutaneous). If after 5 minutes, the rat is still mobile or responsive to toe pinch, give subsequent doses of sodium pentobarbital at 5 mg/kg (intraperitoneal) until the rat is unresponsive to pain. Before performing the first incision, inject lidocaine (5 mg/kg, intradermal) at the incision site for local anesthesia. Six to eight hours after initial injection, inject the rat with 0.05 mg/kg subcutaneous buprenorphine for analgesia. Buprenorphine can be injected every 6-8 hours thereafter if needed, though this is usually not necessary.

It is important to note that other forms of anesthesia can interfere with excitotoxic lesions. For example, although ketamine is a commonly used form of anesthesia in rodents, it can interfere with lesions induced with NMDA because it is an NMDA receptor antagonist. It is important to select a method of inducing anesthesia that does not reduce lesion size. If gas anesthesia is desired, most stereotaxic devices including those described here can accommodate gas mask adaptors.

Note: Materials are described further in the **Table of specific reagents and equipment** below.

1. Preparation of the Pump and Stereotax

1. Fill a 10 μ l Hamilton syringe with sterile water and mount it into the caddy of a 6 syringe programmable pump. Secure the end of the syringe plunger into the clamped holder on the pump.
2. Prefill gas-sterilized PE20 tubing with sterile water using a needle and 1 ml syringe. Slide the open end of the tubing on to the Hamilton syringe, being careful to avoid creating any air bubbles in the tube.
3. Clamp the 30ga, flat-cut, 1 inch infusion needle end of the tubing on to the arm of a stereotaxic device using a barrel-style electrode manipulator. Insert the infusion needle between the clamp and the grooved barrel. Secure the clamp over a piece of the tubing containing metal needle within it to avoid flattening the tubing and restricting fluid flow.
4. Adjust the pump rate to $> 5 \mu$ l/min and turn on the pump. A bead of water should appear at the tip of the infusion needle. Check the length of the tubing and its joints to make sure there are no leaks.
5. Wipe away the water bead with a sterile swab and set the pump to withdraw. Withdraw a 2 μ l air bubble. The top of the bubble should be visible in the tubing.
6. Immerse the infusion needle tip in a 20 mg/ml solution of NMDA in sterile 0.1M PBS and withdraw 4-5 μ l. An air bubble should be visible between the sterile water and the NMDA solution in the tip. It is important to note that NMDA is a potent neurotoxin and caution should be used in the preparation and handling of this solution. Gloves and eye protection are advised at all times.

2. Mount the Rat in the Stereotaxic Device

1. **Figure 1** depicts the basic parts of the stereotaxic device. After shaving the rat's head from the line of the eyes to the ears, load the rat's front teeth over the bite bar, with the rat's body resting on a heating pad set on medium. The tongue should hang below the bite bar, with the lower jaw. If available, a temperature feedback heating pad would be preferable. Monitoring rectal temperature would also be ideal.
2. Holding the rat's neck with thumb and forefinger, move the right horizontal ear canal onto the right ear bar. The bar should feel like it is resting on a solid place, not like it sinks far into the head. Holding the right side of the head steady, slide the left ear bar into the rat's left horizontal ear canal. It should feel like it is resting on a solid place. The pinnae should look symmetrical and should appear to lie flat on the ear bars. Upward flaring of pinnae indicates improper placement of the ear bars. The head should not wobble in response to pressure at the neck.
3. Screw down the nose bar. Be gentle. The bones in the nose are delicate and the nose bar does not need to be tight. Check that the rat is still anesthetized before proceeding.

3. Preparing the Rat for Surgery

1. Using a sterile swab, gently place a small amount of veterinary ophthalmic lubricating ointment into each of the rat's eyes to protect them from drying and debris.
2. Concentrically from the planned incision site to the edge of the shaved region, wipe the rat's scalp with 2% chlorhexidine solution followed 70% ethanol three times. Chlorhexidine scrub may also be used if more effective disinfectant is desired. Finish by wiping with 10% povidone iodine solution. The prepared scalp is now sterile and should only be touched with sterile materials.
3. Don sterile gloves for further steps because you will next deal with the prepared surgical site.
4. Place a sterile drape so that the fenestration exposes the surgical site.

4. Creating the Surgical Window

1. Using a #10 scalpel blade, make an incision (approximately 2 cm in length) along the longitudinal midline of the scalp. The incision should start posterior to the line of the eyes. With the thumb and forefinger of the non-dominant hand, hold the scalp in tension perpendicular to the direction of the incision.
2. Pull the fascia overlying the skull surface to the edges of the surgical site using sterile cotton-tips. It will take some force to clear the skull so it can be viewed.
3. Use four curved, sterile hemostats to create a surgical window. Clamp fascia (NOT skin) on each side of the anterior and posterior aspects of the incision and then lay the clamps alongside the animal, thereby pulling the incision open and fully exposing the skull.

5. Leveling the Skull and Creating the Pilot Hole

1. Locate bregma, the anterior point where the three main plates of the skull meet, as depicted in **Figure 2**. Move the infusion needle so the needle tip just touches the skull at bregma. Note the dorsal-ventral coordinate. **Figure 3** demonstrates how to read coordinates on a Stoelting stereotaxic device. Note, at this point, gloves are no longer sterile and should not touch the sterile surgery site directly. Note: After touching the nonsterile stereotaxic device, the surgeon is no longer sterile and should not touch the surgical site nor parts of equipment which will touch the surgical site.
2. Locate lambda, the posterior point where the three main plates of the skull meet, as depicted in **Figure 2**. Raise the infusion needle slightly and move it straight back to lambda. Lower the needle until it just touches the skull at lambda. Read the dorsal-ventral coordinate. If the skull is level, it will be within 0.1 mm of the bregma reading. If it is further off than this, move the needle up (out of the way), loosen the nose bar, either raise or lower the bite bar platform, depending on which end of the head is too high, and then re-measure.
3. Move the infusion needle over bregma and record the anterior-posterior and medial-lateral coordinates. Calculate where the needle must be moved based on the coordinates of the brain area of interest according to a brain atlas¹². For the BLA, subtract 2.8 mm from the anterior-posterior coordinate and either add or subtract 5.1 mm to/from the medial-lateral coordinate depending on whether you wish to target the left or right. Move the infusion needle to the calculated position.

4. Swing the needle out of the way, taking care to keep track of where it was located and then drill a hole approximately 2 mm in diameter in that location using a Dremel moto-tool with a sterile dental drill bit attached. Drill carefully as when the drill breaks through the skull, it will tend to pull down, possibly damaging the dura and brain tissue underneath. Ream the hole to enlarge it.
5. Absorb any blood with a sterile cotton swab.

6. Infusion of Neurotoxin

1. Swing the needle back into position. Lower the needle until it touches the surface of the brain. Record the dorsal-ventral coordinate. This is the level of dura. Subtract the appropriate value to reach the brain region of interest. For the basolateral amygdala, subtract 6.7 mm.
2. Lower the infusion needle to the calculated level slowly. Then raise it back up, well above the skull. Use a Hamilton syringe cleaning wire to unclog the tip of the infusion needle, being careful not to touch the end of the wire that comes in contact with the infusion needle with non-sterile gloves.
3. Run the pump at $>5 \mu\text{l/min}$ and make sure you see a bead of liquid come out of the infusion needle. If not, unclog with the wire and try again.
4. Lower the infusion needle to the desired dorsal/ventral level slowly.
5. Set the rate on the infusion pump to $0.1 \mu\text{l/min}$ and start the infusion. Infuse for 2 min.
6. Once the infusion has started, mark the two ends of the air bubble in the tubing with a black marker. If both ends do not move, your needle is clogged. Stop the infusion, raise the needle, unclog with the wire, check for flow with a high flow rate, re-lower the needle and try again.
7. Once the infusion is complete, stop the pump and wait 5 min for the NMDA to diffuse away from the needle tip.
8. Raise the needle 0.5 mm, and infuse again for 1 min. Wait 2.5 min for the NMDA to diffuse away from the needle-tip. This extra infusion helps ensure all of the BLA is covered by the infusion. Whether this is necessary for other brain regions must be determined experimentally.
9. Raise the needle out of the brain slowly and swing the stereotaxic arm out of the way.

7. Closing the Incision

1. Don new sterile gloves to close the incision.
2. Wipe away any excess blood on the skull with sterile cotton-tips and remove the clamps from the fascia.
3. Using London forceps, push the two sides of the incision together. The inner edges of the skin should meet and the outer edge of the skin should not be permitted to curl into the incision. While pressing the incision closed, apply wound clips to close the protrusion firmly. Continue to push the skin together and staple along the length of the incision. This usually takes 3-5 wound clips.
4. After placing all the wound clips, use the forceps to pinch the staples again, making sure they are secure. The more secure the clips, the less likely a rat will pull them out before the incision has healed.
5. Swab the stapled incision with 10% povidone iodine solution and return the rat to its home cage and monitor until mobility is restored (usually 1-2 hours after anesthesia, total surgery time from induction to closure being 30-45 min). At this point, the rat may be returned to the colony room. After one week, remove staples with a staple remover tool under isoflurane anesthesia.
6. Use a hot bead sterilizer to sterilize the tip surfaces of all instruments before use on the next rat. Instruments should be immersed in the beads for no longer than 15 seconds, or they may become too hot to handle. Instruments should be fully cooled before used on another animal.

8. Recovery

1. It is possible that rats will exhibit seizure activity in the days following the lesion. Depending on the brain region damaged, other behaviors such as food consumption or grooming may be affected. It is important that animals be monitored on a daily basis to ensure that they remain healthy during recovery. Supportive care, such as liquid food, high-calorie food, or additional analgesia may be required. Mortality due to anesthesia occurs in a low percentage of rats, usually less than 5%. Mortality due to other causes (infection, bleeding) is extremely rare. Any signs of distress after recovery of anesthesia may be cause for exclusion from future studies, but are extremely rare. **Figure 4** depicts the weight loss during the first few days following surgery as it should be expected. Body weight should rebound within 1 week.

9. Representative Results

One week or more after NMDA infusion, the lesion can be visualized using a cresyl-violet counterstain and brightfield microscopy. Brains should be perfused with ice cold 4% paraformaldehyde, equilibrated in 30% sucrose in 0.1M PBS and frozen. They should then be sliced at 30-60 μm , in a series of 1:6 to 1:12 and mounted on to gelatin-coated glass slides before undergoing a standard cresyl violet counterstain. The lesion should correspond to a "bald spot" or an area of decreased cresyl violet stain due to cell death following lesion. **Figures 5A and B** show a representative lesion of the BLA and CeA. This image was obtained using a flatbed scanner with a high resolution scan (1200 dpi). To visualize the lesion, it helps to counterstain a series of sections through much of the tissue surrounding the target area so a misplaced lesion can be located.

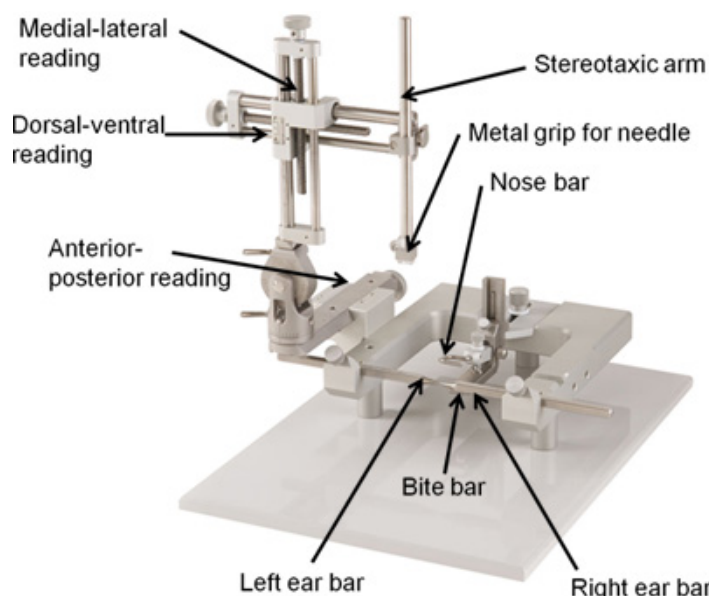


Figure 1. Stereotaxic setup. This figure shows the rat stereotaxic with relevant parts labeled.

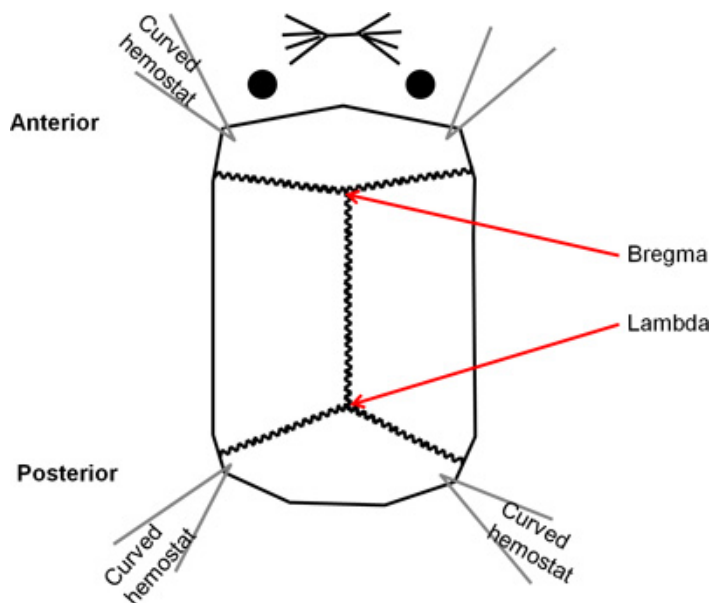
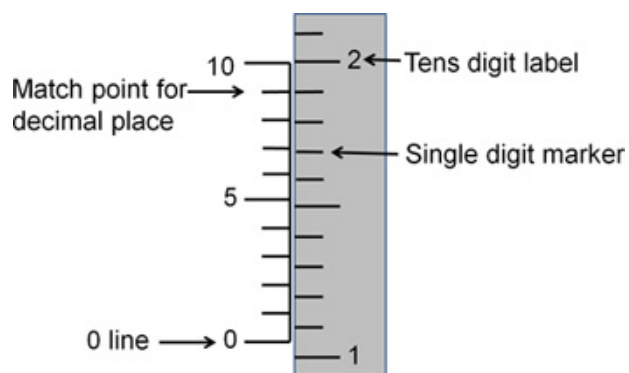


Figure 2. Bregma and lambda on the rat skull. This illustration depicts the locations of bregma and lambda. All coordinates are measured relative to bregma. Lambda is used to ensure that the skull is level.



This reading is 10.9 mm

Figure 3. Reading stereotaxic coordinates. This drawing depicts a standard stereotaxic reading. To obtain the digits to the left of the decimal place, use the markers on the right side. The labeled digit corresponds to the tens place (i.e. 1 = 10, 2 = 20). The hash marks between the

labeled digits correspond to the single units (1-9). The zero line indicates at what integer the stereotax is set. Here, the zero line is between the 10 and 11 markers, indicating that the reading is between 10 and 11. To determine the numbers to the right of the decimal place, use the markers on the left side. The hash marks on the left are numbered 0 to 10, with each unlabeled hash representing one unit difference from those next to it. Whichever of the hash marks on the left lines up best with the hash marks on the right indicates the first decimal place of the coordinate reading. Here, the hash mark on the left corresponding to number 9 lines up best with the right-side hash marks so the decimal place is 9. The final reading is 10.9 mm.

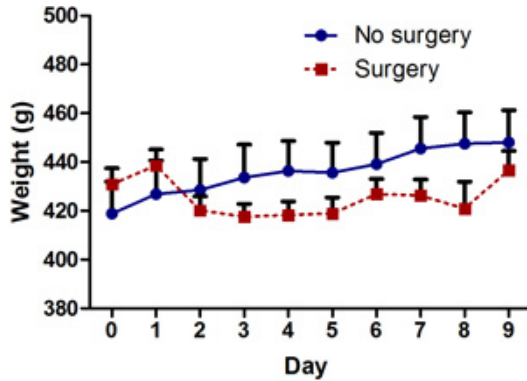


Figure 4. Weight change after surgery. This graph shows the average ($\pm$ SEM) weight of rats who underwent stereotaxic surgery ($n = 17$) or did not have surgery ($n = 6$). Day 0 is the day of surgery. Following surgery, the weight of rats decreased for 2-3 days after surgery and then increased around day 5-6.

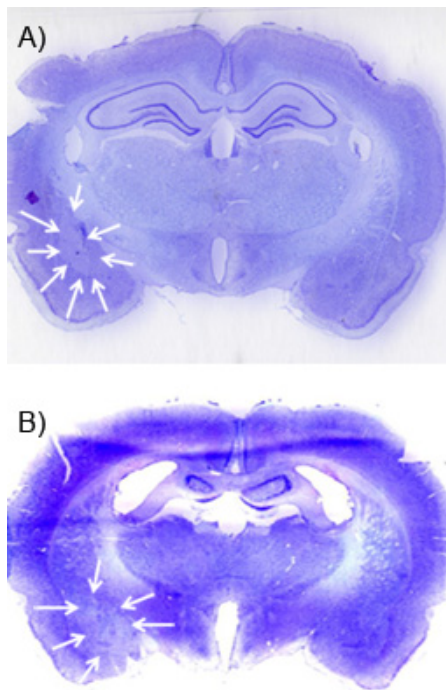


Figure 5. Excitotoxic lesion example. A) This image shows a cresyl violet counterstain on a 30 μ m coronal slice with the "bald spot" from NMDA lesion of the basolateral amygdala. The white arrows outline the lesion. It can be compared to the contralateral side of the brain slice which has no lesion. B) A similar lesion of the central nucleus amygdala.

Discussion

The stereotaxic method presented here allows for excitotoxic lesion of specific brain areas via infusion of NMDA. The basic stereotaxic methods can be adapted to infuse a variety of pharmacological and biological agents in a site-specific manner. It can also be adapted to target a variety of brain areas, defined by their stereotaxic coordinates in a brain atlas¹². Adaptation to other species such as mice can be made with similar equipment built for smaller animals. The present procedures are optimized for 3 mo old adult rats, but they could be used for a variety of ages from juveniles through older adults. If working with younger animals that may not be represented in a standard brain atlas, exploratory surgeries infusing a colorful dye can help determine the proper needle placement empirically.

For investigators who require long-term implantation of a cannula using stereotaxic surgery, Geiger *et al.*¹³ present an alternative protocol with appropriate incision closure and aftercare instructions. If targeting smaller brain regions is desired, however, it should be noted that the

present protocol offers more precise methods for head placement and leveling in the stereotaxic device. The present protocol also offers incision clamping methods that allow for a wider surgical window, often necessary when targeting lateral structures such as the BLA.

Following surgery, weight loss compared to control animals is normal and expected. **Figure 4** shows the change in weight of adult male rats that underwent surgery versus unmanipulated controls from the same cohort. Rats can be expected to show weight loss for the first 2-3 days after surgery and then begin to gain weight again after 5-6 days. More prolonged weight loss could indicate infection or other problems.

Occasionally, a rat will remove its wound clips. If the incision has healed, this does not present a problem. If the incision has not healed and is open, the wound clip must be replaced either with another clip or with sutures, both of which can be performed under isoflurane anesthesia. Including antibiotics (80-96 mg trimethoprim sulfa/kg/day, 240 mg/5 ml water) in the drinking water for the next 5 days may also be advisable to prevent infection if the wound was open to the environment.

Perhaps the most challenging aspect of this protocol is placement of the ear bars. Experimental surgeries with dye infusion followed by immediate sacrifice are recommended to check the accuracy of both ear bar placement and the selected stereotaxic coordinates. If the brain region to be targeted is in the midline, we recommend using an angled approach from either side of the brain as large blood vessels in the midline can prevent a direct midline approach.

Even when performed as well as possible, stereotaxic surgery still entails heavy exposure to analgesics and anesthetics, tissue damage and immune response. All these factors may affect experimental outcomes, particularly close to the time of surgery. Side-effects of surgery can be minimized by allowing for ample recovery time (at least one week) before beginning experimental procedures. Alternative approaches such as targeted genetic knockouts may be possible in mice but can be much more complex than stereotaxic surgery.

In summary, the described method allows for localized lesion of brain areas without damaging passing fibers. It can be adapted for numerous purposes and is highly reliable and verifiable.

Disclosures

No conflicts of interest declared.

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