A Complete Guide to Using the Endothelin-1 Model of Stroke in Conscious Rats for Acute and Long-Term Recovery Studies

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Abstract

Multiple methods exist to model permanent and transient ischemia under anesthesia in animals, however most human strokes occur while conscious. The use of endothelin-1 as a vasoconstrictor applied to the perivascular surface of the middle cerebral artery is one of the only methods for inducing stroke in conscious animals. Here, we describe standard operating procedures for stereotaxic placement of an ET-1 guide probe above the middle cerebral artery, induction of stroke in conscious rats, predictive outcome scoring during stroke, and neurological behavioral tests that we use to monitor transient and continuing deficits. The inclusion of long term neurological assessment is of particular importance when taking into consideration the effects of stroke on brain remodeling.

Key words Standard operating procedure, Stereotaxic surgery, Conscious stroke, Predictive outcome, Neurological assessments

1 Introduction

Given the setbacks associated with neuroprotective drugs in clinical practice, it is clearly important to use animal models of stroke that mimic more closely the human condition. Endothelin-1 (ET-1) is a potent and long-acting venous and arterial constrictor. Originally isolated [1] and generated by endothelial cells, ET-1 exerts its effects through two main receptors, endothelin-A receptor (ETA-R) and endothelin-B receptor (ETB-R) [2, 3]. Targeting the receptor located on the perivascular surface of the middle cerebral artery lead to the development of the first conscious model of stroke in animals [3]; stereotaxic injection of ET-1 adjacent to the middle cerebral artery (MCA) in conscious rats resulted in constriction of the MCA followed by gradual reperfusion. This was the first viable model of conscious focal cerebral ischemia that resulted in cerebral infarctions equivalent to those observed using anesthetized models of MCAo (3 & 13). Since then
application of ET-1 to the MCA to induce focal ischemic strokes has also been adapted for use in primates [4], representing a most relevant platform for clinical translation to investigate ischemic stroke.

ET-1 induced reduction in CBF with gradual reperfusion resembles the majority of human strokes where varying degrees of spontaneous reperfusion occurs in the absence of thrombolysis [5]. The model enables prediction of outcomes based on observed behavioral changes as they occur during stroke induction. Similar prognostic clinical approaches, such as the use of the Scandinavian Stroke Scale, allow prediction of functional outcome and survival of stroke sufferers in order to correctly stratify treatment groups in clinical trials [6]. This same approach has now been developed for use in rats [7] where concurrent observations during ET-1 stroke induction can be used to accurately predict the extent of histological damage and neurological deficits incurred [8]. Such a predictive outcome model enables stratification of rats into treatment groups ensuring that stroke severity is evenly represented across all treatments to be assessed [7, 8]. This holds particular relevance to the clinical setting since humans are not preassigned to treatment groups prior to the onset of stroke, and not all human stroke is the same [6]. Historically, preclinical animal studies randomized rats into treatment groups prior to stroke induction which may have led to groups being unevenly weighted according to stroke outcome.

A high degree of variability in stroke damage is observed between individuals and this too can be accounted for when using the ET-1 model since all degrees of stroke severity can be assessed across treatments. This is particularly important when undertaking a complete characterization of treatments on histological, molecular, and functional outcomes, with differential effects reported across varying stroke severities [7, 8]. Additionally, it is equally important to include appropriate long-term behavioral assessments to detect neurological deficits across stroke outcomes to fully assess a potential treatment on long-term survival.

If used correctly and consistently, a model of stroke induced in the absence of anesthesia, which incorporates gradual spontaneous reperfusion, and can be used reliably in long term studies, is most desirable for the translation of new treatments to prevent the spread of injury and promote recovery in patients. Herein we describe in detail the Subheadings 2 and 3 and forms of analysis used to conduct ET-1 induced stroke in conscious rats.

2 Materials

2.1 Surgery and Stroke

This procedure uses Male Hooded Wistar rats, aged 10–12 weeks (280–360 g). Rats are housed on a 12-h day/night cycle with temperature maintained between 18 °C and 22 °C and maintained on a standard chow diet. Rats are dually housed prior to surgery,
but are housed separately after surgery to ensure no disruption to
the indwelling guide cannula. Be sure not to use sawdust or shred-
dded paper in the cages as dust from these can easily settle in the
ET-1 guide cannula and cause it to become blocked prior to stroke.

1. Rats.
2. Paracetamol (2 mg/kg in drinking water 24 h presurgery,
postsurgery).
3. Anesthetic—Ketamine, Xylazine, Isoflurane.
4. Lignocaine (1% solution).
5. Stereotaxic frame.
6. Sterile surgical instruments including scalpel blade, curved
suture needles and thread, microdissecting scissors, a fine spat-
ula, small curved forceps.
7. 23 gauge stainless steel tubing for guide cannula
(HTX-23-24).
8. 30 gauge stainless steel tubing for microinjector (HTX-30-24).
9. Small stainless steel screws (OPSM glasses frame screws).
10. Glasses frame screw driver set.
11. Hamilton glass syringe (5 μl).
12. 1 ml sterile syringe.
13. 100 μl pipette and pipette tips.
15. Bench coat to line base of plexiglass box.
16. Premade microinjectors (see below).
17. Rectal thermometer.
18. Vaseline.
19. Endothelin-1 (Sigma; 40 pmol stock; dissolved in sterile
saline).
20. 301/2 gauge needle.
21. Endothelin-1 infusion line: OD 0.61 × ID 0.28 mm–20 cm.
22. Cuff: OD 0.96 × ID 0.58 mm–5 mm.
23. Digital timer.
25. Heating pad.

2.2 Neurological Screening

1. Running beam (3 cm wide × 70 cm long, approx. 5 mm thick).
2. Digital Timer.
3. Small 1 cm round sticky labels in colors other than white or
black.
4. Clear plexiglass box (approx. Height 30 cm, width 20 cm, length 40 cm).
5. Plexiglass cylinder (height 30 cm, diameter 20 cm, thickness 7 mm) open top.
6. Mirror (50 cm × 50 cm).
7. Video camera.
8. Rat accelerating Rota-Rod.
10. Bio-Serve Sugar pellets (45 mg each; Able Scientific, Australia).
11. Forceps for pellet positioning during training sessions.

3 Methods

3.1 Construction of Microinjector and Probe

3.1.1 Microinjector (See Fig. 1a, b)

1. To make the microinjector start with HTX-30-24 stainless steel metal tubing and cut to approximately 5 cm in length. This is best achieved by first creating a bevel along the tubing at the desired length with a small file (see Note 10) and then snapping the tubing at the bevel point to create a clean open finish. Only apply light pressure when using the file so as not to dent the lumen of the tubing. If dented ET-1 will not infuse smoothly through the injector.

2. Thread the above piece of tubing through a piece of HTX-23-24 tubing that has been cut ~2 cm in length in a similar fashion. Ensure that one end of HTX-30-24 metal tubing extends 2 cm from the end of HTX-23-24. Again use light pressure when filing as a dented tube will prevent the injector from being inserted.

3. Bend the HTX-23-24 in the middle to a 45° angle thus creating a reinforced elbow for the microinjector. Dents within the lumen of the injector can be checked by infusing distilled water smoothly through. Ensure remaining distilled water within the injector is removed before continuing.

![Fig. 1 Schematic diagram of the micro injector (a), which is threaded through a piece of 2 cm HTX-23-24 tubing to create a reinforced elbow (b). The final injector is later inserted into a second piece of 2 cm HTX-23-24 tubing such that it extends 2 mm from the end (c). This second piece of 2 cm HTX-23-24 tubing makes up the ET-1 guide cannula that is stereotaxically inserted adjacent the middle cerebral artery](image-url)
3.1.2 Probe (See Fig. 1c)

1. Using a second piece of HTX-23-24, file to length so that the microinjector made above will extend 2 mm from the end of the probe once it is inserted through the probe. Hence if the microinjector measures 2 cm from the reinforced elbow then the guide probe will need to be 18 mm exactly. This 18 mm probe will be later stereotaxically positioned to sit ~3 mm dorsal to the middle cerebral artery. This distance from the artery ensures that the artery is not punctured during surgery. When the microinjector is positioned during stroke it will sit ~1 mm dorsal to the artery for endothelin-1 application. Again when filing use light pressure so as not to dent the lumen of the tubing, thus creating clean entry and exist either end of the guide cannula.

2. Place each individual microinjector into a separate clearly labeled container (glass vial) and label for each rat so that each injector made can be matched to the corresponding implanted guide probe. It is very important that each microinjector made is an exact match to the probe implanted in the rat to ensure accurate individual localization of the middle cerebral artery during stroke induction.

3.2 Stereotaxic Surgery

The precision in placing the probe adjacent to the MCA is critical to achieving best outcomes. Attention must firstly be paid to sourcing a high quality stereotaxic frame and then ensuring no parts on the frames arm or probe holder become wobbly when set to position. Human error when reading the frame coordinates is often cause for imprecision. While some misalignment to the MCA can still result in stroke (one reason for obtaining smaller strokes), attention to detail when reading and setting coordinates is key to successful, repetitive outcomes.

1. Prior to surgery all rats should undergo physiological and neurological assessments to ensure no damage is induced as a result of stereotaxic placement of the ET-1 guide probe. This should include recording the rat’s weight presurgery and assessing neurological performance according to behavioral tests described below (see Subheading 3.4).

2. To prevent postoperative pain, pretreat rats with Paracetamol (2 mg/kg in drinking water) 24 h prior to surgical procedure and for a further 24 h postsurgery.

3. Turn on the heating mat to support rat thermoregulation during surgery.

4. Attach the previously made guide probe (see above) to the probe holder on the stereotaxic frame making certain that the guide probe is positioned perfectly vertical once clamped firmly into place.
5. Anesthetize the rat by intraperitoneal injection of Ketamine (100 mg/kg)/Xylazine (10 mg/kg) solution 0.2 ml/100 g. Anesthetization is then maintained throughout surgery by inhalation Isoflurane (95% oxygen and 5% Isoflurane) via a stereotaxic nose cone attachment that features an inlet and outlet port for the flow of gaseous mixture. The oxygen gauge is set to 1.5 l and the Isoflurane gauge is set to 1 l.

6. Mount the rat into the stereotaxic frame making sure that the rat head is perfectly secure and sitting straight in the mounted position. It is important that the rat head does not wobble or move under pressure. Blunt ear probes should be positioned just above the ear canal.

7. Position the nose cone attachment on the stereotaxic frame so that it is fully covering the rat nose and apply isoflurane using an inhalation anesthetic machine as described above.

8. Once the head is secured make a mid-line incision down the center of the scalp starting just behind the eyes and moving back ~2–3 cm.

9. Place two suture threads on either side of the skin flaps and secure to each side of the frame with surgical tape, thus exposing the scalp for surgical access.

10. Gently remove the fine connective tissue layer under the scalp until the skull bone is exposed. Douse with Lignocaine to anaesthetize the surface (this will support recovery).

11. Make an incision ~ 1 cm in length along the right surface of the skull between the bone and the temporalis muscle so as to gently detach the muscle from the skull without damage to the muscle. DO NOT cut into the muscle. Use a pair of small curved forceps to pull and hold the muscle away from the skull, fixing in place with surgical tape. Clear away any residual tissue left behind on the skull bone and pat dry with a cotton bud.

12. Locate Bregma and mark with a fine tip black marker pen (see Fig. 2a)

13. Drill one small dent on top of the skull for placement of one small screw on the ipsilateral side close to where the ET-1 probe is destined to be positioned (see Fig. 2a). Gently wind in the screw such that it fixes into the skull but does not penetrate below the epidural space (about half way). This can be helped by using a set of flat forceps that hold the screw in position between the skull and screw head, thus preventing the screw from physically being pushed in all the way (see Note 1).

14. Once the screw is in place align the base of the guide probe so that it is positioned directly over and sitting on the point of bregma. Record the coordinates on the frame for Anterior/Posterior, Medial/Lateral, and Dorsal/Ventral. Raise the
probe and adjust the frame according to the following coordinates: +0.2 AP; −5.9 ML; −5.2 DV.

15. Gently lower the guide probe until it sits on top of the point of entry into the skull. Mark this point with a black marker pen.

16. Drill a burr hole over this marked point (down the side of the skull, see Fig. 2a) to allow the guide probe to enter the brain and be positioned above the middle cerebral artery (see Note 2).

17. Once the probe is in position use a small amount of bone wax to seal the point of entry and secure with dental cement being sure to cover the previously attached screw to anchor the cement and probe.

18. Once the cement is dry remove the probe from the probe holder and suture wound.

19. Douse the wound with lignocaine and monitor the rat every 30 min until fully awake (approximately 2–4 h). Treat with Paracetamol in the drinking water for 24 h to prevent postoperative pain. Allow rats to recover for 4–5 days before stroke induction.

3.3 Stroke Induction

Stroke induction needs to be performed in a calm and safe environment free from disruption. The experimenter should be familiarized with each rat prior to commencing stroke. This is often best achieved while conducting prestroke physiological and behavioral assessments described below.

1. Take 60 μl of stock ET-1 (40 pmol) and add 60 μl saline to make a final 20 pmol ET-1 solution.
2. Fill a 1 ml sterile syringe with ET-1 using a 30-gauge needle attached to the syringe.

3. Attach the appropriate microinjector to one end of a 20 cm ET-1 infusion line (OD 0.61 x ID 0.28 mm) and fill the line with the endothelin-1 (20 pmol) using the 30-gauge needle attached to the other end of the line, making certain that endothelin-1 freely perfuses without air bubbles, through the line and out the tip of the microinjector as the line is filled.

4. Place a small infusion line “cuff” (OD 0.96 x ID 0.58 mm ~ 5 mm) on the elbow of the microinjector in order for it to be fastened to the implanted guide probe in the conscious rat (see Fig. 3a). This will enable the rat to freely move about the cage without the microinjector becoming dislodged during the study.

5. Fill a Hamilton glass syringe (5 μl) with distilled water and attach this to the infusion line, leaving a small air pocket between the endothelin-1 and the attached syringe so that ET-1 is separated from the dH2O as it is pushed through. This also allows the experimenter to monitor the progression of endothelin-1 through the line by keeping track of the moving air pocket.

6. Prior to stroke use a small animal digital thermometer embalmed with Vaseline to take rectal temperature, weigh the rat, and record each for poststroke monitoring.

7. Induction of stroke itself does not cause pain. Lightly restrain the conscious rat using a soft cloth. First ensure that the external opening of the guide cannula is clear. This can be done by

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**Fig. 3** Schematic diagram of the microinjector positioned into the implanted guide cannula and secured by an infusion line ‘cuff’ (Orange) (a). The external end of the microinjector is attached to the ET-1 infusion line, which is then attached to a 5 μl Hamilton syringe containing saline only. Photo of ET-1 injector correctly positioned in the conscious rat for induction of stroke (b)
inserting a 30 gauge needle into the tip of the cannula first (see Note 12). Then once cleared the microinjector can then be inserted into the previously implanted guide cannula, securing in place with the cuff. Be careful not to rush this process as once the microinjector is in place you do not want to bend it and compromise its final positioning within the guide probe (see Note 11).

8. Once the microinjector is positioned, place the rat in a clear plexiglass box (30 × 20 × 25 cm) for observation during stroke induction.

9. Stroke is induced by slow microinjection of ET-1 (60 pmol in 3 μl over a period of 10 min). Start timer.

10. Record all observed changes in rat behavior according to the time they occur during ET-1 infusion (see Table 1). It is important that during stroke rats are continually monitored and changes in normal rat behavior are observed and recorded. These behavioral changes include contralateral forepaw clenching and continuous circling (see Fig. 4), and can be graded and used to predict stroke outcome (see ref. 8). If the rats appear to be having a large stroke prior to the full 3 μl infusion, STOP INFUSION, to avoid having to euthanize the rat due to extreme stroke.

11. Monitor changes in rectal temperature every 30 min for the first 3 h after stroke. Increases in temperature (~1°C) can occur during stroke. Temperature increases above 3°C may result in seizure (see Note 13).

3.3.1 Monitoring Requirements

Body weight should be assessed daily throughout the duration of the study and animals continuously monitored for changes in activity. In addition to monitoring temperature and weight following stroke induction (described above) rats can also be monitored daily/weekly for neurological deficits using neurological tests specific for this species.

3.4 Assessment of Functional Outcome

When addressing the development of brain injury following stroke or traumatic brain injury it is important to measure any functional deficits that occur as a result of injury. Groups of rats can be routinely scored on a number of commonly used behavioral assessments and each rat acts as its own control preinjury. It is important to use a variety of different tests in order to pick up what are often quite subtle changes in function in rats following injury to the brain. In addition, it has been reported that rats often appear to have no deficits in one test but will still show deficits in others, depending on where the injury occurred. The behavioral tests described below will help to determine conscious limb function, fine motor control and sensorimotor function. These tests are routinely used with the ET-1 model of stroke and have been
Table 1
Changes in behavior upon ET-1 injection in the conscious rat gives an indication of stroke intensity that correlates to the histological outcomes (see ref. 8)

<table>
<thead>
<tr>
<th>Observed behavior</th>
<th>Stroke rating</th>
<th>Stroke Infarct (unstained brain sections)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grooming, teeth chattering</td>
<td>1</td>
<td><img src="image1" alt="Image" /></td>
</tr>
<tr>
<td>Tongue poking, licking, contralateral whisker twitch, raised contralateral forepaw</td>
<td></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>Raised and clenched contralateral forepaw</td>
<td></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>Grooming, biting cage and bedding</td>
<td>2</td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>Spasmodic contralateral turns (not continuous)</td>
<td></td>
<td><img src="image5" alt="Image" /></td>
</tr>
<tr>
<td>Head turned to contralateral direction</td>
<td></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>Head bobbing in contralateral direction</td>
<td></td>
<td><img src="image7" alt="Image" /></td>
</tr>
<tr>
<td>Continuous consecutive contralateral turns</td>
<td>3</td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td>Chin rubbing on base of cage</td>
<td></td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>Contralateral forepaw clench</td>
<td>4</td>
<td><img src="image10" alt="Image" /></td>
</tr>
<tr>
<td>Ipsilateral circling</td>
<td></td>
<td><img src="image11" alt="Image" /></td>
</tr>
<tr>
<td>Forepaw shuffling/digging</td>
<td></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
<td>Tight ipsilateral circling</td>
<td></td>
<td><img src="image13" alt="Image" /></td>
</tr>
<tr>
<td>Loss of balance on rearing/walking</td>
<td>5</td>
<td><img src="image14" alt="Image" /></td>
</tr>
<tr>
<td>Loss of righting reflex</td>
<td></td>
<td><img src="image15" alt="Image" /></td>
</tr>
<tr>
<td>Still circling after 60 min</td>
<td></td>
<td><img src="image16" alt="Image" /></td>
</tr>
</tbody>
</table>
shown to detect immediate deficits, spontaneous recovery of deficits, and persistent long-term deficits (see ref. 10). Current STAIR and RIGOUR guidelines for preclinical assessment of therapeutics to treat stroke state that all neurological assessments should be performed blinded to treatment (see ref. 11). Therefore, after stroke each rat should be assigned a coded number once allocated to treatment so that any further assessment is conducted blind to treatment. This generally requires two experimenters to work together, one to manage treatments and the other to perform assessments blind to treatment.

3.4.1 Neurological Deficit Score

Neurological abnormalities are evaluated with the use of a neurological deficit score based on detection of abnormal posture and hemiplegia, as described by Yamamoto and colleagues [15] (see Fig. 5).

1. Suspend the rat by the tail 10 cm above the bench top or home cage floor for ~5 s and observe any twisting of the thorax defined by the rat reaching up toward its tail. A nonstroke rat will extend both forepaws toward the ground as if reaching for the base of the cage (score 0) (see Fig. 5a). After stroke the contralateral forepaw may not reach to the ground but flex to the contralateral side. Slight flexion (score 1), 45° flexion (score 2), and pronoune 90° flexion (score 3). Often severe flexion is accompanied by obvious twisting of the thorax.

2. In addition to forelimb flexion some thorax twisting is also likely to observed in stroke affected with a wobble to contralateral side (score 1), some contralateral twisting up toward the tail (score 2), or twisting all the way up to touch the tail (score 3) (see Fig. 5b).

3. Set up the running beam so that it is raised approximately 20 cm above the benchtop. Limb dysfunction is detected by
placing the rat across the narrow beam (3 cm wide × 70 cm long) and ability to grip onto the beam is observed. Forelimb and hind limb paralysis is scored by ability to grip and keep all limbs on the beam (see Fig. 4c). Loss of grip and occasional slipping while walking along the beam (score 1), no grip and limb resting over edge of beam (score 2) (see Fig. 5d), no grip and limb dangling from beam with an inability to move along the beam (score 3).

4. Add all scores from the above tests together for a total neurological deficit score with a maximum score of 12. Compare neurological deficit scores poststroke to prestroke scores such that each rat acts as its own control (see Note 3).
5. Results should be presented as box plots for nominal scores and analyzed using a Kruskal–Wallis nonparametric ANOVA followed by Dunn’s posttest for multiple comparisons.

3.4.2 Sensorimotor Hemineglect (Sticky Label Test)

Sensory hemineglect is evaluated by a test developed by Schallert and Whishaw (1984) (see ref. 12) that measures sensitivity to simultaneous forelimb stimulation. This test is based on observations of behavior in humans with unilateral brain damage. If two stimuli are presented simultaneously, one on each side of the body, the contralateral stimulus appears to be masked (“extinguished”) and either remains undetected until the ipsilateral stimulus is removed or feels subjectively weaker. In rats, the test consists of placing adhesive tapes (Avery adhesive labels, 1-cm circles) on the distal-radial region of each wrist (see Fig. 6).

1. Place the rat in a clear plexiglass box and allow that rat to explore the new environment for 2–3 min.

2. Gently restrain the rat and place a small (1 cm diameter) adhesive label (any color except white or black) on the inside surface of each forelimb just above the thumb, on the wrist (see Note 4). Placement of the first tape should be randomized between contralateral and ipsilateral limbs.

3. Place the rat back into the clear plexiglass box and time how long it takes for the rat to first touch each label and to remove each label. Each trial should only last a maximum of 3 min and should be conducted twice in training and then once only on subsequent days.

4. The maximum score if tape is not removed is 180 s. Compare the time to touch the tape from the contralateral forepaw to that of the ipsilateral forepaw, and plot against prestroke scores. Compare the time to remove the tape from the contralateral forepaw.

Fig. 6 Photograph of a rat with adhesive labels positioned on the wrist of the forepaws for assessment of hemineglect
forepaw to that of the ipsilateral forepaw, and plot against prestroke scores.

5. Analyze using two-way repeated measures ANOVA with 2-factor repetition (side × hour after stroke) to compare latencies in the ipsilateral and contralateral forepaws over time. A one sample t-test is used to determine significance of asymmetry from chance or <0.05.

3.4.3 Rota-Rod Performance

Motor performance is commonly assessed in rodents after brain injury using an accelerating spinning wheel (Rota-Rod). While this test is a reliable measure of short term impairment, spontaneous recovery is often reported beyond 3 days and this involves learned compensatory use of the tail to maintain balance while on the wheel. As such it is not considered a reliable measure of long term motor deficits.

1. Rats are pretrained to remain on the Rota-Rod for 3 min (see Note 14). Each rat will be given two training sessions of three trials each, 1 h apart on an accelerating Rota-Rod (spinning wheel).

2. Rats are scored by timing how long they are able to remain on the Rota-Rod compared with prestroke scores.

3. When a rat falls off the Rota-Rod, it lands 20 cm below on a plastic plate which trips and stops the automatic timer. Bubble-wrap is used as a cushioning device under the Rota-Rod so that the rat does not harm itself when falling off.

4. Compare the time to fall off poststroke to that of prestroke scores such that each rat acts as its own control.

5. Analyze using a one-way ANOVA across time within groups and a two-way repeated measures ANOVA between treatment effects followed by Bonferroni post hoc test for multiple comparisons.

3.4.4 Cylinder Test

The cylinder test assesses exploratory weight bearing motor movements of rats against the wall of a cylindrical enclosure (see ref. 14) (see Fig. 7). Rats will voluntarily rear and explore the wall using their forelimbs. Prelesion rats typically distribute the weight bearing movements equally on their ipsilateral and contralateral forelimbs during vertical rearing and upon landing use both forelimbs simultaneously. After stroke, rats favor their nonimpaired limb (ipsilateral forepaw) to support them while rearing and then land on the dangling impaired limb first (see Note 5).

1. Stand cylinder on smooth clean surface.

2. Lean mirror against a wall directly behind the cylinder.
3. Angle the video camera in front of the cylinder so that movements by the rat on all sides of the cylinder can be seen (recommended position: 100 cm in front; 75 cm to side).

4. Start video camera before placing rat in cylinder as many movements can occur during the initial period of exploration.

5. Place the rat in the cylinder. The rat will rear and use forelimbs to explore the walls of the cylinder. Rats will typically explore walls with at least three forepaw touches before landing, therefore 10 landings should be counted in order to be confident of obtaining at least 30 vertical wall touches.

6. Repeat test 24 h after stroke, 72 h and then at 7 days with 7 day intervals thereafter (see Note 6).

7. Analyze the videotaped movements in slow motion.

8. Count only vertical wall touches where the forepaw is flat on the wall with the digits spread apart.

9. Score the first 30 vertical wall touches as either a left forelimb touch, or a right forelimb touch. Right and left forelimb movements are counted independently; if one forelimb remains stationary on the wall while the other moves, the first scores only one until it moves again.

10. Do not score ambiguous movements.

11. Compare poststroke and posttreatment scores to a prestroke baseline such that each rat acts as its own control (see Note 7).

12. Analyze using a two-way repeated-measures ANOVA followed by Bonferroni post hoc test to compare differences between treatment groups over time.
3.4.5 Staircase Test

A novel reaching test for the rat has been developed to assess the independent use of forelimbs in skilled reaching and grasping tasks that allow evaluation of sensory abilities, dexterity, and motor coordination. The apparatus is a plexiglass box with a removable baited double staircase. Food pellets are placed on the staircase and presented bilaterally at seven graded stages of reaching difficulty to provide objective measures of side bias, maximum forelimb extension and grasping skill. Animals should be well handled prior to training. The time required to train the animals for the task will take 2 weeks, with training conducted twice daily at the same time each day, taking 10–15 min each session.

The apparatus into which the animal is placed consists of a clear Perspex chamber (203 mm long × 108 mm high × 60 mm wide) with a hinged lid. A narrower compartment (165 mm long × 108 mm high × 60 mm wide) with a central raised platform running along its length, creating a 19 mm wide trough on either side, connected to the chamber. The narrowness of the side compartment prevents the animal from turning around, so that it can only use its left paw for reaching into the left trough and right paw reaching into the right trough. A removable double staircase is inserted into the end of the box, sliding into the troughs. Each of the seven steps of the staircase contains a small 3 mm deep well into which three food pellets are placed. Therefore 21 pellets are placed into the staircase. The highest step of the staircase is 13 mm below the central platform. A trained animal can collect pellets by reaching into the trough: the number of steps from which pellets have been removed provides an index of how far the rat can reach, and the number of pellets remaining at the end of the test indicates the rat’s success in grasping and retrieving pellets.

Training

1. On the first day, animals are familiarized to the experimental apparatus by placing them into the test box for 15–20 min.

2. On the second day trial the rats twice. During the training period, the experimenter helps the rat into the narrower compartment. For this purpose some pellets are first distributed along the platform to attract the rats into the narrower compartment. Once in the compartment more pellets are then presented to each well with forceps in order to help the rat localize them.

3. Repeat the trials daily until the rats have learned to reach food (usually by the 4–5th training session) (see Notes 8 and 9).

Trail period: By the fifth day commence recording pellet retrieval.

1. Loading each step with three pellets for a total of 21 pellets on either side. Place rats in the test box for 15 min.
2. Count how many pellets are retrieved from each side over the 15-min trial period.
3. The final four tests will be used to determine baseline scores.
4. Only rats that can collect a minimum of 12 pellets from each side will be included in analysis of the staircase test.
5. Results are expressed as a percentage of forepaw performance compared to prestroke scores.
6. Analyze using a two-way repeated-measures ANOVA followed by Bonferroni post hoc test to compare differences between treatment groups over time.

4 Notes

A number of problems can occur during surgery and stroke induction that can affect experimental outcomes. In addition to comments made above relative to each procedure, we now provide some examples with further comments regarding prevention or correction of these problems.

1. The screw is inserted first so that once the MCA coordinates are established very little pressure is then applied to the head.
2. It will be necessary to make a groove down the side of the skull to allow the probe to be inserted vertically into the brain. Test the position of drilling regularly. It is important that the guide probe is not moved out of positioned as it passes through the skull.
3. Occasionally ipsilateral deficits are also observed, particularly in the beam grip test.
4. This is different to placing the tape on the base of each forepaw. True hemineglect involves visual recognition without processing a functional response.
5. For accuracy in scoring, the test should be videotaped and analyzed in slow motion.
6. This test is not sensitive to the time period, thus exploratory movements may be encouraged by creating stimulation such as momentarily turning lights off or sliding the cylinder over a small distance. Caution: Do not overstimulate as the rat may freeze or become agitated.
7. Additional analysis could include horizontal landings scored as right, left, or both. The number of vertical movements per horizontal landing could then be assessed in order to determine the amount of movement the rat can accomplish per exploration.
8. Wipe the floor and walls of the starting chamber between rats, but not the staircase chamber to encourage the next rats to explore this area. Clean the test box more thoroughly at the end of each day.

9. If rats struggle to locate pellets in the wells, try raising the staircase by holding the silver handle up so that the uppermost stair is almost equal with the platform. If rats show no interest in going on to the platform try tapping the end of the staircase chamber or running a finger along the ceiling of this chamber. Poking extra pellets through the gap at the end of the staircase chamber may also encourage uncooperative rats.

10. A dull file can result in denting of the lumen when making injectors and guide cannulas, which can affect the smooth insertion of the injector into the conscious rat. This can be prevented by ensuring the file is sharp and the user has a light touch when filing.

11. When inserting the injector into the implanted guide in the conscious rat, sudden movements while pushing it through can result in bending of the injector. This renders the injector unusable. We therefore recommend that each injector and guide combination is documented for exact length such that a new injector could be made to fit the implanted guide.

12. Occasionally an implanted guide cannula can become blocked (often due to dust or small blot clots as a result of surgery). We recommend using a 30 gauge needle to clear the external end of the guide cannula prior to inserting the injector. If the block appears to be occurring closer to the internal end of the cannula, try infusing a small amount of saline first in an attempt to dissolve the block. If this does not work this rat could be used in a sham surgical group.

13. There are a number of expected risks associated with stroke induction. Temperature increases above 3 °C during stroke may result in seizure and rats displaying these signs should be humanely euthanized immediately (Lethobarbitone 1:2 dilution, ~160 mg/kg i.p.). Any rat showing signs of prolonged loss of righting reflex or seizure activity during or after stroke should also be humanely euthanized (Pentobarbitone 1:2 dilution, ~160 mg/kg i.p.). Body weight should be assessed daily throughout the duration of the study: loss of weight greater than 20% of preweight stroke should be keenly assessed for euthanasia. Rats experiencing a severe stroke may lose some forelimb/hind limb dexterity but stroke does not usually result in complete loss of function.

14. During Rota-Rod training many rats learn to turn around while on the wheel, enabling them to drop off quickly. This
can be avoided during training by taping the nose of the rat with a pen tip each time the rat’s head tries to turn around.

References

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