Animal handling

- Animal supplier:

  Charles river Germany for C57BL6J animals

- Animal characteristics:

  C57BL6J, Male, 10-12 weeks old, 25-28gr

- Housing conditions:
  - Light cycle, room temperature, and ventilation systems to be reported by the different groups
  - Food and water supplier and characteristics to be reported by the different groups
    - Wet food on the cage floor after stroke
  - Housing conditions:
    - Cage size:

      2-5 animals per cage

      Sham and stroke separated after fMCAo stroke surgery
    - Degree of environmental enrichment in the cages:

      Tissue for nest formation

      Red plastic box for nesting
    - 1week of acclimatization when animals arrive before starting the experiment

Anesthetics and analgesics

- Eye ointment (Bepanthene) during surgery
- Anesthesia
    - Surgeries
        - Gases: 100%O_2
        - Inhaled isoflurane (4% induction and 2% for the surgery)
    - Termination (100µl injection)
        - Ketamine 120 mg/kg
        - Xylacin 16 mg/kg
- Analgesia
    - All drugs diluted in Saline
    - 30min before surgery and until day 3 after surgery every 12h.
    - fMCAo: 500µl i.p.
    - PT/dMCAo: 100µl i.p.
        - Carprofen 5mg/Kg
        - Buprenorphin 0,1mg/Kg

- Monitor the animals during the surgery (not for PT):
    - Pulse oximetry
    - Rate breathe

- Animals not shaved for the surgery

Gravity perfusion

Materials:

- PBS + Heparin (2U/ml)
- 4 % Paraformaldehyd (PFA) in PBS, pH 7,4 (Morphisto, 11762)
- 2x 20ml Syringe
- 1ml Syringe
- Ketamine 120 mg/kg
- Xylacin16 mg/kg
- 30% sucrose
- Isopentane (methylbutane)(Sigma-Aldrich, M32631)
- Dry ice
- Small containers for the brains

Perfusion

Two 20ml syringes will be attached to the hood wall, 1m above. One syringe will contain PBS-heparin and the other one will contain PFA.

5min after 100µl injection of ketamine/Xylacin, animals will be perfused with 20ml PBS-heparin or 20ml PBS-heparin and 20ml PFA (perfusion speed: 60µl/s).

After perfusion only with 20ml PBS-heparin, brains will be removed and frozen immediately with dry ice.

After perfusion with 20ml PBS-heparin and 20ml PFA, brains will be immersed in 4%PFA for 24h, immersed in 30% sucrose in PBS until the sample sinks and frozen with cold Isopentane.

All brains will be stored at -80°C.

Shipping

Fresh frozen samples will be ship on dry ice.

PFA perfused samples will be ship in sucrose on ice.

Neuroscore

Material

- 45° ramp
- Surgery clothes to keep „smell“ neutral

Comments

- Perform the Neuroscore at 15h
- Mice rest 30 mins in room with “open” cage before the test
- Time points analysis: BL, 24h, 3d, 7d, 14d
- 30s observation per statement
- Send always raw data for data analysis

	Time-point of scoring		score
General Neuroscore	**Hair**	0. Hair neat and clean	
		1. Localized piloerection and dirty hair in 2 body parts (nose and eyes)	
		2. Piloerection and dirty hair in >2body parts	
	Ears (mouse on an open bench top)	0. Normal (ears are stretched laterally and behind, they react by straightening up following noise)	
		1. Stretched laterally but not behind (one or both), they react to noise	
		2. Same as 1. NO Reaction to noise.	
	Eyes (mouse on OBT)	0. Open, clean and quickly follow the surrounding environment	
		1. Open and characterized by aqueous mucus. Slowly follow the surrounding environment	
		2. Open and characterized by dark mucus	
		3. Ellipsoidal shaped and characterized by dark mucus	
		4. Closed	
	Posture (place the mouse on the palm and swing gently)	0. The mouse stands in the upright position with the back parallel to the palm. During swing, it stands rapidly.	
		1. The mouse stands humpbacked. During the swing, it flattens the body to gain stability.	
		2. The head or part of the trunk lies on the palm	
		3. The mouse lies on one side, barely able to recover the upright position.	
		4. The mouse lies in a prone position, not able to recover the upright position.	
	Spontaneos activity (mouse on OBT)	0.The mouse is alert and explores actively	
		1.The mouse seems alert, but it is calm and sluggish	
		2.The mouse explores intermittently and sluggishly	
		3.The mouse is somnolent and numb, few movements on-the-spot	
		4.No spontaneous movements	
	Total score for general scoring (normal=0 max=18)		

	Time-point of scoring		score
Focal Neuroscore	**Body symmetry** (mouse on OBT, observe the nose-tail line)	0. Normal (Body: normal posture, trunk elevated from the bench, with fore and hindlimbs leaning beneath the body. Tail: straight)	
		1. Slight asymmetry (Body: leans on one side with fore and hindlimbs leaning beneath the body. Tail: slightly bent.)	
		2. Moderate asymmetry (Body: leans on one side with fore and hindlimbs stretched out. Tail: slightly bent).	
		3. Prominent asymmetry (Body: bent, on one side lies on the OBT. Tail: bent)	
		4. Extreme asymmetry (Body: highly bent, on one side constantly lies on the OBT. Tail: highly bent)	
	Gait (mouse on OBT. Observed undisturbed)	0. Normal (gait is flexible, symmetric and quick)	
		1. Stiff, inflexible (humpbacked walk, slower than normal mouse)	
		2. Limping, with asymmetric movements	
		3. Trembling, drifting, falling	
		4. Does not walk spontaneously (when stimulated by gently pushing the mouse walks no longer than 3 steps)	
	Climbing (mouse on a 45° surface. Place the mouse in the center of the gripping surface)	0. Normal (mouse climbs quickly)	
		1. Climbs with strain, limb weakness present.	
		2. Holds onto slope, does not slip or climb	
		3. Slides down slope, unsuccessful effort to prevent fail	
		4. Slides immediately, no effort to prevent fail.	
	Circling behavior (mouse on OBT, free observation)	0. Absent circling behavior	
		1. Predominantly one-side turns.	
		2. Circles to one side, although not constantly.	
		3. Circles constantly to one side.	
		4. Pivoting, swaying, or no movement.	
	Forelimb symmetry (mouse suspended by tail)	0. Normal	
		1. Light asymmetry: mild flexion of contralateral forelimb.	
		2. Marked asymmetry: marked flexion of contralateral limb, the body slightly bends on the ipsilateral side.	
		3. Prominent asymmetry: contralateral forelimb adheres to the trunk.	
		4. Slight asymmetry, no body/limb movement.	
	Compulsory circling (forelimbs on bench, hindlimbs suspended by the tail: it reveals the presence of the contralateral limb palsy)	0. Absent. Normal extension of both forelimbs.	
		1. Tendency to turn to one side (the mouse extends both forelimbs, but starts to turn preferably to one side)	
		2. Circles to one side (the mouse turns towards one side with a slower movement compared to healthy mice)	
		3. Pivots to one side sluggishly (the mouse turns towards one side failing to perform a complete circle)	
		4. Does not advance (the front part of the trunk lies on the bench, slow and brief movements)	
	Whisker response (mouse on the OBT)	0. Normal	
		1. Light asymmetry (the mouse withdraws slowly when stimulated on the contralateral side)	
		2. Prominent asymmetry (no response when stimulated to the contralateral side)	
		3. Absent response contralaterally, slow response when stimulated ipsilaterally.	
		4. Absent response bilaterally	
	Total score for focal deficits (normal=0 max=28)		

Neuroscore

	General deficits							
1.	Hair (0-2)							
2.	Ears (0-2)							
3.	Eyes (0-4)							
4.	Posture (0-4)							
5.	Spontaneous activity (0-4)							
	TOTAL							
	Focal deficits							
7.	Body symmetry OBT (0-4)							
8.	Gait OBT (0-4)							
9.	Climbing Grip surf. (0-4)							
10.	Circling behavior (0-4)							
11.	Front limb symmetry by tail (0-4)							
12.	Compulsory circling front limbs on bench (0-4)							
13.	Whisker response (0-4)							
	TOTAL							
	Composite SCORE							

Body weight and temperature

Day									
Weight									
Temperature									

Phototrombotic stroke model

Material

- Rose Bengal Power (REF: #198250-5G, Sigma Aldrich)
- Laser (Cobolt HS-03, Solna, 561 nm wavelength)

Surgery

1. Place the mouse in the isoflurane chamber 4%.
2. Place the mouse in the stereotactic frame with the isoflurane mask (2%). The depth of anesthesia is verified by foot pinches. If the mouse is adequately anesthetized, there is no response of extremity (flexion or withdrawal).
3. Apply dexpanthenol eye ointment on both eyes
4. The body temperature is monitored and maintained at 37°C by a thermostatically controlled heating pad with a rectal probe.
5. The scalp is longitudinally incised (2.0-2.5 cm) and retracted to expose the skull. To avoid wound complications the skull exposure should be achieved with a single cut.
6. The periost is gently removed and coronal and sutures are identified.
7. Mark bregma +3mm left.
8. A sticker with a 4mm diameter hole placed at the coordinates mentioned above.
9. A laser beam of 1.5 mm diameter and 561 nm wavelength is stereotactically positioned onto the skull +3mm left from Bregma and at 1cm from the skull.
10. Inject the mouse with 1% Bengal Rose (10µl/gr).
11. The skull is illuminated for 20min
12. Suture the wound and place the animal in a recovery chamber at 34°C for 1h to recover from anesthesia
13. After 1h the mice are returned to their cages in a temperature-controlled room

Comments

- Lesion in the left hemisphere lesion
- Optimal duration of the surgery=25min
- Sham:
    - Rose Bengal injection without laser
    - Same surgery time (25min)
- Exclusion criteria:
    - Died during surgery
    - Infection of the suture
    - Bite wound
    - For the stroke animals:
        - No infarct or fore assimetry<2 at 24h after PT
    - For the sham animals:
        - infarct or with fore assimetry>2 at 24h after PT

pMCAo stroke protocol (Coagulation)

Material

- Electrocoagulationdevice (same divice, old and new model)
    - ERBETOM ICC 80/50 HF-Chirurgiegerät, ERBE
    - ERBETOM VIO 100 C HF-Chirurgiegerät, ERBE
- Electrocoagulationforceps (ERBE)
    - straight: #20195-011 (tip: 0,2mm)
    - 30° angled: #20195-013 (tip: 0,2mm)
- Drill (D-34343, Proxxon)
- Drill bits (#28212, Proxxondiamont, Ø1mm)

Surgery

1. Place the mouse in the isoflurane chamber 4%.
2. Transfer the mouse in a lateral position with its nose into the anesthesia mask and maintain isoflurane concentration at 4% for another minute, then reduce and maintain it at 2%.
3. The body temperature is monitored and maintained at 37°C by a thermostatically controlled heating pad with a rectal probe.
4. Apply dexpanthenol eye ointment on both eyes
5. Make a 1 cm skin incision between the ear and eye using operation scissors.
6. Separate the skin and localize the temporal muscle.
7. Select in the high-frequency generator the coagulation function, bipolar mode, select 12 watts and connect the electrocoagulation forceps with the cable.
8. Add a drop of saline and use the forceps to detach the temporal muscle from the skull in its apical and dorsal part, thereby, making a muscle flap without totally removing the muscle.
9. Identify the MCA below the transparent skull, in the rostral part of the temporal area, dorsal to the retroorbital sinus. If the MCA bifurcation is not visible (due to an anatomical normal variation) identify the vessel most rostral.
10. Add some saline on the skull and thin out the bone with the drill right above the MCA branch until it has a thin and translucent texture.
11. Carefully withdraw the bone above the artery with a very thin forceps.
12. Select bipolar mode in the high-frequency generator at 7 watts. Coagulate the artery with the electrocoagulation forceps proximal and distal to the bifurcation. When the bifurcation is not visible due to an anatomical variant, coagulate the correctly identified MCA branch at two sites of approx. 1mm distance. It is not necessary to grasp the artery with the forceps for coagulation, touching the artery carefully with the forceps on both sides from above is sufficient and induces less mechanical damage.
13. Wait 30 seconds and gently touch the artery with a blunted forceps to check for any blood flow due to spontaneous recanalization. In case of recanalization repeat the electrocoagulation once.

14. Relocate the temporal muscle to its position, covering the burr hole.
15. Suture the wound and place the animal in a recovery chamber at 37 °C for 1h to recover from anesthesia.
16. After the surgical procedures, the mice are returned to their cages in a temperature-controlled room

Comments

- Lesion in the left hemisphere lesion
- Without removing meninges
- Optimal duration of the surgery = 15min
- Sham:
    - Same surgery but only thin the skull without open a hole
- Exclusion criteria
    - Died during surgery
    - For the stroke animals:
        - Infarct volume<5mm^3
        - Artery broken during surgery
        - Major bleeding artery and remaining blood between the brain and skull
    - For sham animals:
        - Infarct volume>5mm^3

tMCAO stroke protocol (filament)

Materials

- Laser doppler (PF 5010 LDPM, Periflux System 5000, Perimed)
- Filament (#602112PK5Re, Doccol)
    - 1-2mm coating length

Surgery

1. Place the mouse in the isoflurane chamber 4%.
2. Place the mouse nose in the anesthetic cone (2%). The depth of anesthesia is verified by foot pinches. If the mouse is adequately anesthetized, there is no response of extremity (flexion or withdrawal).
3. The body temperature is monitored and maintained at 37°C by a thermostatically controlled heating pad with a rectal probe.
4. Apply Bepanthene eye ointment on both eyes.
5. Cut between the ear and the eye, to find the middle artery. (retire the temporal muscle to find it).
6. Introduce the Doppler and fix the tip on the artery with glue, close the skin with the glue.
7. Turn the mice upside down, put the mice mouth into the anesthesia cone and fix the animal with tape.
8. Do an incision in the neck, along the trachea.
9. Find the left common carotid artery and do a transient ligation before the bifurcation. Do a ligation in the external carotid artery. Do another ligation, softly, in the external carotid, immediately after the bifurcation and a clip the internal carotid artery 5mm over the bifurcation.
10. Cut, softly, the external carotid artery between the ligation and the soft ligation, and introduce the filament until the common carotid artery.
11. Cut completely the external carotid artery after the permanent ligation, remove the clip and introduce the filament through the internal carotid artery until the end.
12. Tie the filament with the soft ligation done before, to maintain it.
13. Suture the wound and place the animal in a recovery chamber at 37 °C for 1h (until filament removal).
14. Remove the filament and tie the artery with the suture.
15. Open the transient ligation before the bifurcation from the CCA.
16. Suture the wound and place the animal in a recovery chamber at 37 °C for 1h to recover from anesthesia.
17. After the surgical procedures, the mice are returned to their cages in a temperature-controlled room

Comments

- Left hemisphere lesion
- Optimal duration of the surgery = 20min
- Sham
    - All sutures, also CCA during 1h
    - In/out filament
    - Same surgery time
- Inclusion
    - For the stroke animals:
        - >80% reduction of blood flow during occlusion
        - >80% reperfusion rate after 10min
- Exclusion criteria
    - Died during surgery
    - For sham animals:
        - Infarct
        - >20% blood flow reduction when CCA occluded
    - For stroke animals:
        - No infarct
        - <80% reduction of blood flow during occlusion
        - <80% reperfusion rate after 10min
        - >20% blood flow reduction when CCA occluded