Video Article

Paw-Dragging: a Novel, Sensitive Analysis of the Mouse Cylinder Test

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Abstract

The cylinder test is routinely used to predict focal ischemic damage to the forelimb motor cortex in rodents. When placed in the cylinder, rodents explore by rearing and touching the walls of the cylinder with their forelimb paws for postural support. Following ischemic injury to the forelimb sensorimotor cortex, rats rely more heavily on their unaffected forelimb paw for postural support resulting in fewer touches with their affected paw which is termed forelimb asymmetry. In contrast, focal ischemic damage in the mouse brain fails to result in comparable consistent deficits in forelimb asymmetry. While forelimb asymmetry deficits are infrequently observed, mice do demonstrate a novel behaviour post stroke termed “paw-dragging”. Paw-dragging is the tendency for a mouse to drag its affected paw along the cylinder wall rather than directly push off from the wall when dismounting from a rear to a four-legged stance. We have previously demonstrated that paw-dragging behaviour is highly sensitive to small cortical ischemic injuries to the forelimb motor cortex. Here we provide a detailed protocol for paw-dragging analysis. We define what a paw-drag is and demonstrate how to quantify paw-dragging behaviour. The cylinder test is a simple and inexpensive test to administer and does not require pre-training or food deprivation strategies. In using paw-dragging analysis with the cylinder test, it fills a niche for predicting cortical ischemic injuries such as photothrombosis and Endothelin-1 (ET-1)-induced ischemia – two models that are ever-increasing in popularity and produce smaller focal injuries than middle cerebral artery occlusion. Finally, measuring paw-dragging behaviour in the cylinder test will allow studies of functional recovery after cortical injury using a wide cohort of transgenic mouse strains where previous forelimb asymmetry analysis has failed to detect consistent deficits.

Video Link

The video component of this article can be found at https://www.jove.com/video/52701/

Introduction

The goal of neural regeneration strategies is to demonstrate both tissue repair and functional recovery. Functional recovery is typically evaluated with behavioural tests that measure functional deficits, in this case involving motor skills that are associated with damage to the specific brain regions. Traumatic brain injury or ischemic damage to the sensorimotor forelimb area of the cortex can be demonstrated by a number of behavioural tests. One such test, the cylinder test is used extensively in rats to assess functional deficits in forelimb activity[1]. The test has a low set-up cost requiring only a cylinder, camera and table with a transparent top. It is easy to administer as it is based on the natural exploratory behaviour of rodents, so pre-training and food deprivation or rewards are not required. Despite these numerous advantages, the cylinder test is under-utilized to assess forelimb deficits in mice following focal injuries to the forelimb sensorimotor cortex, which we attribute to the analysis of mouse behaviour in the cylinder test. Forelimb asymmetry is the classical measure of analysis for the cylinder test. When placed in the cylinder, rodents naturally explore the walls of the cylinder by rearing onto their hind limbs and touching the cylinder walls with their forelimb paws for postural balance. The number of paw touches with the wall with each forelimb is easily quantified by filming rodents during this exploration of the cylinder. Forelimb asymmetry occurs when the affected forelimb paw makes fewer touches with the wall than the unaffected forelimb paw and is indicative of damage to the contralateral sensorimotor cortex. In rats, intra-cortical injections of the vasoconstrictive agent, Endothelin (ET-1), into the forelimb sensorimotor cortex causes a focal ischemic lesion which results in behavioural deficits in the contralateral forelimb. Deficits in contralateral forelimb use are readily detected as changes in forelimb asymmetry in the cylinder test in rats[1-3]. In contrast to rats however, changes in forelimb asymmetry are variable and less consistent in mice following comparable ET-1 injections[4-6]. Here we demonstrate a novel analysis of forelimb behaviour in the cylinder test – analysis of paw-dragging behaviour. We have previously shown that paw-dragging analysis is a more sensitive measure of damage to the forelimb sensorimotor cortex in mice than the classical forelimb asymmetry analysis and therefore is applicable to a variety of focal cortical injury models.

Examination of how the forepaw contacts the cylinder wall following ischemic damage to the forelimb sensorimotor cortex revealed a novel behaviour in mice - paw-dragging[4]. A paw-drag occurs when a mouse stands on its rear legs to explore the cylinder wall then drags its affected (contra-lesional) paw along the cylinder wall towards its midline or down the wall while its unaffected forepaw provides postural support against the wall. Paw-drags rarely occur in uninjured mice therefore the appearance of a paw-drag is a positive indicator of injury to the forelimb sensorimotor cortex[4]. We have previously quantified paw-dragging behaviour in mice following ET-1 ischemic damage to the forelimb sensorimotor cortex and have shown sustained paw-dragging behaviour in mice up to two weeks post-stroke[4]. Here we show that paw-dragging behaviour is sustained up to four weeks post-stroke. Analysis of paw-dragging behaviour provides a novel and sensitive tool for assessing focal

ischemic damage to the forelimb sensorimotor cortex in mice. Its inexpensive set-up, ease of administration and scoring make this a simple, yet useful tool to rapidly assess forelimb behavioural deficits in mice.

Protocol

Ethics statement: All experiments were approved by Memorial University of Newfoundland's Animal Care Ethics Committee according to the guidelines of the Canadian Council on Animal Care.

1. Mice

1. Use adult mice. In this study, adult male FVBN mice (n=10) between 2-4 months were used. House mice on a 12:12 hr reverse light-dark cycle and provide standard rodent chow and water *ad libitum*.

2. Materials Required for the Cylinder Test

1. Obtain a table with a transparent top to film the cylinder test. The dimensions of the table are irrelevant, the top must be plexiglass or glass, and there must be enough room to position a mirror below the table. This allows for the mouse to be videotaped from below. Use a mirror below the table to reflect the image through the cylinder. As an alternative, use an upside-down camera if available. The dimensions of the table used in this protocol are 54 x 56 x 66.5cm (w x l x h) with a 51 x 51cm top (w x l).
2. Obtain a mirror. The dimensions of the mirror used in this protocol are 34 x 58cm (w x l).
3. Obtain a transparent/plexiglass cylinder for the mouse to perform in. The dimensions of the cylinder used in this protocol are 17.5cm high, 8.8cm I.D, 9.5cm O.D with a wall thickness of 0.35cm. A taller cylinder may be required for more active mouse strains.
4. Place the cylinder on the tabletop and film the reflection in the mirror below.
5. A videocamera and tripod are required. Record videos at approximately 650Kb/s, which is approximately 190Mb per 5 min of cylinder video. Ensure that the camera has a zoom functionality to ensure that the cylinder encompasses the entire field of view.
   NOTE: The videocamera used in this protocol is a Sony DCR-SR42, 40x optical zoom, 2,000x digital zoom, 680kpix which uses standard definition, NTSC interlaced video.)
6. Obtain software for analysis – a media player with video support and playback speed modulation. The media player used in this protocol is the VLC Media Player v2.1.2.
7. Obtain a computer with an operating system capable of running the media player and a monitor.
8. Electronic storage for videos is required. Download videos to an external hard drive or copy it onto DVDs for long-term storage.
   NOTE: At 190Mb per video, 84 sessions will fit on a 16 GB SD card and 168 sessions will fit on a 32GB SD card. Due to the relative inexpensiveness of SD media, and the uncertainty in how much time some mice require to complete 20 rears, a 32 GB card is recommended. In the current study, the videos were copied from the camera to a 2TB external hard drive and then recopied onto DVDs as backup.

3. Experimental Setup of the Cylinder Test

1. Fasten mirror below the table at a 45 degree angle to the tabletop. Do this using two support brackets attached to the table legs to support the top and bottom of the mirror, respectively. Bracket locations are noted in a side-view of the table (**Figure 1A**) and a face-on view of the table (**Figure 1B**).
2. Place cylinder on the center of the table. Draw four equidistant lines where the cylinder sits on the table with a black marker (Sharpie) so that the cylinder can be lifted and returned to the same position (**Figure 2**). Drawing them on the underside of the transparent tabletop allows the tabletop to be cleaned between animal tests without dissolving the marker ink.
3. Attach the camera and tripod. Aim the camera at the mirror so that the picture is looking directly through the barrel of the cylinder. Ensure that the full inner wall of the cylinder is visible and unobstructed by the base (**Figure 3**). See a side-on view of the setup, including the relative angle of the camera and mirror setup, from above (**Figure 4A**) and from the level of the table, including a mouse rearing (**Figure 4B**).
4. Prepare cue cards to identify each mouse prior to filming. Ensure that the cards typically include an identification number for each mouse, the time-point of the test (*e.g.*, 3 days after treatment) and the date of the filming session. Do not include the treatment group on the cue card to ensure the experimenter is blinded.
5. Film in standard indoor lighting conditions as this level of light is required to clearly see the mouse movements around the cylinder.
   NOTE: If available filming in the dark with a red light camera may suffice, however one would first need to test whether paw touches and drags are clearly visible for quantification.

4. Execution

1. Start filming. Display the appropriate mouse cue card in front of camera lens.
2. Lower the mouse into the cylinder from the open top immediately after filming the cue card.
3. Begin filming the mouse. Minimize noise during this time, as mice may lose interest in exploring if startled.
4. Observe the mice rear to explore the cylinder. Capture the video until the mouse performs a minimum of twenty rears.
   NOTE: A rear occurs when both forepaws lose contact with the floor and the mouse stands on its hind legs.
5. Wipe tabletop and cylinder with an appropriate cleaning solution between each mouse to sanitize and remove mouse scents.
6. Freezing is when mice stop exploring and remain quietly sitting on all fours for approximately 5 min. If mice freeze before twenty rears occur, it may be necessary to remove them from the cylinder for 10-20 min before resuming the test. If mice do not perform twenty rears, they are removed from the study.
   NOTE: In our experience, mice have never needed to be excluded due to failure to explore the cylinder.

5. Evaluation of the Cylinder Test using Paw-Dragging Analysis

1. Play back the video at a rate of between 0.25x and 0.67x regular speed depending on how quickly the mouse explores the cylinder. Use a media player that offers slower playback speeds.
2. Quantify the total number of paw touches. Paw touches occur when the mouse rears (**Figure 5A**), touches the side of the cylinder (**Figure 5B**), subsequently dismounts with both paws simultaneously (**Figure 5C**) and lands (**Figure 5D**). The paw may or may not contact the cylinder wall with a full palm, but some contact with the cylinder wall must occur. Assess paw touches by counting the number of times the mouse makes contact (no matter how brief) with the cylinder wall with either or both forepaws while standing on its hind limbs during a rear. Note that contact with the cylinder wall is only counted as a “paw touch” or “paw-drag” if the mouse is in a rear position – standing on its hind limbs with both forepaws off of the tabletop. If the mouse remains in a 3-point stance – both hind limbs and one forepaw on the tabletop and proceeds to touch the wall with the free paw – this is not counted as a paw touch. Mice may rear and touch the cylinder wall with a single forepaw and this is counted as a paw touch. Note: a mouse may also move its body around the cylinder during a rear, making more than two contacts. These contacts are tallied – one for each left forepaw touch and one for each right forepaw touch.
3. Quantify the number of paw-drags. Paw-dragging behaviour is distinct from normal paw touches.
    1. If the paw contacts the cylinder wall with a full open palm (**Figure 6B**), it will slowly fall away from the wall, often with a slight tremor. The movement begins with the digits dragging against the cylinder wall either in a medial or downward direction, (**Figure 6C**) before falling away completely (**Figure 6D**). The mouse will then dismount with its unaffected paw (**Figure 6E**) before landing on all fours (**Figure 6F**). This is considered a paw-drag and should be counted in a tally.
    2. If the paw does not contact the cylinder wall with a fully open palm, it will graze the cylinder wall with its digits before falling away from the cylinder wall. Similarly, a mouse may drag its paw against the cylinder wall but not release it entirely before dismounting. These are both considered paw-drags as well as touches and should be counted as both in a tally.
    3. The paw may also drag along the cylinder wall while a mouse explores the cylinder. In this case, the paw will follow the twisting of the mouse’s torso as it explores left or right of its original position (**Figure 7A-D**) before dismounting (**Figure 7E**). This is not considered a paw-drag, as it depends on the mouse randomly choosing a direction to explore and does not depend on which cortical hemisphere was damaged.
4. Paw-drags are expressed as a percentage of paw-drags per total number of paw touches during a session. Express the number of paw-drags as a percentage of total paw contacts for each forelimb separately.
5. Touches resulting in a paw-drag count as a paw-drag and a touch simultaneously. Thus, if a mouse drags its paw each time its paw contacts the cylinder wall, the paw-dragging percentage is expressed as 100%.

6. Additional Experimental Design Suggestions

1. To minimize extraneous variables:
    1. Test the mice at the same time on each testing day. Test the mice during their wake cycle. Keeping mice on a 12 hr reverse light cycle facilitates performance.
    2. Mice may be reluctant to explore the cylinder if stressed either by noise or a novel environment. Testing mice in their animal holding room or a room they have been familiarized with reduces stress. This can occur if the room is noisy, if the mouse has been jostled prior to entering the cylinder or due to habituation.
    NOTE: Testing should be performed once before experimental manipulation to serve as a baseline reading. After manipulation, testing days are at the experimenter’s discretion, though it is advised to avoid an excessive number of exposures to the cylinder over a short period of time.
    3. Mice may become reluctant to rear after 6-7 exposures to the cylinder. For the current study, mice were tested in the cylinder for a total of seven times, prior to ischemia and on days 1, 3, 7, 14, 21 and 28 post-surgery.
    NOTE: In this study we used the FVBN mouse strain. We have previously tested C57Bl/6 mice in the cylinder and observed paw-dragging behaviour following an ET-1 ischemic injury (data not shown). C57BL/6 mice were more active than FVBN mice when rearing and frequently jumped onto the rim of the cylinder before climbing out. Taller cylinders should be used if mice attempt to escape by jumping.

7. Endothelin-1 Surgery and Infarct Volume Measurements

1. Perform Endothelin-1 surgery and infarct volume measurements according to previously published protocols[4] . To target the anterior forelimb motor cortex, each mouse should receive three ET-1 injections at the following coordinates: (i) +0.7 anteriorposterior (AP)/1.5 medial-lateral (ml)/-1.2 dorsal-ventral (DV), (ii) +0.4 AP/1.25 ml/- 1.2 DV and (iii) +0.1 AP/1.75 ml/-1.2 DV[4] .

8. Statistical Analysis

1. A two-way repeated measures analysis of variance (ANOVA) is recommended to analyze the percent of paw-dragging for the affected and unaffected paws across different time points.

Representative Results

We have previously demonstrated that paw-dragging behaviour appears following a focal ischemic injury to the forelimb sensorimotor cortex and is a positive indicator of damage[4]. Intra-cortical injections of ET-1 into the forelimb sensorimotor cortex were used to induce an ischemic lesion (**Figure 8A,B**). This study examined whether paw-dragging behaviour extended for longer than 14 days post-injury for its potential use to assess functional recovery. Mice were tested in the cylinder test on the day prior to ET-1 injections for the pre-surgery time point, and on days 1, 3, 7,

14, 21 and 28 post-injury. At each time point, the number of paw touches and paw drags were quantified for both the affected and unaffected paw. Two way repeated measures ANOVA on the number of paw touches revealed significant main effects of time [F(6,108) = 3.59, *P*=0.0028] and subjects [F(18,108) = 2.38, *P*=0.0032] but no effect of treatment (Table 1). Whereas, using the standard analysis of forelimb asymmetry for the cylinder test which quantifies affected paw touches versus total paw touches revealed inconsistent forelimb behavioural deficits. A one-way repeated measures ANOVA on the percent of affected paw use revealed a significant main effect of time (p=0.015) which followed by Dunnett's post hoc test showed significant reductions in the percent affected paw use at 7, 14 and 21 days post-surgery and recovered by 28 days post-surgery (**Figure 8C**). In contrast, a two way repeated measures ANOVA on the number of paw drags revealed significant main effects of time [F(6,108) = 7.09, *P*<0.0001], treatment [F(1,108) =33.02, *P*<0.0001], interaction [F(6,108) = 9.89, *P*<0.0001] and subjects [F(18,108) = 4.84, *P*<0.0001]. Further Bonferroni post hoc analysis showed significant increases in the number of paw drags at each time point following surgery (Table 1). Similarly, a two way repeated measures ANOVA comparing the number of affected paw-drags versus total affected paw touches revealed significant main effects of time [F(6,108)=6.63, p<0.0001], treatment [F(1,108)=20.46, p=0.0003], interaction [F(6,108)=8.21, p<0.0001] and subjects (matching) [F(18,108)=7.35, p<0.0001]. Further Bonferroni post hoc analysis showed significant paw-dragging behaviour up to 28 days post surgery (**Figure 8D**). The paw-dragging behaviour was specific to the affected limb as no increase or change in paw-dragging was observed with the unaffected limb. Paw-dragging behaviour with the affected forepaw was significantly elevated at 1, 3, 7, 21 and 28 days post-surgery (**Figure 8D**). Paw-dragging behaviour peaked at 1 day post-surgery with >30% of all paw touches with the affected forelimb resulting in a paw-drag then dropped to ~15% at 3 days post-surgery where it remained up to and including 28 days post-surgery. At 28 days post-surgery, mice were euthanized and infarct volumes assessed. The mean infarct volume for the group was 3.2 ±0.4 μm^3 (n=10 mice). These results show that small cortical infarcts can result in significant and sustained behavioural deficits as measured in the cylinder test. In summary, these data demonstrate that not only is paw-dragging highly responsive to damage to the forelimb sensorimotor cortex, but that paw-dragging also persists over time and can be used to assess functional recovery.

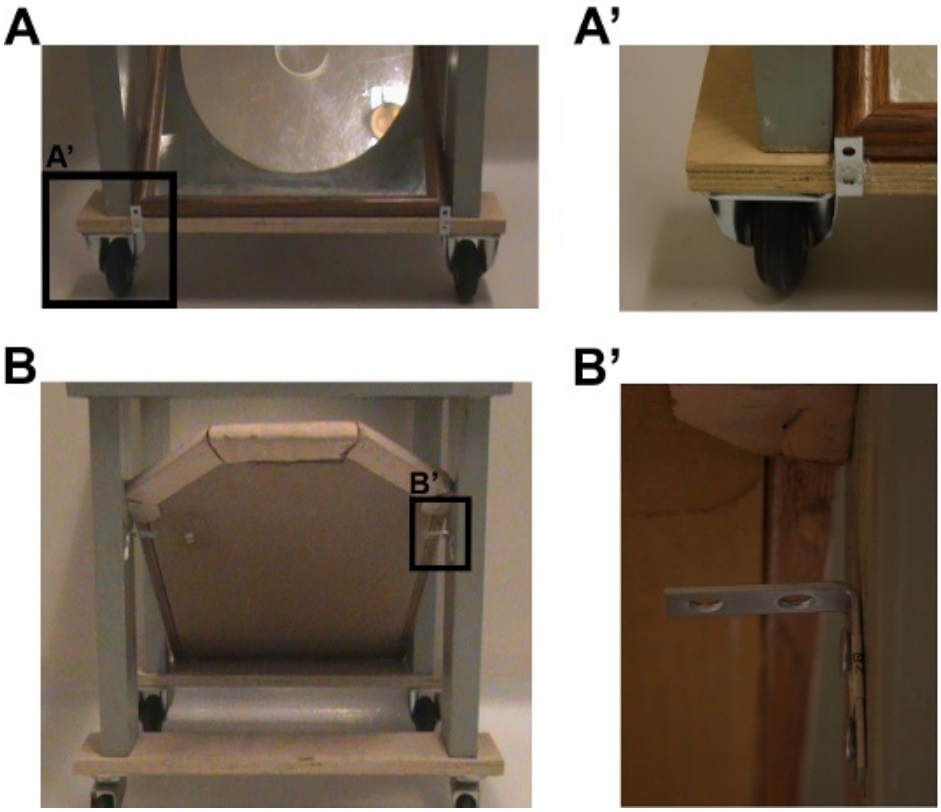

Figure 1. Bracket locations to fasten mirror in place below the table. (**A**) Front view of table showing bracket locations on front legs of the table. (**A'**) Higher magnification of inset in A showing location of brackets on front legs. (**B**) Rear view of table showing rear leg bracket locations. (**B'**) Higher magnification of inset in B indicating bracket location on rear legs of table.

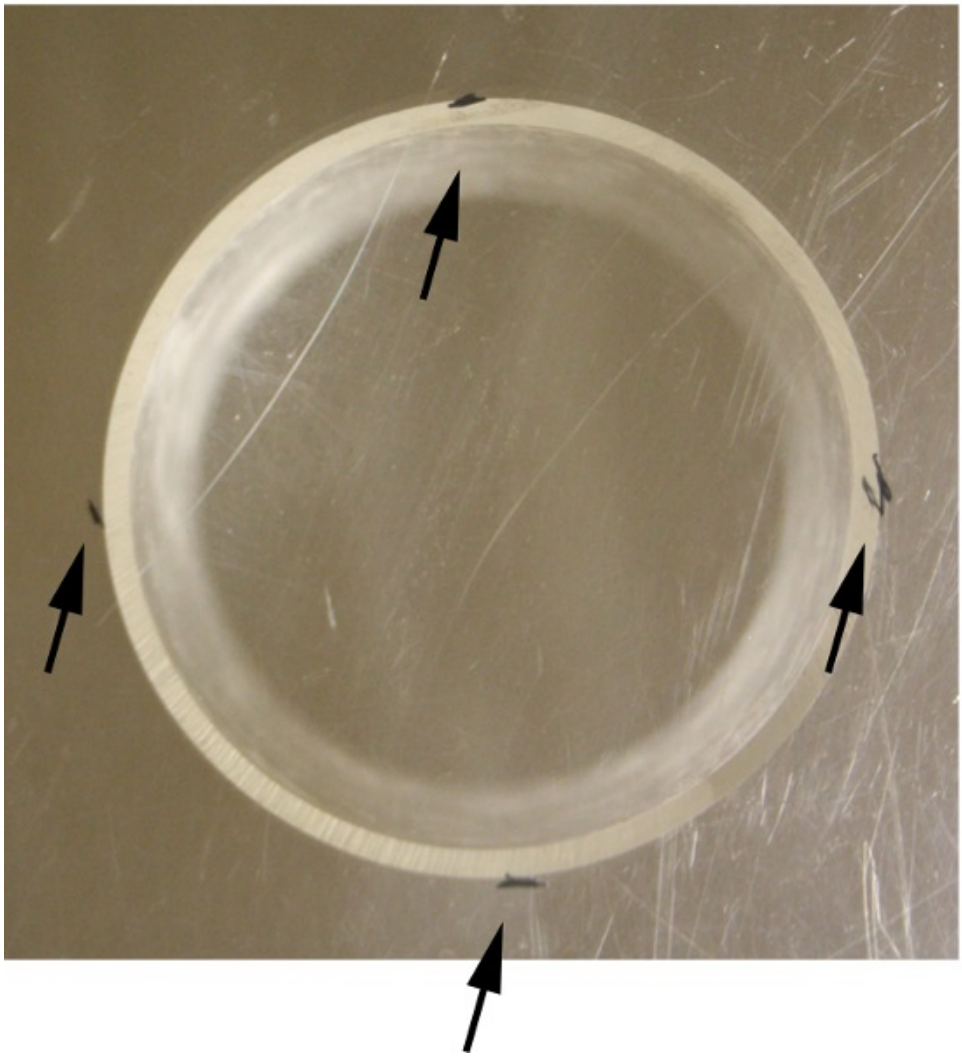

Figure 2. Marking the location for cylinder placement on the table. Photo of the tabletop indicating placement of the cylinder with black lines drawn around the perimeter of the base. Arrows point to the black lines drawn on the underside of the Plexiglas used for centering the cylinder on the tabletop.

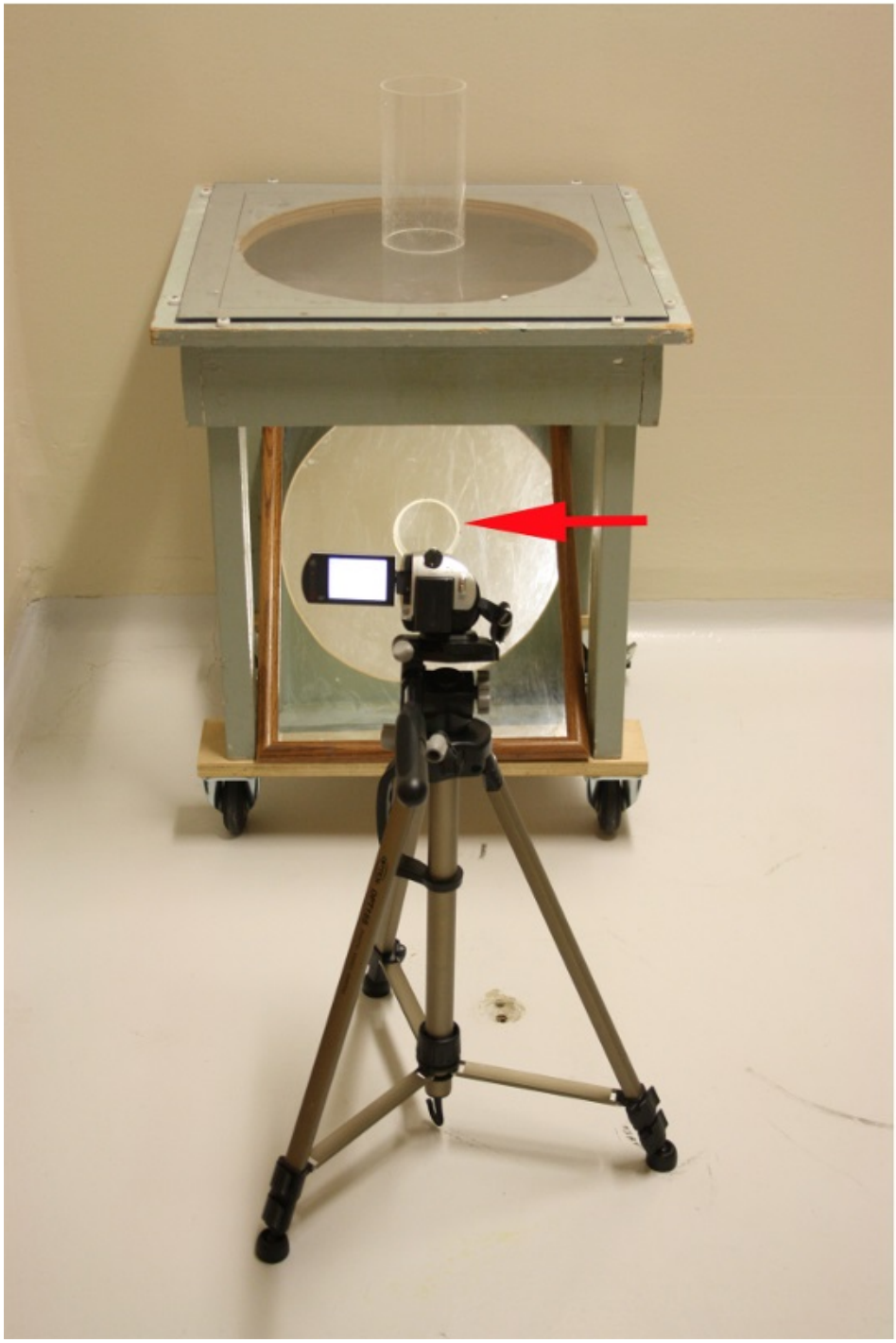

Figure 3. Front view of camera and tabletop set-up. Photo of the tabletop demonstrating the line of sight directly through the cylinder barrel (red arrow).

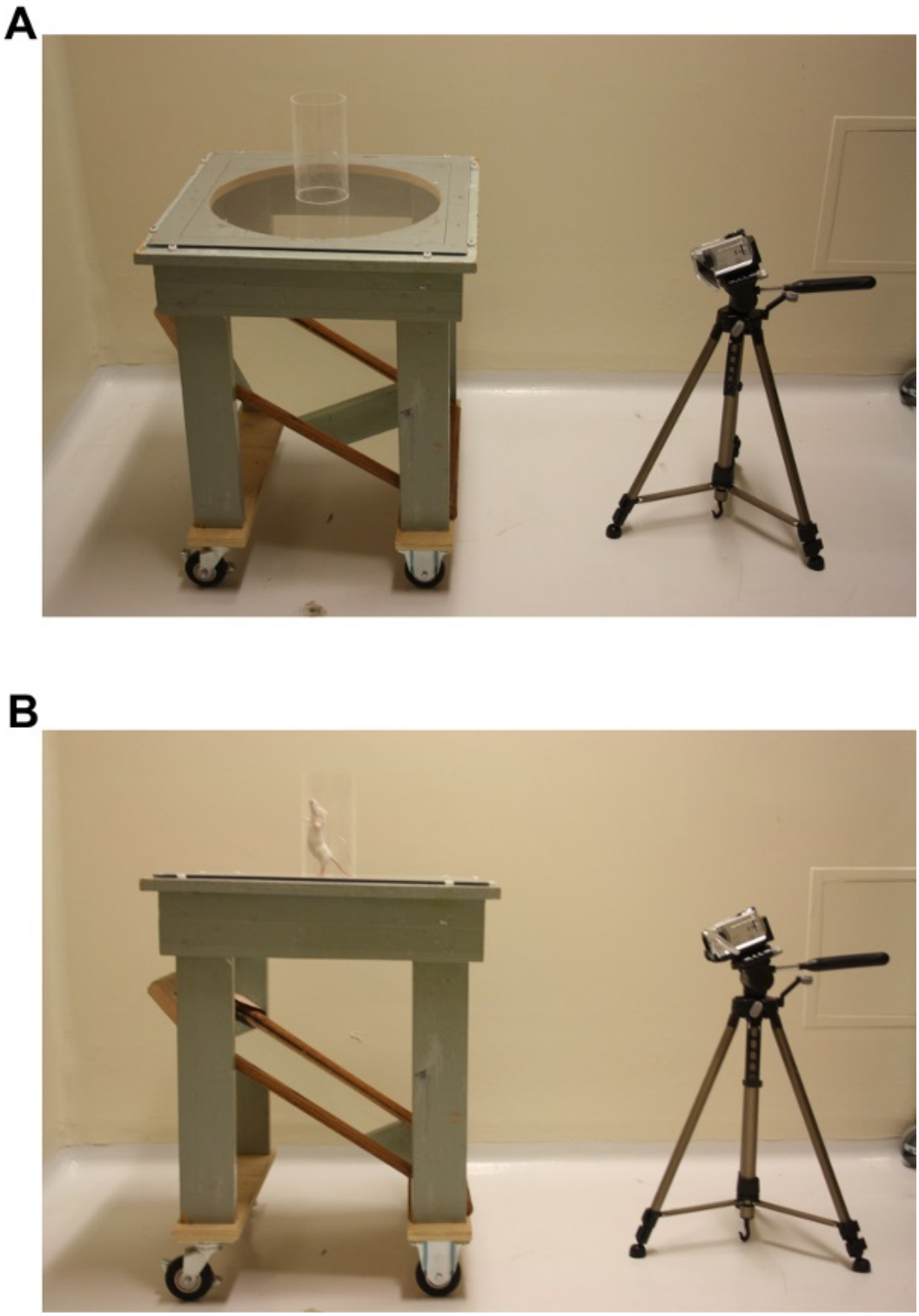

Figure 4. A side view of the camera and table set-up. The camera is aimed directly at the base of the cylinder. (**A**) Table and camera setup taken from above. (**B**) Table and camera setup taken at the level of the table, showing a mouse rearing in the cylinder.

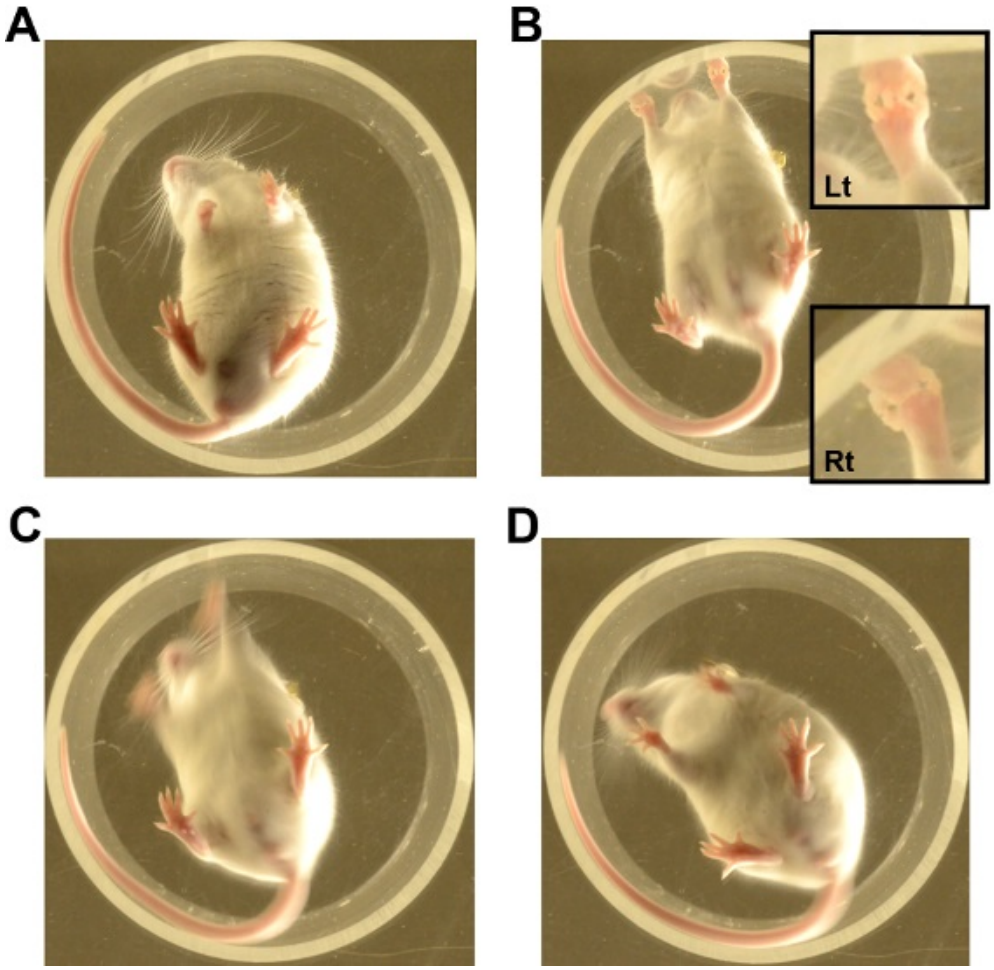

Figure 5. A sequence of photos demonstrating an uninjured mouse rearing. (**A**) Photo of a mouse prior to a rear. (**B**) The mouse touches the cylinder wall with both paws. (**C**) To dismount, the mouse will push against the cylinder wall using both paws, and (**D**) land on all four paws. Lt = mouse's left paw, Rt = mouse's right paw.

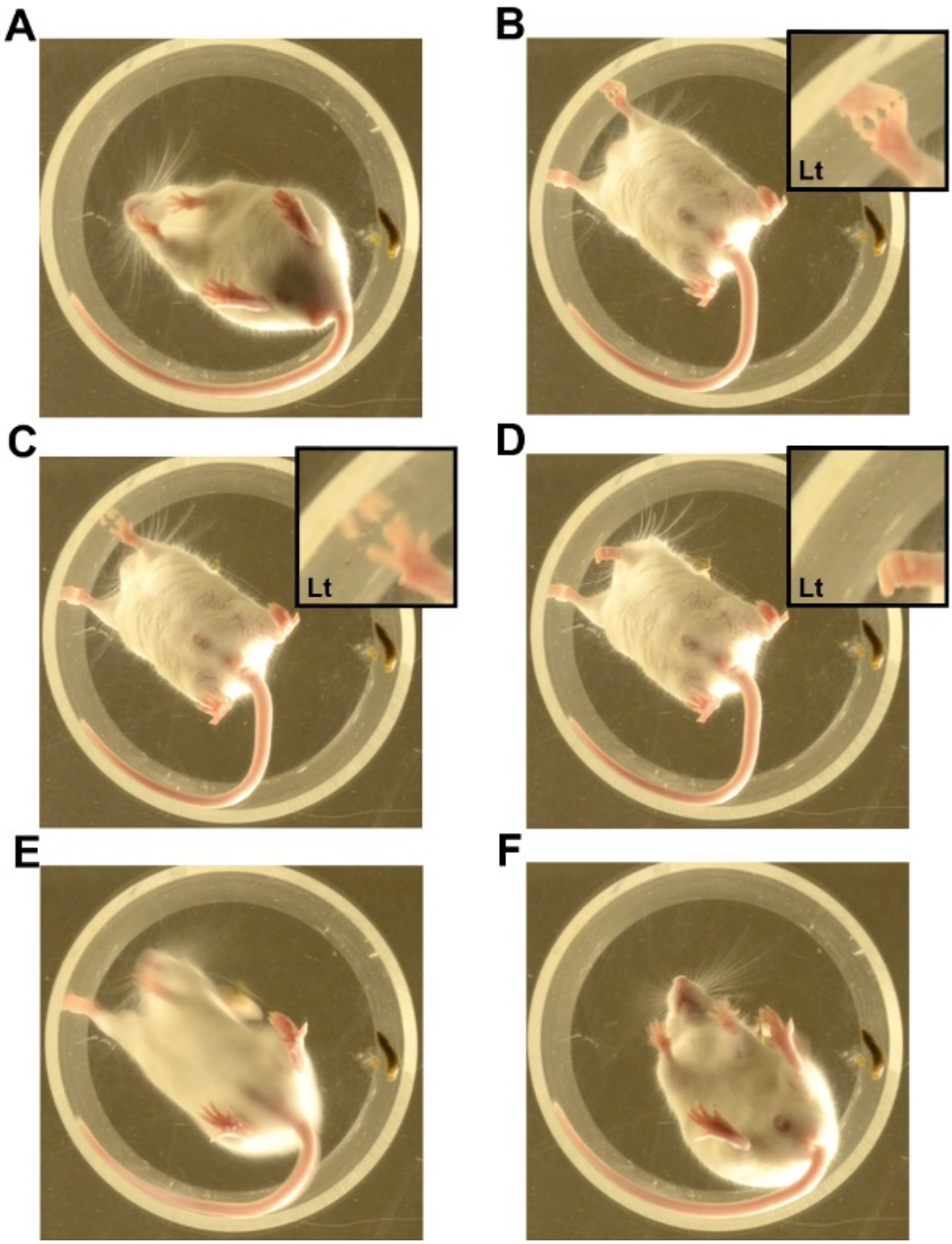

Figure 6. A sequence of photos demonstrating an injured mouse paw-dragging. (**A**) Photo of a mouse prior to a rear. (**B**) The mouse will touch the cylinder wall with both paws; (**C**) then slowly let the digits on the affected paw drag vertically down the cylinder wall; (**D**) before letting the paw fall away from the wall. (**E**) The mouse will then dismount with their unaffected paw and (**F**) land on all four paws. High magnification insets in B,C and D demonstrate how the affected forepaw contacts the cylinder wall. Lt = mouse's left paw.

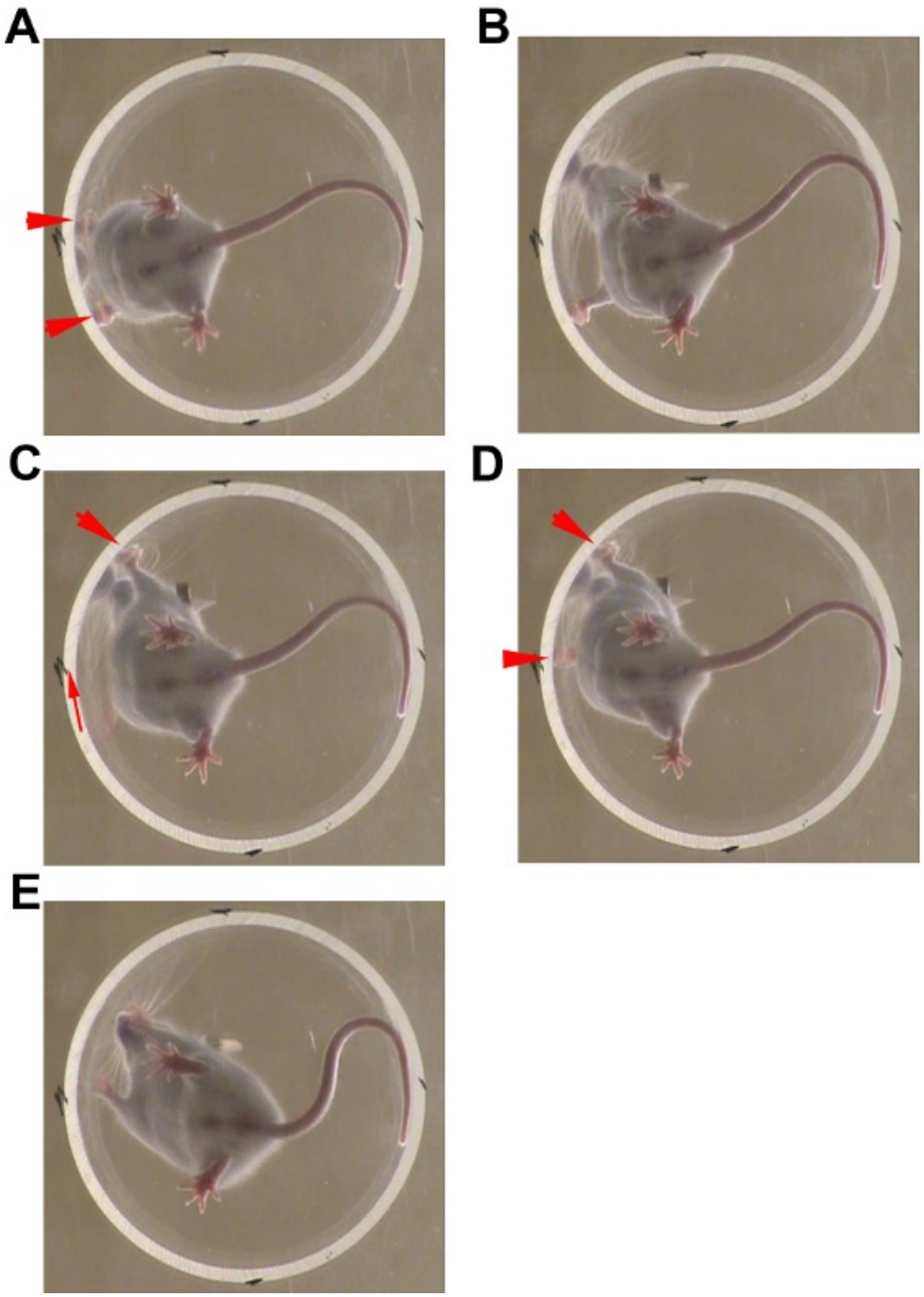

Figure 7. A 'non paw-drag'. Lateral exploratory movement during a rear is not considered a paw-drag. (**A**) The mouse touches the cylinder wall with both paws. (**B**) The mouse twists its torso laterally to explore the cylinder wall. (**C**) The mouse re-positions its leading forepaw to a new position laterally and drags its trailing paw in the same direction. (**D**) The trailing paw is planted firmly in its new location, and both paws are used to dismount (**E**) to return to all four paws. Red arrowheads indicate location of paws at start and end positions. Red arrow indicates movement of trailing forepaw along the cylinder wall.

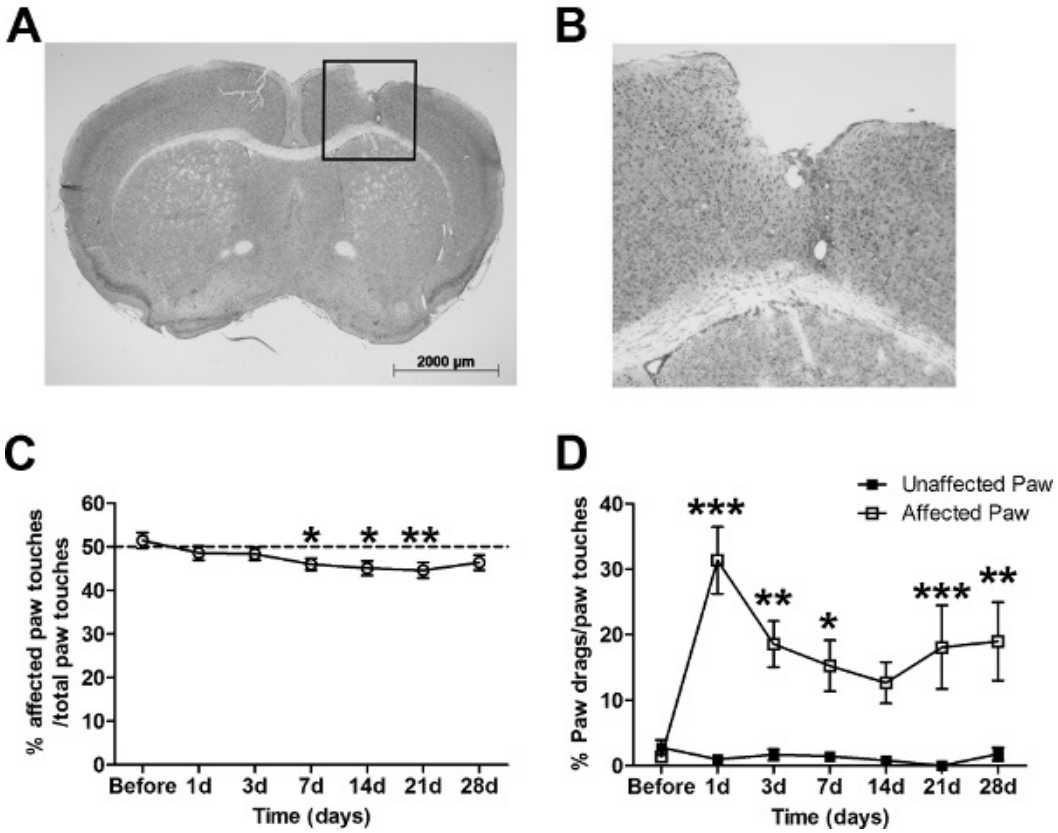

Figure 8. Paw-dragging behaviour is sustained for 4 weeks following a focal cortical, ischemic lesion. (**A**) Representative photomicrograph of a cresyl violet-stained coronal brain section through an ET-1 ischemic lesion at 28 days post-surgery. (**B**) Higher magnification of the ET-1 lesion of boxed area in **A**. (**C**) Analysis of forelimb asymmetry in the cylinder test following an ET-1 ischemic injury to the forelimb sensorimotor shows variable behavioural deficits. Data is expressed as mean ± SEM. Means were analysed by one-way repeated measures ANOVA revealing a significant main effect of time (p=0.015) then followed by Dunnett's post hoc test comparing all means to the means before treatment. (**D**) Analysis of paw-dragging behaviour in the cylinder test reveals a forelimb behavioural deficit is sustained up to four weeks following an ET-1-induced ischemic injury. Means were analysed by two-way repeated measures ANOVA followed by Bonferroni posthoc test. (n=10) **P*<0.05, ***P*<0.01, ****P*<0.001. Please click here to view a larger version of this figure.

Discussion

The key points to establish when quantifying paw-dragging behaviour in the cylinder test are the following: i) quantify the number of paw-drags versus total paw touches for each paw before brain injury to establish a baseline; ii) quantify the number of paw-drags versus total paw touches for each paw following the ischemic injury; and iii) discriminate between a paw-drag and the lateral motion of the paw along the cylinder wall during lateral rotation of the mouse's torso.

Paw-dragging is a novel behaviour that appears following injury to the forelimb sensorimotor cortex. The appearance of paw-dragging behaviour therefore can be used as a positive indicator that the forelimb sensorimotor cortex has been damaged. The representative results show that small ET-1 infarcts approximately 2-4 mm^3 in volume and localized to the forelimb sensorimotor cortex result in paw-dragging behaviour. This is in contrast to forelimb asymmetry analysis which fails to detect consistent deficits in the percent of affected paw touches versus overall touches following ET-1 ischemic cortical injuries[4-6]. Analysis of paw-dragging behavior therefore is more sensitive in detecting damage to the forelimb sensorimotor cortex. Furthermore, because paw-dragging was maintained up to four weeks post-injury it may also be suitable for analyzing recovery of function. As we have previously shown that paw-dragging behaviour correlates with damage to the forelimb sensorimotor cortex[4], any number of injury models may benefit in having this analysis of the cylinder test. Although large injuries, such as middle cerebral artery occlusion and traumatic brain injury[7,8] show deficits on the classical forelimb asymmetry analysis of the cylinder test, these deficits often resolve over time. In these instances, paw-dragging, being a more sensitive measure of damage to the forelimb sensorimotor cortex would be useful in detecting chronic, more subtle deficits. Similarly in injury models which show less consistent results with the classical forelimb asymmetry analysis, paw-dragging analysis would be useful in detecting more consistent behavioural deficits. Paw-dragging analysis of the cylinder test has broad applications for a variety of ischemic injury models including middle cerebral artery occlusion, photothrombosis, pial stripping and ET-1, as demonstrated here.

There are a variety of behavioural tests used to analyze forelimb motor and sensory deficits following injury to the sensorimotor cortex. The Montoya staircase test assesses forelimb reaching and grasping behaviours[9,10]. Similarly single pellet reaching and pasta eating tests analyze the fine motor activity of the paws and digits[11,12]. Forelimb asymmetry analysis of the cylinder test is associated with postural support when the mouse is up on its hind limbs[1]. Only the number of contacts each paw makes with the cylinder wall is quantified. How the paw makes contact is not examined and may be further indicative of damage. Previous studies have quantified the duration of support of each forepaw touch and

found more consistent deficits in mice following photothrombotic stroke[13,14]. Our results show that paw-dragging in the cylinder appears following injury to the forelimb sensorimotor cortex and may be related to a reduced ability to support its weight with the affected paw and/or due to a loss of sensory reception in the paw. The paw is observed to make contact with the wall but does not appear to maintain a supportive stance or assist in pushing off from the wall but rather slips off in what we call a paw-drag. We have observed that paw-dragging behaviour occurs in nearly every animal with an injury to the forelimb sensorimotor cortex and involves a very unique pattern of behaviour, making it quite strong in predicting cortical injury in its own right. In this sense, paw-dragging is a useful tool in a battery of behavioural analyses. It is the combination of a low start-up cost, ease of administration of the test, and the reliability of the paw-dragging analysis that makes paw-dragging analysis of the cylinder test such an attractive choice in predicting focal ischemic injury in the mouse.

Disclosures

The authors have no competing financial interests.

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