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REVIEW ARTICLE

Assessing post-stroke behavior in mouse models of focal ischemia

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Experimental treatment strategies and neuroprotective drugs that showed therapeutic promise in animal models of stroke have failed to produce beneficial effects in human stroke patients. The difficulty in translating preclinical findings to humans represents a major challenge in cerebrovascular research. The reasons behind this *translational road block* might be explained by a number of factors, including poor quality control in various stages of the research process, the validity of experimental stroke models, and differences in drug administration and pharmacokinetics. Another major difference between animal studies and clinical trials is the choice of end point or outcome measures. Here, we discuss the necessity of poststroke behavioral testing to bridge the gap between clinical and experimental end points. We review established sensory-motor tests for outcome determination after focal ischemia based on the published literature as well as our own personal experience. Selected tests are described in more detail and good laboratory practice standards for behavioral testing are discussed. This review is intended for stroke researchers planning to use behavioral testing in mice.

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INTRODUCTION

Stroke represents a major socioeconomic burden in developed countries and is a life-changing catastrophic event for patients and their families. From a therapeutic perspective, neuroprotective drugs have been a major focus for scientists and pharmaceutical companies. There are an extraordinary number of studies that report experimental drugs or interventions, which are beneficial for stroke treatment in rodent models. More than 150 of these were tested in clinical trials with little success. Thus, despite decades of intensive research, specific stroke treatments are still not available, apart from thrombolytic agents. The apparent failure in translating animal research into humans requires a reappraisal of our understanding of stroke pathophysiology, animal models, as well as the standards and criteria used when conducting and reporting preclinical research.[1]

The reasons behind this 'translational road block' might range from low quality in various stages of the research process to problems in the validity of experimental models and differences in drug administration and pharmacokinetics.[2] To evaluate the potential therapeutic efficacy of an agent in experimental *in vivo* stroke models, typically, histologic determination of lesion volume is performed. In fact, many experimental studies rely solely on lesion size determination as evidence for neuroprotection. While certainly an objective way of comparing outcome, this procedure differs substantially from the end points used in the clinical setting. In the latter, assessment of functional outcome using neurologic scores, such as the modified Rankin Score, serves as the primary end point. In addition, based on numerous rodent studies that report lesion size does not necessarily correlate with functional deficits and relevant behavioral outcome, lesion size alone is likely insufficient as evidence for neuroprotection.[3–8] Therefore, functional end points need to be included in all preclinical drug testing, which has long since been suggested by the STAIR consortium.[9]

Rats have been widely used as laboratory animals, mainly because of their easy handling, good performance in complex tasks, and relatively short breeding cycles. Thus, almost all behavioral assays for mice have originally been validated in rats. The development of genetically modified animals resulted in the increased use of mice in experimental stroke research. However, it is not always possible to transfer what we know about rats to mice; they exhibit fundamental differences in behaviour.[10] Still, over the years, more and more behavioral tests have been successfully used in the mouse. Yet despite these advances, behavioral testing still is not a standardized procedure of routine outcome evaluation in experimental stroke research. As a poignant example, the efficacy of NXY-059 for improvement of function was merely assessed by using the Bederson score.[11] The Bederson score was developed to assess successful middle cerebral artery occlusion (MCAo) after 24 hours and is neither intended nor validated for behavioral outcome assessment.[12,13] NXY-059 failed to show any functional improvement in its clinical phase III study.

For reasons such as the smaller body size, and lesser intelligence of mice, behavioral testing in mice can be more challenging than in rats. In addition, native mice display a tremendous ability for spontaneous recovery after ischemic manipulations. Together, this makes long-term behavioral testing of mice a challenging task. But, as mice are increasingly being used in stroke research, behavioral tests have to be further evaluated for reliability and sensitivity in different settings.

The focus of this review will be on behavioral tests used to assess functional outcome after focal cerebral ischemia in the mouse. As deficits of sensory-motor function are most prominent in the clinical evaluation of stroke outcome, we will primarily

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consider behavioral tests of sensory-motor function in the mouse. Undoubtedly, the views expressed will have been shaped by our own experience with the various testing paradigms presented here. Nonetheless, we hope that by introducing the training regimes and experimental protocols that we use and by explaining the rationale behind them, we can promote behavioral testing in murine stroke models and facilitate consistency across different laboratories to improve reproducibility. In general, we will first review the literature concerning each test (ischemia model, mouse strain, time windows, treatment) before describing its performance in more detail. Advantages and pitfalls of the respective test, as we have experienced them, will be recounted and hopefully be of use to investigators contemplating the use of behavioral tasks after ischemic injury.

GENERAL CONSIDERATIONS

When planning an experiment involving behavioral testing after ischemia, various variables affect testing and need to be considered beforehand. Deficits differ in various stroke models because of differences in lesion size and localization, just as tests differ in their selectivity and sensitivity for various deficits (e.g., Wire Hanging tests primarily motor strength, while Corner test is a combined test of postural, sensory, and motor function). Moreover, the mouse strain used influences both behaviour[14] and ischemia susceptibility.[15] A specific behavioral test that shows significant differences between groups, for example, within 3 to 7 days after stroke may not be sensitive at earlier or later time points. Optimal timing of testing is therefore critical for sensitive results and needs to be considered for each test in the context of the above information. Accordingly, there is no 'golden bullet' for behavioral testing. Instead, tests performed should complement each other for sensitivity over a prespecified time window, as well as for the detection of distinct deficits (i.e., motor coordination versus sensory dysfunction in cortical damage).

Other factors such as aged animals,[16] and comorbidities like hypotension/hypertension or diabetes[17] may influence behavioral test performance. Studies examining the effects of focal ischemia in aged mice show inconsistent effects.[18,19] Manwani *et al*[20] compared young and aged C57 mice in a battery of behavioral tests and concluded that recovery is delayed but complete in aged mice after 60-minute MCAo. Although the general approach to behavioral testing will not change in those mice (Manwani *et al* recommend a battery of Pole test, Corner test, Hanging Wire, and Open Field), it should be considered that age-related decline in cognition, learning, explorative behavior, and sensory-motor performance may occur.[16,21] In general, more extensive baseline characterization may be required to rule out fundamental differences in test performance, especially if knock-out animals are used.[22]

ROTAROD

The Rotarod is one of the most frequently used tests of motor function after ischemia in the mouse. It has been used in a variety of stroke models and mouse strains species at various time points. Short-term sensitivity for detection of intervention effects during the first 4 days has been shown in various models of proximal MCAo, ranging from 30 minutes to permanent in duration, in 129 Sv, swiss, ddY, C57Bl/6 as well as transgenic mouse strains.[6,23–26]

Long-term sensitivity at 28 days was reported after embolic MCAo in naïve and treated C57Bl/6 mice, when tested singularly on day 28 and performance compared against preoperative values.[27] Similar long-term sensitivity was also reported in C57Bl/6 mice subjected to photochemically induced cortical ischemia.[28] However, in this study a control group was not included and the results were compared against preoperative values obtained after 3 days of training, whereas subsequent testing intervals were large and testing singular. Moreover, in a study of swiss mice subjected to distal MCAo, the Rotarod was unable to detect significant differences between MCAo and sham even within the first 5 days.[5]The Rotarod also failed to detect treatment effects after distal MCAo in another study.[29]

The Rotarod was first described[30] for rats and later adapted to mice[31] for evaluation of motor function and ataxia. The set-up consists of a rotating cylinder (3.0 to 3.2 cm in diameter) and an attachment with several compartments that are separated by thin walls to test multiple mice simultaneously. Mice placed on the rotating rod will walk and try to remain on the rod to avoid falling onto a platform below. Hence, the 'latency to fall' is typically used as a quantitative end point to evaluate motor function. The Rotarod is either used at a constant speed or with gradual acceleration, accelerating paradigms are widely preferred in stroke research.

Motor learning is an important factor in the Rotarod testing. Animal performance on the accelerating Rotarod gradually improves over time and eventually reaches a stable plateau over several days.[32] This improvement is not permanent, if mice are left untrained for several days their scores will decline. Therefore, preoperative testing and baseline determination is especially crucial to differentiate between motor learning and actual recovery after stroke. This is most evident with milder models of stroke. Training is also important to exclude the effect of motivational factors on the test score—animals that fall off very early (e.g., for healthy C57Bl/6N mice before 40 seconds) are likely to lack motivation. This is best recognized and remedied before actual testing takes place by applying mildly aversive stimuli (e.g., a pinch in the tail or through the use of a foot shock) if animals repeatedly fall off early or only by selecting only those animals that show a certain minimum proficiency in the test.[26]

In summary, the Rotarod is a relatively simple and well-evaluated test for short-term evaluation of deficits after proximal MCAo in a variety of mouse strains and interventions. Long-term sensitivity is uncertain but may depend on the testing routine. It is unlikely to detect deficits because of cortical damage after distal MCAo.[5]

POLE TEST

In stroke research, the Pole test has been used to detect differences between MCAo- and sham-operated mice for up to 3 weeks after 30 to 60 minutes of proximal MCAo in C57Bl/6 and 129/SV mice.[33,34] In swiss mice subjected to 60 minutes of proximal MCAo, sensitivity has been shown for at least 8 days after ischemia.[4] In a 60-minute transient MCAo model, the acute neuroprotective effects of rosuvastatin were shown via the Pole test.[35] It appears likely that striatal involvement is important for Pole test performance (see below), and reduction of up to 50% in dopamine levels has been reported after proximal occlusion.[36] Consequently, the Pole test has not been found useful in two studies of distal MCAo (producing cortical damage and sparing the striatum) in swiss and C57BL/6 mice.[5,29]

The original Pole test was developed by Ogawa to evaluate bradykinesia in Parkinson models and was later shown to predict the extent of lesion in 6-hydroxydopamine-lesioned mice.[37] The Pole test evaluates simple motor function and requires little equipment. Animals are placed on top of a 50- to 55-cm vertical pole with a diameter of 8 to 10 mm and trained to turn around and descend the pole (snout first). Scoring starts when the animal initiates the turning movement. The time to make a complete 180° turn (i.e., T_{turn}) and latency to reach the ground (i.e., T_{total}) are recorded. If the animal cannot turn but instead descends with a lateral body position, then T_{total} is usually attributed to T_{turn}. When an animal makes a turn, descends halfway and falls, the recorded time stops when the animal reaches the floor. However, if an animal falls immediately, a maximum duration (e.g., 20 seconds or

the longest T_{total} of that group) for the experiment is assigned to T_{turn} and T_{total}. During a successful run, the animal must not pause, if the animal stops, then the trial is excluded and repeated. To avoid sliding, the surface of the pole should be rough. That can be achieved with adhesive tape, if needed. Some animals may climb over the tip of the pole instead of making a turn. In that case, a small piece (i.e., 7×7 cm) of cardboard can be placed at the top of the pole to prevent climbing.

WIRE HANGING

In 129/SV mice, the Wire Hanging test successfully distinguishes between sham and MCAo animals and detects neuroprotective effects of different treatments at early and late time points (up to 3 weeks) in 30- and 60-minute transient MCAo.[33,35,38] Studies using C57Bl/6 mice failed to detect any differences between stroke and sham groups at days 13 to 19, even when a more severe MCAo paradigm was used such as 45, 60, and 90 minutes occlusions.[3,34] However, a study by Abe *et al*[39] however reported a significant difference between treatment and vehicle groups till day 8 after 25 minutes of MCAo, and studies by De Silva and Broughton used the Wire Hanging test successfully to detect group differences 24 hours after 30-minute MCAo.[40,41] The C57Bl/6 strain may be less suited for Wire Hanging because of training problems (in our experience, they struggle more and are more likely to escape/let go of the wire) and low endurance making it difficult to evaluate gross deficits at early time points. We personally have found it difficult to distinguish true motor impairment from motivational factors in C57 mice.

Wire Hanging is a simple test that evaluates grip strength, balance, and endurance. Mice are trained to suspend their body by holding on to a single wire stretched between two posts 50 to 60 cm above the ground. To prevent the animal from using all four paws, the hind limbs are gently covered with adhesive tape. Between the two posts is a pillow to avoid injury when the mice fall. 'Latency to fall' is the primary end point used to assess motor performance. Focal ischemia causes sensory-motor impairment in the contralateral paw and significantly reduces the hanging ability of animals.

Compared with the 'inverted grid hanging test' (often also referred to as the 'Wire Hanging test' where the animals hold on to a cage lid with four paws) mice are in a more stressful and challenging position in the wire hanging test. This makes the animals more likely to let go and try to escape, which may be further exacerbated in the acute phases of surgery. Consequently, pretraining to increase motivation to hold on as long as possible is necessary. During training, mice are forced to grasp the wire with their fore paws and slowly released by the experimenter until they must support their own body weight. If animals are moving along the wire and reaching the posts, then small pieces of card boards may be placed on the wire to prevent escape. The experimenter should let the animal hang for short intervals (such as 10 to 20 seconds) for several trials and then return the animal to its cage. If the animal falls before the end of training, then it should be immediately returned and forced to grasp the wire. Animals should only be brought back to their respective home cage when the trial is finished. After initial habituation sessions, animals are trained to hang for longer durations.

There is no fixed number of training sessions since each animal performs differently. As a rule of thumb, a well-trained animal should (1) not hold on to the wire with four paws, (2) not move back and forth on the wire, and (3) readjust holding posture repeatedly until exhaustion. In our experience, 129/SV mice can be trained easily after only a couple of sessions and well-trained naïve animals can hold on for >300 seconds.

In summary, wire hanging appears to be a suitable test for evaluating poststroke deficits at early and late time points especially in the 129/SV strain.

CORNER TEST

The Corner test is increasingly being used in murine stroke studies. In the initial paper by Zhang *et al*,[42] the Corner test was successful in detecting functional deficits as late as 90 days after embolic MCAo. Other studies of swiss and C57Bl/6 mice after 60 and 90 minutes of MCAo confirmed the observation at earlier time points.[4,13] The Corner test has shown functional improvements as a result of therapeutic treatment for up to 2 weeks after proximal MCAo of various intensity (from 25 minutes transient to permanent occlusion) in C57Bl/6 and CD1 mice.[39,43,44] In swiss mice, the Corner test was sensitive for at least 8 days after 60 minutes of proximal MCAo.[4] Use of the Corner test has not been described for 129/SV mice and indeed, as this strain shows low exploratory motivation, it has been deemed unsuitable for Corner test performance.[23] After cortical damage, the Corner test did not detect differences between sham and stroke in both swiss and C57Bl/6 mice.[5,45,46] Thus, striatal damage again appears to be central for impaired Corner test performance.

Indeed, the Corner test was first developed by Schallert *et al*[47,48] for testing sensory-motor deficits and asymmetries in rats with unilateral nigrostriatal damage and later adapted to mice.[42] Multiple sensory and motor asymmetries (i.e., vibrissae, limb use, and postural biases) because of striatal and cortical damage might affect outcome in the Corner test.[42] The testing apparatus consists of two connected cardboard walls ($30 \times 20 \times 1$ cm) forming a 30° angle. At the junction of the walls, a small opening is left to motivate the mice to enter deep into the corner. To start the trial, animals are placed halfway into the set-up facing the corner. Mice will then typically walk into the corner at which time their vibrissae will be stimulated. In response, animals will rear and turn to either side (left or right).[4,13,42] Turning behavior is recorded, any turn that is not a part of the rearing movement is discarded. In naive mice no side preference is observed, but mice subjected to proximal MCAo of varying duration have been found to turn more often toward the nonimpaired (ipsilateral) side.[42,43] Interestingly, after distal MCAo, i.e., cortical damage, animals preferentially turn contralateral.[45] Typically, the session ends after 10 to 20 successful turns left or right.

The Corner test requires no pretraining, however, preoperative testing is advised to compare prestroke versus poststroke values, as well as to identify a baseline. A major pitfall of the Corner test is that animals can be too sick and unmotivated to perform at early time points. They also rapidly lose motivation to perform when tested continuously. As the animals are introduced to the apparatus repeatedly, it is more difficult to observe a 'proper' spontaneous turn since the animals lose their exploratory interest and become overly anxious by continuous handling. To avoid that, both intertrial intervals and breaks between successive test sessions should be as long as possible. There are only a few papers that present data obtained from daily Corner test experiments for up to 2 weeks. However, in our hands this was only achievable by forcing the mouse into the corner by pushing or pulling the tail. The effect of handling on the outcome of the Corner test has not been not examined. If limiting stress is a major concern, and acquiring data from only a few time points is sufficient, then a closed box set-up may be helpful.[43] Here, the animal is allowed to explore the set-up freely under red light and rears and turns are observed and scored via a video camera.

In conclusion, despite some experimental difficulties, the Corner test is one of the very few tests that is sensitive in detecting sensory-motor asymmetry at early and late time points after stroke, especially in models where the striatum is severely damaged.

ADHESIVE REMOVAL TEST

The Adhesive Removal test has been successfully used to distinguish swiss mice subjected to 60 minutes of MCAo from

sham-operated mice for up to 6 weeks.[4,49] In a model of 30-minute MCAo using C57Bl/6 mice, we could confirm sensitivity for detection of MCAo deficits over 4 weeks (Balkaya *et al*,[50]). The test was also sensitive in models of cortical damage induced by distal MCAo.[5] The adhesive tape has also shown sensitivity for differences between knockout strains ($-/-$ versus $+/-$) and treatment effects in swiss mice in a short interval of 3 days after 60-minute MCAo.[51,52] However, sensitivity was more modest in a model of chemically induced cortical lesions after 20 days in C57Bl/6 mice.[46]

The Adhesive Removal test was established in rats[47,48] and has been widely used in rats to determine long-term effects of brain injury on sensory-motor behavior before being adapted to mice. It evaluates sensory-motor impairments after unilateral lesions involving the sensory-motor cortex, the corticospinal tract, and the striatum. During the test, a small adhesive patch (rectangular 0.3×0.4 cm or circular 0.3 cm diameter) is applied to each forepaw.[4] The order of placement (right or left) alternates between each animal and session. Directly after the placement of both patches the experimenter presses both forelimbs simultaneously to minimize bias. The mouse is then placed in its home cage or, if not practical, a transparent Perspex box for 120 seconds, and the time to contact and remove each adhesive tape is recorded. Contact occurs when the paw is shaken or the mouth is used to touch the patch. Mice with a unilateral lesion typically contact and try to remove the adhesive patch attached to the ipsilateral paw first. The mouse senses the patch on the contralateral paw later, and it generally takes longer to remove it because of impairments in forelimb movement and coordination. Thus, sensory bias in animals after MCAo manifests itself in the order of contact and removal. The latency to contact the patch on the impaired paw is used to assess sensory impairment. However, the latency to remove is influenced by both sensory and motor systems.

To achieve optimum performance, animals should be trained for 4 to 5 days before surgery. A protocol description by Bouet *et al*[4] recommends one trial per training day for swiss mice. To achieve 'plateau' and ensure reliable results when using C57Bl/6 and 129SV mice, we may however increase training to 2 to 3 trials per day. When home cage testing is not feasible, animals should be habituated to the observation box for a period of ~ 60 seconds before the adhesive tape placement.

Ideally, two experimenters take part; one can hold and stabilize the animal while the other applies the patches. Two people can score the contact and removal times more precisely by observing the animals from different locations. Animals can turn and it might be difficult to spot which hand has contacted a patch or when the patch is removed, especially if animals are fast. Testing can begin as early as 24 hours after the operation (depending on the severity of the model) and may easily be repeated daily without any major difficulty.

Testing is usually performed for one trial per day. However, when it is critical to minimize random effects, we prefer to do three trials and average the results of the best two for the final score.

In summary, the Adhesive Removal test is a valuable test that excels in its detection of sensory deficits in both the short-term and long-term after both proximal and distal MCAo, but requires experience and time.

OPEN FIELD TEST

The Open Field test is widely used to evaluate motor function and normal exploratory locomotion in rodents.[53,54] During the acute phases of focal ischemia, mice develop a hypoactive phenotype.[36,55] The exact duration of this hypoactivity period varies and probably depends on several factors including the ischemia model, severity of the procedure and mouse strain. Interestingly, first described in rats but also shown in mice, a number of papers report hyperactivity starting several days after operation that may last up to months.[36,56] An interesting feature of this hyperactive phase is that the animals may have apparent motor deficits and still show high locomotor behavior with an apparent rotation preference in the Open Field. Consequently, this initial hypoactivity and subsequent hyperactivity can potentially be used to evaluate the effects of therapeutic interventions. In our laboratory, we have observed that acute hyperactivity can be attenuated by a number of therapeutic drugs that confer neuroprotection and reduce lesion volume (unpublished data). Kilic *et al*[56] has also shown the attenuating effects of melatonin on the hyperactivity observed in Open Field.

The Open Field is a simple method to assess the locomotor activity of mice, but patterns of exploration are influenced by the emotional state of the animals.[57] The Open Field is an unobstructed square or circular test arena composed of wood or plastic. The floor of the Open Field, in its simplest form, is divided into equally spaced regions and surrounded by relatively high walls to keep the mice in the arena. The Open Field should have an adequate size (e.g., at least 50 by 50 cm wide or 50 cm in diameter).

Often, an automated Open Field apparatus is used and the locomotor activity is assessed by photocell beams or cameras and video tracking software. The use of automated systems minimizes disturbing effects that can be induced by a human observer. These systems may detect several facets of locomotor behavior. Open Fields with photocell arrays adjusted to different heights, e.g., direct above the ground and several centimetres higher, can detect horizontal movement and vertical exploration (rearing), which occur when the animals raise their body and the front paws leave the ground.

Video tracking systems allow the determination of the same parameters, combined with the possibility of subsequent reanalysis since the data are stored.

When performing the Open Field test, it has to be considered that several external and animal-derived factors influence the behavior of the mice in the arena. In general, an open field is an unfamiliar and mildly aversive environment for the mice, and increased anxiety reduces exploratory behaviour.[53] Low lighting conditions are generally associated with reduced anxiety and increased exploratory behavior, while bright light is associated with increased aversion and reduces exploration. Repeated exposure to the Open Field apparatus leads to habituation of the animals and changes the movement patterns of untreated mice. Habituation decreases the movement of the animals during the observation period. This decrease in exploration could mask a recovery of motor function after an ischemic insult. Additionally, automated Open Fields are often smaller, which leads to increased habituation to the novelty of the Open Field.

GAIT ANALYSIS

Improving gait and gait-related activities is a major clinical goal after stroke and analyzing gait in stroke survivors is a crucial part of poststroke therapy.[58] Gait analysis has provided a more specific understanding of locomotion and is now a cross-species tool that is sensitive to changes associated with disease, injury, or rehabilitation. It is used as a progress measurement in humans undergoing rehabilitation after stroke and it represents the face validity (phenomenological similarities) of the animal model.[59,60]

In the most basic version of gait analysis, ink is applied to the paws and the animal has to walk over a length of paper.[61,62] The resulting footprints allow the determination of static gait parameters (e.g., stride length and hindpaw drag). This approach is fairly simple and reasonably sensitive, but has practical limitations such as difficulties in getting good paw prints and stressful testing procedure. This primary method has been refined using videos of the moving animals and subsequent slow motion gait analysis.

Computer aided systems, like the 'Catwalk' or 'DigiGait', are video-based systems designed to detect locomotor deficits or movement alterations in mice or rats, while the animal walks on a transparent surface. It is generally thought that the tunnel-like walkway eases the assessment of gait in freely moving animals, and elicits an unforced and natural walking pattern. Analysis allows the discrimination of each paw, and ideally, an estimation of the amount of weight that each of the individual paws bears. This allows the detection of even slight impairments in gait. The main difference of the above mentioned systems is that walking speed is fixed in the 'DigiGait' system, while in the 'Catwalk' animals walk at leisure. Automated gait analysis has been used frequently in models of musculo-skeletal disorders, spinal cord injury, and traumatic brain injury in rodents. A possible drawback of the automated systems is the rather large amount of data that is generated, all of which requires selection and thorough evaluation. In some cases, the low body weight of young mice and rapid weight loss after MCAo[63,64] can cause detection problems and artefacts, which also requires careful analysis. In systems where the animal is freely moving, speed variations between trials may increase standard deviation of several parameters and have a considerable impact on stance and break durations. Setting clear and stringent exclusion criteria for speed is therefore strongly advised.

Despite their potential, computer-assisted gait analysis systems have not been extensively used in stroke research because they are relatively new and costly. To our knowledge, there are only two papers using this technology in mice with focal ischemia.[45,65] The paper by Lubjuhn *et al* compared the traditional paw-inking method to Catwalk and DigiGait systems in a model of distal MCAo and found differences in stance and break durations in both systems at days 1 and 2 post stroke. In the study by Hetze *et al*, animals were tested on the Catwalk before and 10 days after 60-minute proximal MCAo without controls with significant differences in a number of parameters. We recently performed a more exhaustive characterization of Catwalk performance after 30-minute transient MCAo in C57Bl/6 mice and found sustained impairments for up to 28 days in a number of parameters such as *Stand duration, SwingSpeed, StrideLength, StepCycle*, and *DutyCycle*.[50]

CYLINDER TEST

The Cylinder test has frequently been used in the rat, but reports in mouse models of focal ischemia are less common. Li *et al*[13] reported a significant difference in asymmetry scores of C57Bl/6 mice subjected to 90 minutes of transient MCAo when compared with controls up to 15 days post stroke. However, the Cylinder test failed to distinguish between sham and ischemic animals in models of cortical infarction using distal MCAo or chemical induction.[5,46] After 30 minutes of MCAo in C57Bl/6 mice, we could not obtain reliable results using the Cylinder test.[50] All in all, this suggests that the Cylinder test, despite being a very sensitive test in rats, should only be used in mouse models of focal ischemia when the damage to the brain is relatively severe.

Unilateral damage to the sensory-motor cortex, corticospinal tract, and striatum may cause chronic forelimb use asymmetry in rats and mice which is somewhat comparable to disuse that can develop in some stroke patients.[66] The Cylinder test, also called the spontaneous forelimb use asymmetry test, is a relatively easy but time-consuming test that can be used to evaluate poststroke limb use asymmetries in mice. In this test, an animal is placed inside a glass cylinder with a diameter of 9 to 10 cm and a height of 15 cm. The forelimb use during vertical exploration of the cylinder is evaluated by recording the forelimb contacts. A mirror is placed behind or under the cylinder to ensure both forelimbs can be seen at all times by the observer or the camera. The criteria used in scoring the forelimb placements was described by Li *et al*[13]: (1) The first limb to touch the cylinder during a full rear is scored as an independent placement for that limb. (2) If the second forelimb makes contact while the first is still in place, then the first paw is scored as another independent placement, while the second paw is scored as both placements. (3) If both paws are placed simultaneously on the wall during a full rear, or if the animal uses both paws during lateral movements, then each placement is scored as both movements. (4) If the animal explores the wall by alternating between forelimbs it is scored as both. A total of 20 vertical movements are recorded and subsequently analyzed by an observer in slow motion. The asymmetry score that was initially described by Schallert *et al* in rats is obtained as follows (nonimpaired forelimb contact − impaired forelimb contact/nonimpaired forelimb contact + impaired forelimb contact + both contacts).

HANDEDNESS TEST

Impairment of the contralateral paw is a common finding in stroke models where motor cortex is damaged. Hence, a handedness test that examines paw preference of animals in a food grabbing task[67] may prove useful to detect deficits of forepaw fine-motor coordination in focal ischemia models. Although clinically highly relevant (i.e., hand impairment), there are few such tests for the mouse (the Staircase test involves forepaw coordination yet involves several other modalities as well). Lubjuhn *et al*[45] were the first to show the utility of the Collins' handedness test in C57Bl/6 mice after distal MCAo to detect changes in paw preference between sham and stroke animals for up to 8 days. After 30 minutes of proximal MCAo, we found that handedness or 'paw preference' changed significantly in MCAo animals as compared with sham over 4 weeks.[50] For this test, animals are placed in clear plexi-glass cells containing a small opening in front, from which crunched food pellets may be retrieved using the forepaws. It has been suggested that sucrose may be added to the crushed food to motivate animals to reach forward. We have, however, found that animals have sufficient motivation for performance of the test after being food restricted for one night. Animals should be habituated to the testing apparatus and gradually trained to reach for food before operation on 3 to 4 training days. Access to food should be immediately restored after completion of testing. After ischemia, mice are videotaped for retrospective analysis whether reaching occurred with the left, right, or both paws. Usually, 50 reaches are evaluated for characterization of handedness, in the setting of stroke however, less may be sufficient.

GRIP STRENGTH

This test has only rarely been used in mice after stroke. Kilic *et al*[56] found that the test was sensitive for treatment differences in C57Bl/6 mice after 30-minute MCAo at days 7 and 30. Ferrara *et al*[23] however reported that they could not detect any significant differences between stroke and sham animals in the first week after 60 minutes of MCAo in 129S2/SvPas mice.

During testing, mice are trained to hold a grid or wire that is attached to a force gauge. They are then gently pulled back from the base of the tail and the maximum force that is exerted before they let go is measured. Because of a weakness that may be present after stroke in the contralateral paw, grip strength may distinguish between sham and stroke animals. A pitfall of the Grip Strength test is the fact that the values obtained are highly influenced by the motivation of the animal as well as speed that the experimenter pulls.

STAIRCASE TEST

The Staircase test is a skilled reaching task, which is sensitive at detecting deficits up to 26 and 28 days after 60 minutes of MCAo or photothrombosis (inducing cortical lesions) in swiss and

C57Bl/6 mice compared with sham.[4,28] It was however not successful in detecting deficits after distal MCAo in swiss mice.[5] This test consists of a chamber that contains an elevated central platform with two staircases placed on either side.[68] The staircases have six steps and can each be baited with foot pellets. Animals are food restricted to motivate them to climb the platform and collect the pellets.[4,69] Animals are trained preoperatively for 3 weeks. Although an interesting test of sensory function, motor dexterity, and spatial attention, it has, to our knowledge, found little use in stroke research (with the above exceptions), possibly because of the complex training and the required food restriction schedule.

FOOT FAULT TEST

The Foot Fault test was successfully used to asses differences between sham and stroke animals, and the effects of a therapeutic substance up to 7 days after 60-minute transient MCAo, and for up to 14 days after permanent MCAo in C57Bl/6 mice.[44,70] It however failed to show differences between knockout and wild-type mice over 2 weeks after 60-minute MCAo in another study.[71] It is however a simple test and requires minimal equipment. Animals are placed on an elevated grid (30L $\times$ 30W $\times$ 31H cm) with squares sized $\sim$2.5 cm^2, and the number foot faults (failure to step on the wire grid) made by ipsilateral and contralateral limbs are counted during locomotion.

LADDER RUNG TEST

The Ladder Rung test was used to assess deficits in C57Bl/6 mice up to 26 days post surgical or chemical induction of sensory-motor cortex lesions.[46,72] But it has, to our knowledge, not yet been applied in models of proximal MCAo.

This test relies on measuring foot faults during locomotion on a horizontal ladder, thus resembling the Foot Fault test. It was first developed for use in rats[73] and later adapted for use in mice.[72] The apparatus consists of an elevated ladder (70 to 80 cm long and raised 12 to 15 cm off the ground). The rungs are 1 mm in diameter and spaced irregularly or regularly 5 mm apart. Animals walk across the ladder to reach their home cages and foot slips during locomotion are scored in slow motion from video recordings. A few days of training are required. Video analysis and scoring of foot faults however appears to be rather labor intensive (as by the original description, scoring each step on the ladder on a scale from 1 to 7) and the test has not yet found more wide-spread use.

GOOD LABORATORY PRACTICE AND BEHAVIORAL TESTING

The lack of translation that has plagued preclinical stroke research over the course of the last few decades has initiated much debate as to how we can improve. Attempts have been made to set quality control standards for preclinical and clinical stroke research, such as STAIR and CAMARADES.[2] Several key concepts and recommendations for animal studies (STAIR I[9]) stand out as necessary among all these checklists and proposals. These criteria include (1) reporting the precise species, strain, substrain, and the source of animals used, (2) proper sample sizes and sample size calculations, (3) inclusion and exclusion criteria, (4) randomization, (5) allocation concealment, (6) reporting of excluded animals, (7) blinded assessment of outcome, and (8) reporting of potential conflict of interest and study funding. These are all discussed in detail elsewhere.[74]

Behavioral tests, by their very nature, are prone to influence. As such, proper study design and execution are paramount to obtain good, reliable results and quality control standards are even more crucial. Therefore, we discuss the STAIR criteria in relation to behavioral testing. (1) We have mentioned numerous times that different mouse strains behave differently during testing, which in turn impacts the selection of appropriate tests and the time window that a test may yield meaningful results. Even the substrain and animal supplier/vendor used may have significant effects on the baseline behaviors of the animals.[14,75] Therefore, it is imperative to be aware of, and report these characteristics. (2) A major difficulty in poststroke functional testing is establishing tests that are sensitive enough to detect a therapeutic drug effect, especially at relatively late time points. Furthermore, interindividual differences between animals can be great, so much so, that it may be necessary to subgroup animals into good and poor performers before any intervention is made. Making appropriate sample size decisions is a crucial step in experimental planning and wherever possible a priori sample size calculations should be used. (3 and 6) Excluding animals from experimental groups is undesirable, and may become a major source of bias if it is performed without predefined, clear criteria. It is relatively common practice in behavioral testing to exclude animals that do not execute the test 'properly'. An animal may simply be too sick to perform the test, or it may never learn to comply with the test requirements. An animal can actively conflict with the test requirements or develop aberrant behavior at some point during testing (i.e., stopping in the middle of the pole in the Pole test, 'fake' drops in the Rotarod). It should be clearly stated when, why, and how many animals are excluded. (4) Proper randomization is extremely important for experiments that include behavioral testing. Despite best efforts to minimize the impact of the emotional and motivational states of the animals (i.e., intensive habituation and pretraining), these factors still affect sensory-motor testing. For example, an anxious animal may be more hesitant to descend the pole during the Pole test, thus increasing T_{down}. Training a highly anxious and agitated animal on the Rotarod is much more challenging when compared with calmer animals, this can affect performance. Proper randomization ensures that anxious animals will not be grouped together. Furthermore, simply assigning certain cages to specific groups should also be avoided because of in-cage dynamics. For example, fighting and particularly aggressive alpha males will strongly affect the anxiety and motivational status of the cage mates. Picking animals at random can also introduce bias because it is very likely that the first animal to be picked will be either less anxious or dominant. (5 and 7) Many sensory-motor tests rely on the experimenter for scoring. Knowing which animal belongs to which group is likely to introduce bias in two ways. Experimenter decisions during scoring may unconsciously be in favor of the desired effect, or, the experimenter may try to overcompensate for potential bias and end up introducing it against the desired effect.

CONCLUSION

Functional testing, especially at late time points, is crucial for improved assessment of therapeutic interventions in animal stroke models. Behavioral testing not only provides an alternate evaluation of therapeutic interventions, but will also serve to bridge the gap between the evaluation criteria used in animal studies and clinical trials. However, only a few studies reported treatment difference in functional outcome after $>$2 to 3 weeks, which is however to time point most relevant for clinical research. Another major challenge in the field is the fact that most of the sensory-motor tests that are used for mice are actually adaptations of tests developed for rats and the number of reports that use poststroke behavioral testing in mice with focal ischemia is relatively limited. Should the reader be persuaded to use behavioral testing in the mouse, it is critical to select adequate tests and appropriate time windows for the respective species and strain as well as type and severity of the stroke model (see Table 1). Based on our personal experience, we also present a guide and a list of protocols for the tests, models, and strains that we are most familiar with (Supplementary information). Overall,

Table 1. Summary of tests literature: the stroke models (proximal MCAo (occlusion time/minute)/distal MCAo) and strains in which they have been used, whether and at what time (Time Window) they have been successfully to distinguish sham versus stroke, and whether and at what time (Timing) effects of treatment were found in the test

Test	*Stroke model*	*Strains*	*Sham versus stroke*	*Time window*	*Treatment effect*	*Timing*
Rotarod	Prox. MCAo (30-perm.)	129Sv, C57, swiss, ddY, transgenic	Yes	~7 days[a]	Yes	~4 days
	Distal MCAo	C57, swiss	No	—	—	—
Pole test	Prox.MCAo (30–60)	C57, 129Sv, swiss	Yes	Up to 20 days[a]	Yes	Day 5
	Distal MCAo	C57, swiss	No	—	—	—
Wire Hanging	Prox. MCAo (30–60)	129Sv	Yes	3 weeks	Yes	5, 20 days
	Prox. MCAo (45–90)	C57	No	—	No[b]	—
Corner test	Prox. MCAo (25-perm.)	C57, swiss, CD1	Yes	4 weeks up to 90 days[a]	Yes	2 weeks
	Distal MCAo	C57, swiss	No	—	—	—
Adhesive Removal test	Prox. MCAo (30–60)	Swiss, C57	Yes	4–6 weeks	Yes	3 days
	Distal MCAo	Swiss	Yes	20 days	?	?
Catwalk/DigiGait	Proximal MCAo (30–60)	C57	Yes	24 days	?	?
	Distal MCAo	C57	Yes	1–2 days	?	?
Cylinder test	Proximal MCAo (90)	C57	Yes	15 days	?	?
	Distal MCAo, Proximal MCAo (30)	C57, swiss	No	—	—	—
Handedness test	Proximal MCAo (30)	C57	Yes	4 weeks	?	?
	Distal MCAo	C57	Yes	8 days	?	?
Grip strength	Proximal MCAo (30)	C57	Yes	Day 7 + 30	?	?
	Proximal MCAo (60)	129S2/SvPas	No	—	—	—
Staircase test	Proximal MCAo (60)	Swiss	Yes	26 days	?	?
	Photothrombosis (cortical damage)	C57	Yes	28 days	?	?
	Distal MCAo	Swiss	No	—	—	—
Foot Fault test	Proximal MCAo (60-perm.)	C57	Yes	7–14 days	Yes[b]	7–14 days
Ladder Rung test	Cortical lesion models	C57	Yes	26 days	?	?

For detailed information, please refer to the text. [a]Time window may be shorter for swiss mice. [b]One conflicting report.

further research is necessary to characterize many of these tests in different stroke models, and the development of more sensitive tests is imperative for the future translational success of preclinical stroke research.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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