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Functional testing in a mouse stroke model induced by occlusion of the distal middle cerebral artery

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ABSTRACT

Reducing post-stroke disability is the major goal of stroke therapy. Consequently, functional testing is essential in experimental stroke studies to increase the predictive value of animal models. We used several sensory and motor tests to assess functional disability in a mouse model of permanent distal middle cerebral artery occlusion (pdMCAO) that induced mainly cortical infarcts. Gait dynamics were transiently disturbed after pdMCAO as measured by different analysis techniques. Stance and brake duration were shorter after pdMCAO. Consistent with sensory and motor deficits the latency to move was prolonged up to 14 days after pdMCAO and the performance in the corner test and handedness were affected on day 1 or 2 after pdMCAO. Heart rate was decreased and heart rate variability were increased after pdMCAO indicating sympathetic–parasympathetic imbalance. In summary, pdMCAO-induced cortical infarcts lead to clinically relevant sensory, motor and cardiac autonomic dysfunction in mice. The present study provides a basis to explore the potential of functional testing for neuroprotection and neuroregeneration after stroke.

1. Introduction

In stroke, disability is the main target of clinical therapy. In the past, many studies in experimental animals analyzed almost exclusively infarct volumes as a surrogate parameter of ischemic brain damage but neglected functional parameters. Indeed, infarct size is easy to measure but does not reliably reflect functional impairment. This is due to the fact that neurological function may be affected in noninfarcted tissue, or function may recover in spite of the infarction due to brain plasticity (Hurtado et al., 2006). Most experimental studies focus on morphological parameters; this is probably an important reason why preclinical stroke studies do not reliably predict clinical efficacy, which is assessed by functional outcome (Fisher et al., 2009; Sena et al., 2007). Therefore, future experimental stroke research should incorporate functional aspects of the disease.

Nonetheless, functional testing is a challenging task, especially in mice, the preferred species for investigating molecular mechanisms of stroke because of the transgenic tools that are available. Proximal occlusion of the middle cerebral artery (MCAO) in mice for a prolonged time causes large infarcts and a severe deficit that can be readily assessed by the Bederson score (Bederson et al., 1986). Mortality after proximal MCAO, however, is usually high, constraining long-term studies (Groger et al., 2005). A solution to this problem is to reduce the occlusion time to less than 1 h (Winter et al., 2004) resulting in striatal lesions, or to occlude the MCA distally, which induces mainly cortical lesions. Mortality is low with both modifications. Functional impairment in these two models, however, differs due to the different lesion sites. Many studies have evaluated the functional consequences of proximal MCA occlusion, but only few studies have investigated the functional consequences of distal MCA occlusion in mice (Baumann et al., 2006; Freret et al., 2009; Guegan et al., 2006; van Lookeren Campagne et al., 1999). In the present study, therefore, we evaluated functional impairment after permanent distal MCAO (pdMCAO) using different tests to investigate locomotion, especially gait disorders. Gait is often severely impaired in patients who have suffered a stroke in the territory of the MCA. However, only a single study of gait in a rat

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model of stroke has been conducted so far (Wang et al., 2008). Here, we employed three different methods to analyze gait in mice after pdMCAO. The conventional ink technique measures static gait parameters. In addition, we used two video-based systems to determine static and kinetic gait parameters, one in which mice walk at a preset speed and one in which they walk at their spontaneous speed. The autonomic nervous system is also affected by stroke (Korpelainen et al., 1996; Tokgozoglu et al., 1999). We therefore recorded electrocardiograms in the mice after pdMCAO to determine changes in heart rate and heart rate variability indicating autonomic nervous system disturbances. Taken together, this study reports several functional consequences of pdMCAO in a murine model that may provide a basis for improved assessment of potential therapies for stroke.

2. Methods

2.1. Animals

Ten-week-old male C57BL/6 mice from Charles River Laboratories Inc. were housed individually in institutional standard cages (15 cm × 21 cm × 13.5 cm) on a normal 12-h light–dark cycle with ad libitum access to water and food. Each cage contained a cardboard tube for environmental enrichment. Mice were randomly assigned to either pdMCAO or sham surgery. Altogether 59 mice were investigated. The time point of behavioral testing is given relative to the day of surgery. The first test was conducted 1 day before surgery (day −1). A subgroup of 39 mice was repeatedly tested in the following paradigms: gait analysis by paw-inking (n = 15; day 1), Catwalk (n = 22; day 1), corner test (n = 39; days −1, 1, 8, 15, 22), latency to move (n = 18; days −1, 1, 2, 7, 14, 21) and handedness (n = 39; days −1, 2, 9, 16). In another subgroup of 11 mice gait was analyzed by DigiGait and electrocardiography was performed. A third subgroup of mice (n = 19) was euthanized on day 2 to determine the infarct volume. The behavioral testing was performed without knowledge of the treatment group.

2.2. Distal MCA occlusion

Mice were anesthetized by intraperitoneal injection of 25 μl 1.5% tribromoethanol per g of body weight. We applied panthenol eye ointment (Bepanthen, Roche, Mannheim, Germany) to prevent eye dryness. A skin incision was made between the lateral corner of the left eye and the ear. The temporal muscle was removed by electrical coagulation (Modell ICC 50, Erbe, Tübingen, Germany). Then, the stem of the dMCA was exposed through a burr hole and occluded by microbipolar coagulation (Erbe, Tübingen, Germany). The skin was sutured after occlusion of the artery. Surgery was performed under a microscope (Hund, Wetzlar, Germany). Mice were kept at a body temperature of 37.5 °C using a heating pad. Mice were placed under a heating lamp during the recovery phase (1 h). Mortality was less than 10%. Sham-operated mice were treated in the same way but the dMCA was not occluded.

2.3. Gait analysis

We analyzed gait in the mice by three different methods. The paw-inking method: the front paws of mice were labeled with non-toxic ink (Faber, Neumarkt, Germany). Mice were placed in front of a dark tunnel (length 30 cm, width 10 cm), the bottom surface of which was lined with white paper. We analyzed three to four strides to determine stride length, step angle, stance width, and variability of stride length (the longest minus the shortest stride length).

2.3.1. Catwalk overground gait analysis

Mice were studied in a dark room (<20 lux of illumination). The apparatus consists of an elevated, 1.3-m-long glass plate with dim fluorescent light beamed into the glass from the side. The light is reflected downwards and the images of the footprints are captured by a high-speed digital video camera positioned under the walkway when the animal's paws make contact with the glass surface (Noldus Information Technology, Wageningen, The Netherlands). The mice walked spontaneously at their own speed. Only uninterrupted runs were saved for analysis. Gait parameters were generated after each footprint was identified and labeled.

2.3.2. The DigiGait treadmill gait analysis system

Ventral plane videography was applied to generate digital paw prints from which indices of gait were determined, as previously described (Hampton et al., 2004). Briefly, mice walked on a motor-driven treadmill with a transparent treadmill belt. The treadmill belt speed was set to 24 cm/s for all of the mice. Mice were trained to treadmill walking immediately before the test period. An acrylic compartment, ∼5 cm or ∼10 cm wide by ∼25 cm long, the length of which is adjustable, was attached on top of the treadmill to keep the mice within the view of the camera while walking on the treadmill belt (DigiGait, Mouse Specifics, Inc., Boston, MA). Digital video images of the underside of the animals were captured at 150 frames per second. Plotting the area of each digital paw print (paw contact area) imaged sequentially in time provided a dynamic gait signal, representing the temporal record of paw placement relative to the treadmill belt. Multiple postural and kinematic metrics from the gait signals were determined.

2.4. Corner test

Sensorimotor function was tested in mice using the corner test (Schallert et al., 1982; Zhang et al., 2002). The test device consists of two vertical boards (each 30 cm × 20 cm × 1 cm) attached on one side at an angle of 30°. A food pellet in a small opening between the two boards encourages the mice to enter the corner. The mice rear on their hindlimbs and turn to the right or left side after reaching the corner. Turns were only recorded if mice fully rose on their hindlimbs. A total of 12 turns were counted. The laterality index (LI) was calculated according to the formula: LI = (turns to the left side − turns to the right side)/total number of turnings.

2.5. Handedness test

The handedness of mice was determined using the paw preference test (Collins, 1968). The device consists of a transparent plexiglass box (10.5 cm × 6 cm × 6 cm). One side of the box has a feeding opening of 0.9 cm in diameter that is equally accessible from the right or the left side. Mice were accustomed to food pellets (size 2, Bioserv Biotechnologies Inc., Frenchtown, NJ, USA) for 1 week before the first test on day −1. After 12 h of fasting, mice were placed in the box and the food pellets were placed in front of the opening enabling the mice to reach the pellets using the right or left paw. Twenty paw reaches for food were observed and recorded for each mouse. The handedness index (HI) was calculated according to the formula: HI = (use of the left paw − use of right paw)/20.

2.6. Latency to move

Mice were placed on a plate and the time to move one body length (7 cm) was recorded.

2.7. Electrocardiography

Electocardiograms (ECGs) were recorded noninvasively in conscious mice using the ECGenie electrocardiography system (Mousespecifics, Inc., Boston, MA) as described previously (Chu et al., 2001). ECG signals were recorded passively from the underside of the animal's paws as it rested atop of a platform embedded with conductive electrodes. The electrodes were connected to a custom-designed amplifier and analog-to-digital converter. The signals were digitized at a sampling rate of 2 kHz. Data from continuous recordings of 20–30 ECG signals were used in the analyses. Heart rate variability metrics were determined in the time domain and in the frequency domain. ECGs were recorded 3–4 days after pdMCAO or sham surgery.

2.8. Infarct volume

Two or 23 days after pdMCAO the mice were deeply reanesthetized with tribromoethanol, perfused intracardially with Ringer's solution and euthanized. Coronal cryosections of the brains (20 μm in thickness) were cut every 400 μm and stained with a silver technique as described previously (Herrmann et al., 2005). The size of the ischemic lesion was determined using Scion Image software (Scion Corporation, Frederick, MD). To map the distribution 2 days after MCAO, infarcts from 19 mice were overlaid on a coronal scheme and the infarcted cortical surface was marked on a lateral brain view. Twenty-three days after MCAO the infarcted tissue was largely removed and the ischemic damage was reflected by a reduced volume of the ischemic hemisphere as compared to the non-ischemic hemisphere (Herrmann et al., 2005).

2.9. Statistical analysis

Data are expressed as mean ± SEM. Statistical tests were performed with SimgaStat 2.0 software. Statistical significance was evaluated using one-way repeated measures ANOVA, followed by Student–Newman–Keuls post hoc test, or Student's *t*-test with Bonferroni–Holm correction, as appropriate. The latency to move did not show a normal distribution and was therefore analyzed by repeated measures ANOVA on ranks (Friedman test). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Body weight

MCAO led to a statistically significant reduction of body weight on day 1 (24.3 ± 0.3 g before MCAO, 22.7 ± 0.3 g on day 1 after MCAO, $p < 0.001$). A loss of body weight was less marked in the sham group (24.5 ± 0.4 g before sham surgery, 23.9 ± 0.5 g on day 1 after sham surgery, $p < 0.001$). On day 8 after MCAO mice had nearly regained their original body weight and sham-operated mice had put on weight.

3.2. Gait analysis

We used three different methods to quantify gait in mice after pdMCAO. Stride length, stride length variability, stance width, and step angle (for definition of parameters see Table 1) did not differ significantly between pdMCAO mice and sham-operated mice as determined by the paw-inking method, the Catwalk system and the DigiGait system (Table 2). pdMCAO mice and sham-operated mice walked at a preset speed of 22 cm/s using the DigiGait system, thereby eliminating differences in walking speed that could affect gait characteristics. Using the Catwalk system the mice walked spontaneously at their own speed, which tended to be faster after MCAO (36.9 ± 6.5 cm/s) than sham surgery (23.0 ± 2.8 cm/s) without reaching statistical significance between the groups ($p = 0.068$). Both systems provide kinetic and spatial parameters of gait. The spatial parameters of gait were not affected by pdMCAO using the Catwalk and the DigiGait system which was in line with the results using the paw-inking method. The stance duration was significantly shortened in pdMCAO mice compared to sham-operated mice determined by the Catwalk and the DigiGait system (Table 2). The shortened stance duration in pdMCAO mice was partly due to a shorter brake duration (Table 2). In contrast, the propulsion duration was only marginally affected.

Table 1
Definition of gait parameters.

Parameter	Definition
Stride length	The spatial length that a paw traverses through a stride
Stride length variability	Standard deviation of stride length for the set of strides recorded (DigiGait, ~12 strides); difference between longest and shortest stride out of ~4 strides (ink technique)
Stance width	The perpendicular distance between the center points of either set of axial paws during peak stance
Step angle	Angle between left and right hind paws as a function of stride length and stance width
Paw angle	Angle that the paw makes with the long axis of the direction of motion of the animal
Stance duration	Time of paw contact with walking surface
Relative stance duration	Stance duration as percent of total stride duration
Braking duration	Time between initial paw contact and maximum paw contact
Propulsion duration	Time between maximum paw contact to beginning of swing phase
Swing duration	Time the paw is not in contact with walking surface

The stance duration is influenced by the walking speed (Vrinten and Hamers, 2003). We calculated the relative stance duration as the percentage of stride duration to correct for the effects of the walking speed on stance duration obtained by the Catwalk and the DigiGait system (Fig. 1A and B). The relative stance duration of the left forelimbs was significantly reduced in pdMCAO mice walking at a preset speed (DigiGait system) and of the left hind limbs of mice walking at a spontaneous speed (Catwalk system). The measurements became more robust if both fore- and hindlimbs were combined (Fig. 1C–F).

3.3. Latency to move

Latency to move was significantly longer in pdMCAO mice on days 1, 2, 7 and 14 after surgery than before surgery but shorter on days 14 and 21 than on day 1. Latency to move in pdMCAO mice returned to pre-surgery levels on day 21 (Fig. 2A).

3.4. Corner test

Mice subjected to pdMCAO turned significantly more often to the right contralateral side on day 1 than sham-operated controls resulting in a negative laterality index (Fig. 2B). The right side preference of pdMCAO mice was already lost on day 8.

3.5. Handedness test

In accordance with published data, male C57BL/6 mice had not shown a paw preference before surgery in our study (Collins, 1991) (Fig. 2C). On day 2 after left-sided pdMCAO mice used the

Table 2
Gait parameters in mice subjected to sham surgery or pdMCAO. Gait was evaluated by three different methods [inking of paws, automated overground via CatWalk, and automated treadmill via DigiGait].

	Inking (day 1)		Gait at spontaneous walking speed (Catwalk, day 1)		Gait at defined walking speed (DigiGait, day 2)	
	Sham ($n=8$)	pdMCAO ($n=7$)	Sham ($n=11$)	pdMCAO ($n=11$)	Sham ($n=6$)	pdMCAO ($n=6$)
Speed (cm/s)						
	nd		23.0 ± 2.8	36.9 ± 6.52	22.0 [set by operator]	
Stride length (cm)						
FR	5.6 ± 0.4	5.4 ± 0.3	5.7 ± 0.5	7.1 ± 0.5	5.9 ± 0.2	5.5 ± 0.1
FL	5.5 ± 0.3	5.3 ± 0.3	6.1 ± 0.4	7.1 ± 0.5	5.7 ± 0.2	5.4 ± 0.1
HR			6.3 ± 0.4	7.1 ± 0.5	5.7 ± 0.2	5.4 ± 0.1
HL			6.1 ± 0.3	7.2 ± 0.5	5.7 ± 0.2	5.5 ± 0.1
Stride length variability (cm)						
FR	1.8 ± 0.3	0.7 ± 0.1			0.87 ± 0.11	0.81 ±
FL	1.2 ± 0.2	1.2 ± 0.3			0.90 ± 0.09	0.66 ±
HR					0.66 ± 0.08	0.71 ± 0.11
HL					0.76 ± 0.12	0.71 ± 0.12
Stance width (cm)						
FR	1.7 ± 0.03	1.7 ± 0.01	1.10 ± 0.05	1.23 ± 0.04	1.85 ± 0.08	1.86 ± 0.09
FL						
HR			2.01 ± 0.07	2.14 ± 0.07	2.60 ± 0.17	2.38 ± 0.21
HL						
Step angle (°)						
FR	68.7 ± 0.9	67.1 ± 1.9			61.8 ± 1.0	57.6 ± 2.0
FL						
HR					54.3 ± 4.2	59.6 ± 4.6
HL						
Stance duration (ms)						
FR			122 ± 17	88 ± 10	**156 ± 5**	**140 ± 3***
FL			**130 ± 15**	**84** ± 10*	**150 ± 4**	**135 ± 2****
HR			**141 ± 16**	**101** ± 9*	**160 ± 4**	**145 ± 3***
HL			**160 ± 14**	**104** ± 8**	157 ± 8	151 ± 5
Brake duration (ms)						
FR			45 ± 8	30 ± 4	**57 ± 4**	**44 ± 3***
FL			**49 ± 5**	**24** ± 3**	47 ± 6	37 ± 4
HR			51 ± 7	34 ± 4	**32 ± 2**	**24 ± 3***
HL			**61 ± 8**	**32** ± 2**	34 ± 5	39 ± 6
Propulsion duration (ms)						
FR			77 ± 11	58 ± 7	100 ± 5	96 ± 4
FL			81 ± 11	61 ± 9	103 ± 6	099 ± 3
HR			90 ± 10	67 ± 6	128 ± 3	121 ± 3
HL			**99 ± 9**	**73** ± 6*	123 ± 11	118 ± 5
Swing duration (ms)						
FR			137 ± 11	123 ± 11	111 ± 5	110 ± 6
FL			143 ± 13	129 ± 11	108 ± 6	113 ± 4
HR			139 ± 19	110 ± 11	101 ± 5	99 ± 4
HL			122 ± 10	111 ± 10	103 ± 4	95 ± 4

FR, right forelimb; FL, left forelimb; HR, right hind limb; HL, left hind limb. nd, not detected. Mean ± SEM. Values that differed significantly between groups are bold.
* $p<0.05$ vs. sham-operated mice.
** $p<0.01$ (t-test).

left front paw significantly more often to reach the food pellets than before surgery (positive handedness index). On days 9 and 16 the two front paws were used almost equally to reach the food pellets. Handedness was not affected in sham-operated mice (Fig. 2C).

3.6. Electrocardiography

We recorded ECGs noninvasively in conscious mice 3 days after pdMCAO and sham surgery. Heart rate was significantly decreased in pdMCAO mice compared to sham-operated mice (Table 3). Heart rate variability determined in the time domain and in the frequency domain was significantly higher in pdMCAO mice than in sham-operated mice. The low-frequency component of heart rate variability was significantly higher in pdMCAO mice than in sham-operated mice as was the high-frequency component.

3.7. Infarct distribution

Twenty-three days after MCAO the infarcted tissue was largely removed. Based on a volume deficit of the left ischemic hemisphere we deduced an infarct volume of 31.4 ± 3.7 mm^3 ($n=21$).

Table 3
Electrocardiographic parameters 3–4 days after sham surgery ($n=5$) or pdMCAO ($n=6$).

Parameter	Sham	pdMCAO	p
Heart rate (bpm)	773 ± 5	665 ± 13	<0.05
Heart rate variability (bpm)	20 ± 2	64 ± 7	<0.05
Total power variability of RR intervals (ms^2)	1.4 ± 0.3	38.1 ± 8.8	<0.05
Low-frequency component (ms^2)	0.2 ± 0.1	29.9 ± 7.1	<0.05
High-frequency component (ms^2)	1.4 ± 0.3	4.9 ± 1.8	<0.05

Means ± SEM. bpm, beats per minute.

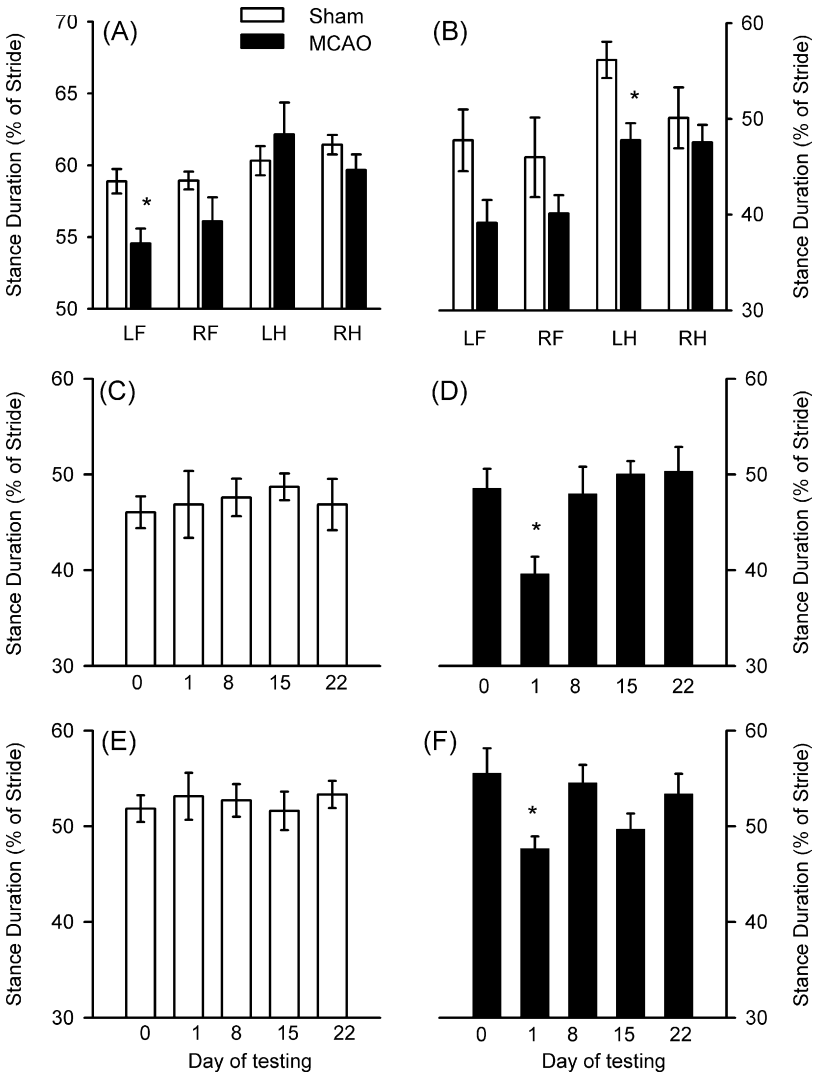

Fig. 1. (A) Relative stance duration of individual limbs in mice subjected to sham surgery or left-sided MCAO as measured on day 2 with the DigiGait system. Values are means ± SEM (n = 5–6). *p < 0.05 (t-test with Bonferroni–Holm correction for multiple testing). (B) Relative stance duration of individual limbs in mice subjected to sham surgery or left-sided MCAO as measured on day 1 with the Catwalk system. Values are means ± SEM (n = 11). *p < 0.05 (t-test with Bonferroni–Holm correction for multiple testing). LF, left forelimb. RF, right forelimb. LH, left hindlimb. RH, right hindlimb. (C) Relative stance duration of forelimbs in mice subjected to sham surgery as measured by Catwalk. The average of left and right forelimbs are depicted. Values are means ± SEM (n = 7–9). $F(4/28) = 0.214$, $p = 0.928$. (D) Relative stance duration of forelimbs of mice subjected to MCAO as measured by Catwalk. Values are means ± SEM (n = 9–10). $F(4/35) = 3.568$, $p = 0.015$. (E) Relative stance duration of hindlimbs in mice subjected to sham surgery as measured by Catwalk. Values are means ± SEM (n = 7–9). $F(4/28) = 0.225$, $p = 0.922$. (F) Relative stance duration of hindlimbs in mice subjected to MCAO as measured by Catwalk. Values are means ± SEM (n = 9–10). $F(4/35) = 2.731$, $p = 0.045$. *p < 0.05 compared to basal level at day 0 (Dunnett's test).

The infarct volume did not correlate with the motor performance in the latency to move, corner test, or handedness (data not shown). In order to localize the infarct the distribution of cerebral infarctions after pdMCAO was mapped on day 2 in a separate group of 19 mice. pdMCAO affected mainly the cerebral cortex (Fig. 3). The infarct extended to the S1 field and the barrel cortex and less often to the S1 forelimb cortex. The S1 hindlimb cortex and M1 area were only affected in <10 out of 19 mice.

4. Discussion

We assessed functional deficits in a mouse model after cortical infarction was induced by permanent distal MCAO (pdMCAO). Quantitative and detailed tests are needed to investigate functional impairment in the murine pdMCAO model since neurological deficits are not overtly obvious. Our investigation may help to define the contribution of the distal MCA territory and the cerebral cortex to deficits caused by proximal MCA occlusion. The following tests or parameters were sensitive to distal MCAO: kinetic gait parameters, corner test, handedness, latency to move, and heart rate variability. In another study we have also found an impaired performance in the latency to move and corner test after pdMCAO demonstrating the reproducibility of these tests (Bargiotas, Schwaninger, manuscript in preparation). In contrast, the chimney test, the pole test, and the cylinder test did not detect deficits 2 days after distal MCAO although they were found to be useful after proximal MCAO (Bouet et al., 2007; Gibson et al., 2005; Hunter et al., 2000; Li et al., 2004). Future application of the presented tests would benefit from a reduction of the test variability. With current protocols of the gait analysis, latency to move, corner test, and handedness we would need a sample size of 20 and larger to detect a 50% improvement with a power of 0.8 and a p-value of 0.05. In this respect, heart rate variability is superior to the other tests as a sample size of about 10 would be sufficient to detect a 50% improvement.

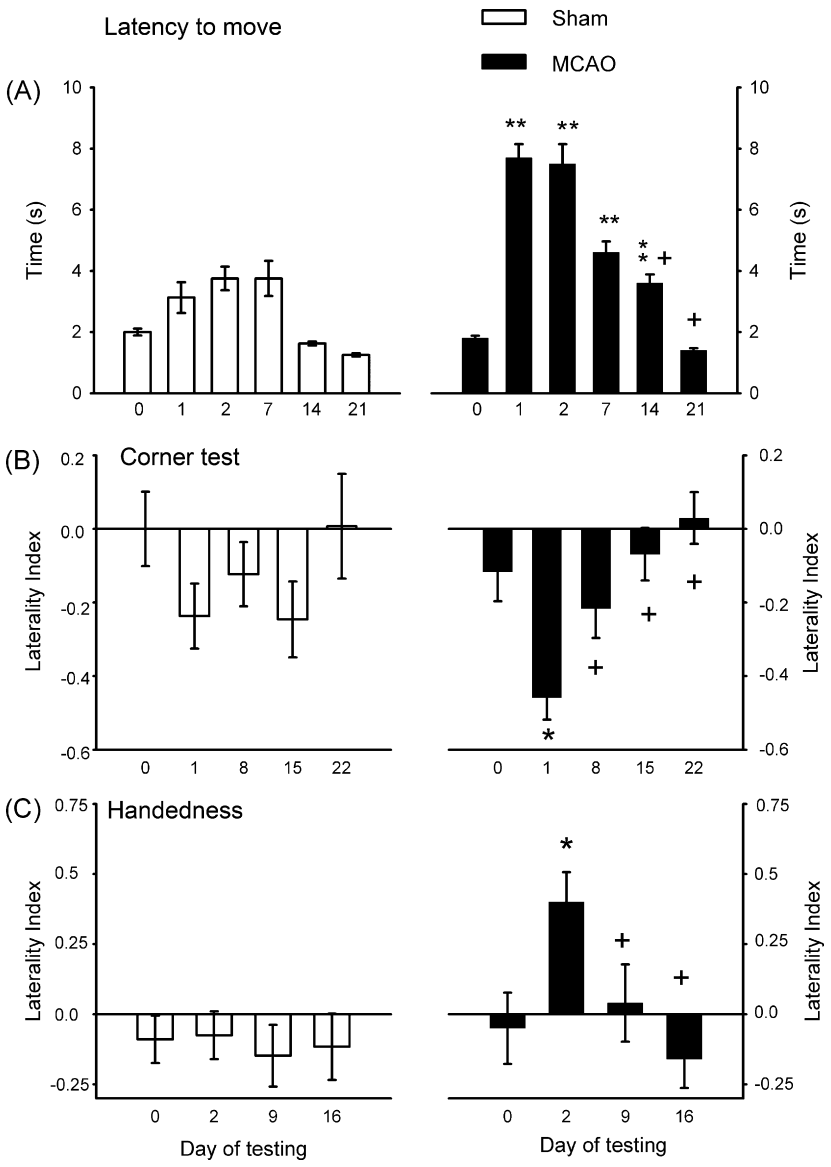

Fig. 2. (A) Latency to move one body length at several time points after sham surgery (Friedman's Chi-Square = 13.968, DF = 5, $p = 0.016$) or MCAO (Friedman's Chi-Square = 27.562, DF = 5, $p < 0.001$). $^{**}p < 0.001$ (Student–Newman–Keuls test compared to day 0), $+p < 0.05$ (Student–Newman–Keuls test compared to day 1). $n = 8$–10. (B) Turning in corner test after sham surgery ($F(4/66) = 2.520$, $p = 0.049$) or left-sided MCAO ($F(4/70) = 7.359$, $p < 0.001$). A negative laterality index denotes turning to the right side. $^{*}p < 0.05$ (Student–Newman–Keuls test compared to day 0). $^{+}p < 0.05$ (Student–Newman–Keuls test compared to day 1). $n = 19$–20. (C) Paw preference in the Collins test after sham surgery ($F(3/51) = 0.188$, $p = 0.904$) or left-sided MCAO ($F(3/57) = 3.758$, $p = 0.016$). A positive laterality index denotes preferred use of the left paw. Values are means ± SEM. $^{*}p < 0.05$ (Student–Newman–Keuls test compared to day 0). $^{+}p < 0.05$ (Student–Newman–Keuls test compared to day 2). $n = 19$–20.

4.1. Gait analysis

In contrast to the key role of gait disorders in clinical stroke care, gait has been largely neglected in experimental stroke research, although a recent study analyzed gait in a rat model of cerebral ischemia (Wang et al., 2008). Gait disorders have probably only rarely been investigated in experimental stroke research because direct observation usually does not indicate any striking gait deficits after MCAO, and the conventional ink technique fails to reveal any gait disturbance. Foot prints on a sheet of paper represent spatial coordinates but not the kinetic aspects of gait. For kinetic assessment of gait a video-based analysis is advantageous; with commercially available systems numerous spatial and kinetic parameters can be measured. We applied two automated gait analysis systems that operate on different principles. Mice run spontaneously at their own speed on a glass plate using the Catwalk system. Mice run on a motorized treadmill at speeds selected by the operator using the DigiGait system. In both systems mice are video-taped from below. With Catwalk, the intensity of paw prints are evaluated, whereas DigiGait provides a ventral view of the entire animal. With both systems, using two independent groups of mice, we found a reduction in stance duration after pdMCAO, mainly due to a decrease in the brake phase of stance. Stance duration is known to change with changes in walking speed (Gillis and Biewener, 2001). It was therefore advantageous to control the walking speed with the DigiGait system to prevent differences in walking speeds from affecting the postural and kinematic metrics. The effect of walking speed on stance duration may explain why a recent study of traumatic brain injury in mice came to the opposite conclusion. Neumann et al. recently reported that mice subjected to unilateral controlled cortical impact spontaneously ran at a lower speed than sham-treated mice, leading to a prolonged stance duration (Neumann et al., 2009).

Wang and coworkers observed a reduction in the intensity and the area of paw prints of rats after MCAO (Wang et al., 2008). In mice we did not detect any change in these parameters despite the

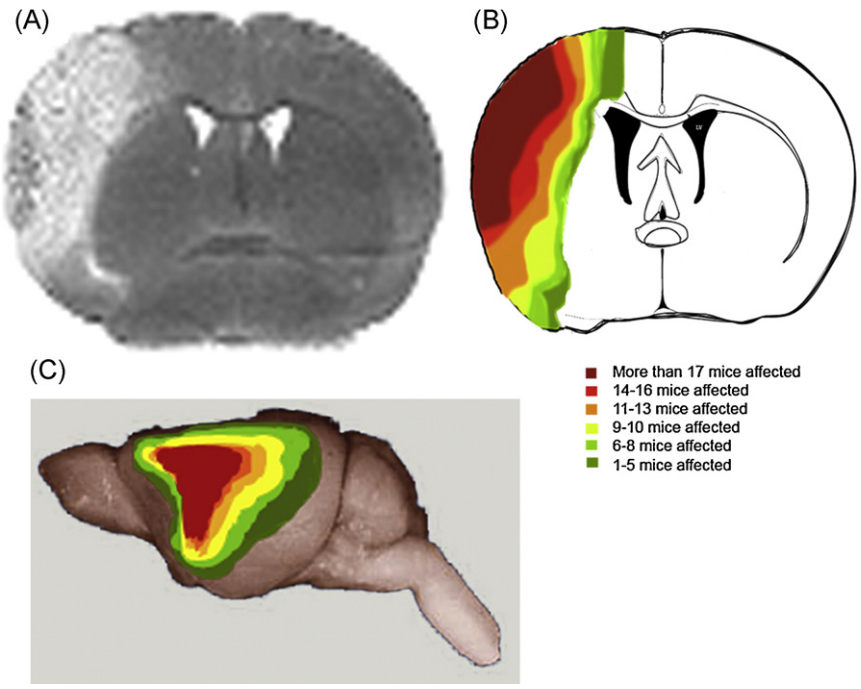

Fig. 3. (A) Silver-stained coronal brain section of a mouse 2 days after pdMCAO. The infarcted tissue is unstained. (B) Density map of infarcts on day 2 after pdMCAO. The distribution of infarcts in 19 mice was mapped on a coronal section (0 mm relative to Bregma). (C) Density map of surface area of infarcts on day 2 after pdMCAO.

reduced body weight of mice after pdMCAO, which should also tend to reduce the paw print intensity and area. As reported previously (Arsenijevic et al., 2005), body weight transiently drops after MCAO. This weight loss is due to a transient decrease in food intake on the day of surgery but hyperphagia compensates for this on days 3–5 after pdMCAO (Arsenijevic et al., 2005).

In human stroke patients gait disorders are characterized by asymmetry. In contrast, the stance and brake duration was bilaterally reduced in mice after pdMCAO. It may have been that the decrease in cortical control after pdMCAO in mice alters the form of gait regulated by spinal networks. Even so, the effect is transient. Already 8 days after pdMCAO stance duration had normalized, which is in agreement with a previous study of unilateral corticospinal tract transsection in rats (Muir and Whishaw, 1999).

4.2. Latency to move

We found an increased latency to move after pdMCAO that affects mainly the cortex. The latency to move was found to be elevated in mice after MCAO in some (Hattori et al., 2000), but not all studies (Craft et al., 2005; Li et al., 2004). In previous investigations, the MCA was occluded at a proximal site in mice to induce both cortical and subcortical infarcts. The finding that pdMCAO and cortical lesions are sufficient to increase the latency to move is supported by previous work in rats (van der Staay et al., 1996). Latency to move probably reflects locomotor activity of mice that is altered after MCAO (Hunter et al., 2000) but may also be affected by increased levels of anxiety after MCAO (Kilic et al., 2008). This test is valuable for functional testing after pdMCAO in mice because it is easy to perform and detects a behavioral effect even 14 days after cerebral ischemia.

4.3. Corner test

The corner test evaluates sensorimotor function and behavioral symmetry. It is thought that stimulation of the neck and the vibrissae prompt animals to rise on the hindlimbs, which tests both cortical and subcortical functions (Schallert et al., 1982). After proximal MCAO, mice turn preferentially to the side of the lesion (Bouet et al., 2007; Li et al., 2004; Zhang et al., 2002) (data not shown). In contrast, following pdMCAO the animals showed a tendency to turn to the contralateral side of the lesion. We have confirmed this finding in two other experiments (data not shown). Since sham-operated mice were subjected to the same surgical procedure except for occlusion of MCA, we can exclude that the contralateral turning effect is due to some peripheral tissue damage. Probably, the contralateral turning is caused by the almost selective cortical lesion site in our model, whereas proximal MCAO induces cortical and striatal lesions. In accordance with this hypothesis striatal lesions have been shown to induce an ipsilateral turning in the corner test (Seyfried et al., 2008). The opposite direction of the turning may be due to an over-compensatory mechanism in pdMCAO as compared to proximal MCAO. pdMCAO did not affect the results of the corner test in the recent study by Freret and colleagues (Freret et al., 2009). This apparent discrepancy may be due to different mouse strains that were used in the two studies or other unclear reasons.

4.4. Handedness

To our knowledge, handedness in mice has not yet been tested after cerebral ischemia. Our study showed that focal cerebral ischemia affects handedness of mice. We used male C57BL/6 mice, 50% of which are right- or left-handed (Collins, 1968). Before cerebral ischemia both the right and left front limb were almost equally used. One day after MCAO mice showed significantly less use of the contralateral right paw than before surgery. This effect was observed transiently; handedness normalized by day 9 after pdMCAO.

4.5. Heart rate

We determined that focal cerebral ischemia affects the autonomic regulation of the heart in mice. Electrocardiographic changes, autonomic nervous system disturbances, and myocardial lesions have been observed in stroke patients. Whereas in human stroke patients a decreased variability of the heart rate has been described (Korpelainen et al., 1996; Tokgozoglu et al., 1999), we

found an elevated heart rate variability in our mouse model of stroke. This discrepancy may denote a species difference or may be attributed to vascular risk factors in stroke patients that are not present in the healthy mice we used in our study (Stein and Kleiger, 1999). Power spectral analysis demonstrated a significantly higher total power variability of the R–R intervals in the pdMCAO mice than in sham-operated mice, which was due to both a higher low-frequency and a higher high-frequency component. The low- and high-frequency components have been shown to reflect cardiac sympathetic and parasympathetic activity, respectively (Gehrmann et al., 2000). Thus, our data suggest that cerebral ischemia enhances the parasympathetic tone. A recent study demonstrated that the vagus nerve mediates the anti-inflammatory and neuroprotective effect of a melanocortin derivative after cerebral ischemia (Ottani et al., 2008). Thus, the enhanced parasympathetic tone supported by our data may indicate an endogenous neuroprotective process. On the other hand, the increased heart rate variability in the low-frequency range may be due to an increased sympathetic tone (Akselrod et al., 1981; Gehrmann et al., 2000; Stein and Kleiger, 1999). Indeed, stroke is often associated with activation of the sympathetic nervous system (Myers et al., 1981; Sander et al., 2001). Apart from cardiac effects, the increased sympathetic tone is responsible for stroke-induced immunodeficiency (Prass et al., 2003). Our findings demonstrate that neuronal injury caused by brain ischemia affects the regulation of the cardiac autonomic nervous system in a murine model of stroke, mimicking some of the changes seen clinically in stroke patients.

5. Conclusion

Detailed quantitative analysis of functional parameters revealed several abnormalities in mice after distal MCAO. Gait disorders and altered handedness are of particular interest due to their clinical relevance. We found that the treadmill gait analysis system is a practicable device for gait analysis in mice after pdMCAO because the walking speed can be set by the investigator. The latency to move proved to be also a robust test in pdMCAO, still reflecting cerebral deficits 14 days after pdMCAO. In addition, analysis of autonomic nervous disturbances due to pdMCAO by electrocardiography may provide opportunities for advancing therapies for stroke.

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References

Akselrod S, Gordon D, Ubel FA, Shannon DC, Berger AC, Cohen RJ. Power spectrum analysis of heart rate fluctuation: a quantitative probe of beat-to-beat cardiovascular control. Science 1981;213:220–2.

Arsenijevic D, de Bilbao F, Plamondon J, Paradis E, Vallet P, Richard D, et al. Increased infarct size and lack of hyperphagic response after focal cerebral ischemia in peroxisome proliferator-activated receptor [beta]-deficient mice. J Cereb Blood Flow Metab 2005;26:433–45.

Baumann CR, Kilic E, Petit B, Werth E, Hermann DM, Tafti M, et al. Sleep EEG changes after middle cerebral artery infarcts in mice: different effects of striatal and cortical lesions. Sleep 2006;29:1339–44.

Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke 1986;17:472–6.

Bouet V, Freret T, Toutain J, Divoux D, Boulouard M, Schumann-Bard P. Sensorimotor and cognitive deficits after transient middle cerebral artery occlusion in the mouse. Exp Neurol 2007;203:555–67.

Chu V, Otero JM, Lopez O, Morgan JP, Amende I, Hampton TG. Method for non-invasively recording electrocardiograms in conscious mice. BMC Physiol 2001;1:6.

Collins RL. On the inheritance of handedness. I. Laterality in inbred mice. J Hered 1968;59:9–12.

Collins RL. Reimpressed selective breeding for lateralization of handedness in mice. Brain Res 1991;564:194–202.

Craft TK, Glasper ER, McCullough L, Zhang N, Sugo N, Otsuka T, et al. Social interaction improves experimental stroke outcome. Stroke 2005;36:2006–11.

Fisher M, Feuerstein G, Howells DW, Hurn PD, Kent TA, Savitz SI, et al. Update of the stroke therapy academic industry roundtable preclinical recommendations. Stroke 2009, STROKEAHA.108.541128.

Freret T, Bouet V, Leconte C, Roussel S, Chazalviel L, Divoux D, et al. Behavioral deficits after distal focal cerebral ischemia in mice: usefulness of adhesive removal test. Behav Neurosci 2009;123:224–30.

Gehrmann J, Hammer PE, Maguire CT, Wakimoto H, Triedman JK, Berul CI. Phenotypic screening for heart rate variability in the mouse. Am J Physiol Heart Circ Physiol 2000;279:H733–40.

Gibson CL, Bath PM, Murphy SP. G-CSF reduces infarct volume and improves functional outcome after transient focal cerebral ischemia in mice. J Cereb Blood Flow Metab 2005;25:431–9.

Gillis GB, Biewener AA. Hindlimb muscle function in relation to speed and gait: in vivo patterns of strain and activation in a hip and knee extensor of the rat (*Rattus norvegicus*). J Exp Biol 2001;204:2717–31.

Groger M, Lebesgue D, Pruneau D, Relton J, Kim SW, Nussberger J, et al. Release of bradykinin and expression of kinin B2 receptors in the brain: role for cell death and brain edema formation after focal cerebral ischemia in mice. J Cereb Blood Flow Metab 2005;25:978–89.

Guegan C, Braudeau J, Couriaud C, Dietz GP, Lacombe P, Bahr M, et al. PTD-XIAP protects against cerebral ischemia by anti-apoptotic and transcriptional regulatory mechanisms. Neurobiol Dis 2006;22:177–86.

Hampton TG, Stasko MR, Kale A, Amende I, Costa AC. Gait dynamics in trisomic mice: quantitative neurological traits of Down syndrome. Physiol Behav 2004;82:381–9.

Hattori K, Lee H, Hurn PD, Crain BJ, Traystman RJ, DeVries AC. Cognitive deficits after focal cerebral ischemia in mice. Stroke 2000;31:1939–44.

Herrmann O, Baumann B, De Lorenzi R, Muhammad S, Zhang W, Kleesiek J, et al. IKK mediates ischemia-induced neuronal cell death. Nat Med 2005;11:1322–9.

Hunter AJ, Hatcher J, Virley D, Nelson P, Irving E, Hadingham SJ, et al. Functional assessments in mice and rats after focal stroke. Neuropharmacology 2000;39:806–16.

Hurtado O, Pradillo JM, Alonso-Escolano D, Lorenzo P, Sobrino T, Castillo J, et al. Neurorepair versus neuroprotection in stroke. Cerebrovasc Dis 2006;21(Suppl. 2):54–63.

Kilic E, Kilic Ü, Bacigaluppi M, Guo Z, Abdallah NB, Wolfer DP, et al. Delayed melatonin administration promotes neuronal survival, neurogenesis and motor recovery, and attenuates hyperactivity and anxiety after mild focal cerebral ischemia in mice. J Pineal Res 2008;45:142–8.

Korpelainen JT, Sotaniemi KA, Huikuri HV, Myllya VV. Abnormal heart rate variability as a manifestation of autonomic dysfunction in hemispheric brain infarction. Stroke 1996;27:2059–63.

Li X, Blizzard KK, Zeng Z, DeVries AC, Hurn PD, McCullough LD. Chronic behavioral testing after focal ischemia in the mouse: functional recovery and the effects of gender. Exp Neurol 2004;187:94–104.

Muir GD, Whishaw IQ. Complete locomotor recovery following corticospinal tract lesions: measurement of ground reaction forces during overground locomotion in rats. Behav Brain Res 1999;103:45–53.

Myers MG, Norris JW, Hachniski VC, Sole MJ. Plasma norepinephrine in stroke. Stroke 1981;12:200–4.

Neumann M, Wang Y, Kim S, Hong SM, Jeng L, Bilgen M, et al. Assessing gait impairment following experimental traumatic brain injury in mice. J Neurosci Methods 2009;176:34–44.

Ottani A, Giuliani D, Mioni C, Galantucci M, Minutoli L, Bitto A, et al. Vagus nerve mediates the protective effects of melanocortins against cerebral and systemic damage after ischemic stroke. J Cereb Blood Flow Metab 2008.

Prass K, Meisel C, Hoflich C, Braun J, Halle E, Wolf T, et al. Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke t helper cell type 1-like immunostimulation. J Exp Med 2003;198:725–36.

Sander D, Winbeck K, Klingelhofer J, Etgen T, Conrad B. Prognostic relevance of pathological sympathetic activation after acute thromboembolic stroke. Neurology 2001;57:833–8.

Schallert T, Upchurch M, Lobaugh N, Farrar SB, Spirduso WW, Gilliam P, et al. Tactile extinction: distinguishing between sensorimotor and motor asymmetries in rats with unilateral nigrostriatal damage. Pharmacol Biochem Behav 1982;16:455–62.

Sena E, van der Worp HB, Howells D, Macleod M. How can we improve the preclinical development of drugs for stroke? Trends Neurosci 2007;30:433–9.

Seyfried DM, Han Y, Yang D, Ding J, Savant-Bhonsale S, Shukairy MS, et al. Mannitol enhances delivery of marrow stromal cells to the brain after experimental intracerebral hemorrhage. Brain Res 2008;1224:12–9.

Stein PK, Kleiger RE. Insights from the study of heart rate variability. Annu Rev Med 1999;50:249–61.

Tokgozoglu SL, Batur MK, Topcuoglu MA, Saribas O, Kes S, Oto A. Effects of stroke localization on cardiac autonomic balance and sudden death. Stroke 1999;30:1307–11.

van der Staay FJ, Augstein KH, Horvath E. Sensorimotor impairments in rats with cerebral infarction, induced by unilateral occlusion of the left middle cerebral artery: strain differences and effects of the occlusion site. Brain Res 1996;735:271–84.

van Lookeren Campagne M, Thibodeaux H, van Bruggen N, Cairns B, Gerlai R, Palmer JT, et al. Evidence for a protective role of metallothionein-1 in focal cerebral ischemia. Proc Natl Acad Sci USA 1999;96:12870–5.

Vrinten DH, Hamers FFT. CatWalk automated quantitative gait analysis as a novel method to assess mechanical allodynia in the rat; a comparison with von Frey testing. Pain 2003;102:203–9.

Wang Y, Bontempi B, Hong SM, Mehta K, Weinstein PR, Abrams GM, et al. A comprehensive analysis of gait impairment after experimental stroke and the therapeutic effect of environmental enrichment in rats. J Cereb Blood Flow Metab 2008;28:1936–50.

Winter B, Bert B, Fink H, Dirnagl U, Endres M. Dysexecutive syndrome after mild cerebral ischemia? Mice learn normally but have deficits in strategy switching. Stroke 2004;35:191–5.

Zhang L, Schallert T, Zhang ZG, Jiang Q, Arniego P, Li Q, et al. A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. J Neurosci Methods 2002;117:207–14.